IN VIVO MODULATION OF INNATE IMMUNITY BY DIETARY SPICES AND HERBS

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By

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DEDICATION

I dedicate this thesis to my parents who have encouraged, supported and inspired me along the way.

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CHAPTER I

INTRODUCTION TO THE STUDY

1.1 Historical and current use of natural products

Natural products such as dietary herbs and spices are considered to have formed the foundation of modern medicine.[1-5] It has been estimated that at least 50% or more of current pharmaceutical drugs available today are of plant based origin.[1,5] The use of natural products such as herbs and spices as therapeutic tools to promote health and manage disease is largely based on their health promoting effects documented in traditional medicine practiced by many ancient cultures.[6-13] Civilizations across the world have relied on natively grown plants for medicinal purposes for thousands of years. The use of herbs and spices has been estimated to have origins as far back as 5,000 BC in ancient Egypt.[10,14] During this time, Sumerians used herbal remedies of garlic, coriander, cumin and cinnamon to treat a variety of health conditions ranging from the common cold to gastrointestinal disorders and urinary tract infections.[7,10,15] The earliest organized literature about therapeutic benefits of herbs and spices comes from ancient, but still practiced, traditional Chinese and Indian medicine, and are centered on the use of natural products to promote health as well as prevent as manage disease.[7,10,16] Even today an estimated 80% the world's population relies on the use of natural products as the primary medicinal treatment, according to the World Health Organization.[17] In the United States

alone, approximately 38% of adults and 12% of children use complementary or alternative medicine, according to the National Institutes of Health (NIH) in 2007.[18]

The discovery of important bioactive compounds found in nature has had implications for the management of infectious disease and for the therapeutic treatment of a range of chronic diseases including cardiovascular and neurodegenerative disease as well as cancer, obesity, and diabetes mellitus.[1,2,5] Many well known and commonly used drugs such as aspirin, originally derived from bark of the White Willow tree (*Salix alba*), morphine derived from Poppy seeds (*Papaver somniferum*) and the cholesterol lowering statins derived from oyster mushrooms (*Pleurotus ostreatus)* are representative of the widespread use of natural products to manage health.[7,19]

Additionally, metabolites in plants are often highly bioactive and exhibit high specificity which may be a result of evolutionarily conserved molecular patterns on protein binding sites across different species facilitating a more targeted drug design modeled around them.[5] However, recent discoveries related to side effects and toxicity associated with the use of highly potent synthetic drugs and the development of multi-drug resistance in infectious pathogens due to indiscriminate use of antibiotics has resulted in a renewed interest in the use of natural products as novel therapeutic approaches to disease management.[4,20,21]

Though lacking in rigorous scientific credibility, it is generally agreed upon that the long history and widespread use of natural products such as herbs and spices as supplemental or complementary treatment may not be without merit. Epidemiologic data have consistently shown that diets high in plant foods, including dietary herbs and spices,

are inversely related to risk for chronic disease.[22-25] Specifically, consumption of diets high in plants has been associated with increased longevity and a decreased risk for developing cardiovascular disease, obesity, cancer, and Alzheimer's disease.[22,26,27] It is believed that the health benefits associated with dietary herbs and spices may be in part due to the presence of bioactive phytochemicals.[13,26,28] Herbs and spices generally are concentrated sources of a variety of plant secondary metabolites including phenolic compounds, carotenoids, saponins, and alkaloids. Many of these phytochemicals because of their chemical structure and electrochemical properties can function by modulating the intracellular redox environment by stabilizing free radicals and by increasing the activity and/or expression of important antioxidant enzymes.[24,29-31] Additionally, certain phytochemicals can interact with cell surface receptors, proton pumps and important signaling pathways mediated by their ligands as many share structural similarities with hormones and neurotransmitters. The ability of these phytochemicals to modulate biologically important pathways is believed to be responsible for their many observable properties such as antioxidant, antimicrobial, antiviral, and anti-inflammatory activity. [29-31]

1.2 Innate immunity

Among the many benefits of dietary herbs and spices, the ability to modulate the innate immunological properties has received renewed attention recently.[32,33] The innate immune system is often described as the first line of defense against modified self and non-self (foreign) entities such as viruses, bacteria, parasites and their associated metabolites.[32,34-37] Innate immunity is phylogenetically conserved between species and is a rapid, consistent, non-specific and non-anticipatory system.[34,38] It is comprised of

anatomic and physiological barriers as well as cellular and humoral components, which collectively serve to protect the integrity of the host.[39] A key role of the innate immune response involves the detection, uptake and destruction of altered or non-self threats to the organism via phagocytosis.[40] This primordial system, which has been estimated to have evolved more than 700 million years ago, is efficiently executed primarily by macrophages and neutrophils as well as the humoral components secreted by them.[41,42] Because challenges to the immune system are presented on regular and continual bases, survival and overall health is dependent upon the ability of the organism to rapidly recognize and eliminate challenges using the humoral and cellular components of the innate immune system.

Though evolutionarily primitive, new discoveries into identifying the cellular and humoral components of the innate immune system have been made continuously and have been found to be remarkably conserved among many species.[34,38] An important distinction between the innate and adaptive branches of the immune system is the ability and efficiency, or diversity, of non-self recognition by cellular and humoral components of the innate immune system, which is mediated by constant, predetermined cell surface receptor specificities.[35,40] The number of non-self epitopes that can be recognized by pattern recognition receptors (PRR's) such as the toll-like receptors (TLR) are coded by genes that follow germ-line inheritance.[34,35,43] Conversely, when cellular and humoral components of the adaptive immune system are presented with non self antigens, they undergo clonal differentiation to produce cells and secrete antibodies which have a much higher degree of specificity towards non self components and therefore participate in a much more specific and efficient removal of foreign epitopes.[34,35] In addition, certain

cells of the adaptive immune system also are transformed into memory cells, which can rapidly promote cell differentiation when the same epitope is encountered again. This process of clonal selection, differentiation and memory development can result in specificity towards an almost unlimited number of foreign antigens.[44] However, this process is fairly time intensive and can take approximately one to three weeks to complete.[35] The initial protection of the organism against the invading entity is therefore mediated by the innate immune system which can mount a response within hours after recognition of pathogen associated molecular patterns (PAMPs).[34, 35,45,46]

A consequence, however, of germline inheritance of the innate immune system is its fairly broad specificity and its inability to develop specificity towards new molecular targets. However, though broad in specificity, cells of the innate immune system are involved in initial processing and presentation of antigen to the adaptive immune system.[34,35] In addition, the innate immune response has also been shown to be important for removal of modified self epitopes, such as oxidized or glycated proteins and lipids generated during normal cellular processes such as apoptosis and tissue remodeling. Recently, many of these modified self epitopes have been identified in association with many chronic diseases including Alzheimer's disease, diabetes mellitus and cardiovascular disease.[47,48] Accumulation of these epitopes has been linked to an inability of the innate immune system to process them either due to a weak quantitative (i.e. lower number of cells) or a poor qualitative, less robust, response (diminished clearance).[49-52] Therefore, having an efficient and robust innate immune system may not only improve the initial removal of the foreign antigen, but also accelerate the adaptive immune response and prevent the progression of these pathologies.[48,50] Thus, stimulation of the

innate response by biological agents may have important implications in the prevention and management of multiple diseases.[32,50,53]

Though an exciting approach, this strategy of stimulating the innate immune system has encountered many hurdles. A primary consequence of a highly active or overactive immune system is potential for increased oxidative assault in the different tissues (blood, muscle, etc) of the body as a result of leakage of reactive oxygen and nitrogen entities following phagocytosis and oxidative degradation of the engulfed phagosome by rapid enzymatic production of free radicals.[54,55] This is especially true for phagocytic cells of the innate immune system which exhibit an unregulated ability to phagocytose antigens.[56] Engorgement of intracellular phagosomes by superoxide generated through cytosolic assembly of NADPH oxidase can often result in the release and leakage of reactive oxygen and nitrogen species which have many collateral detrimental effects such as inflammation and sepsis.[32,54-56] While an uncontrolled, self destructive process is often times seen only during a non-self removal, this is highly controlled during removal of apoptotic cells and during tissue remodeling.[57,58] This is primarily due to a lower target to cell ratio and a more controlled oxidative response resulting from a fewer number of phagosomes per cell. As a result, there is a lower requirement for production of reactive oxidative species overall and potentially an increased ability of these cells to manage a pro-oxidant redox shift due to enhanced antioxidant response. Thus, dysregulation of the innate immune system at both ends of the spectrum can have negative implications for overall health.[55]An underactive response increases susceptibility to infection and potentially augments Alzheimer's disease and

diabetes mellitus. Conversely, an over stimulated response may potentiate development of chronic disorders propagated by uncontrolled oxidative insult.[32,59]

1.3 Modulation of innate immunity by natural products

Agents that are capable of increasing the number of phagocytic cells by promoting a controlled polarized differentiation of myeloid progenitor cells, thereby modulating the phagocytic and redox response may have a high therapeutic potential. Plant secondary metabolites from dietary herbs and spices have been shown to exert multiphasic effects on the immune response. For example, several studies have indicated increased protection against pathogenic challenge via increased phagocytosis and oxidative degradation while others indicate an anti-inflammatory and even immunosuppressive effect.[17,60] Additionally, although there is substantial evidence that natural products can affect the immune response, there is a relative lack of mechanistic and *in-vivo* data on the overall effect of natural products on the innate immunologic parameters making it difficult to appreciate the true functionality of these compounds.[17]

Dietary spices of the *Zingiberaceous* family, including turmeric and ginger, have exhibited immunostimulatory effects in several *in vitro* studies.[50,51,60,61] These spices contain complex mixtures of flavonoids, terpenes, alcohols, ketones, carotenoids and phytoestrogens. *Zingiberaceaous* spices have been used as antimicrobial, antifungal, insecticidal and antioxidant compounds for many years.[62,63] Recently, multiple studies have shown that treatment with curcumin, one of the major bioactive components of turmeric, has been shown to significantly increase phagocytosis and cell viability *in vitro*.[50,51,61,64,65] Additionally, Kim (2007) observed significant increases in not only

phagocytic activity but also respiratory burst, nitric oxide, TNF-α and inducible nitric oxide synthase (iNOS) in raw 264.7 macrophages treated with polysaccharides extracted from *Curcuma zedoaria*, also known as white turmeric, at concentrations of 5, 10, 30 and 50 μg/ml for 24 hours.[64]

1.3.1 Immunomodulation by root spices

Several studies have indicated a therapeutic role for turmeric (*Curcuma longa*) in the treatment of Alzheimer's disease.[50,51,65,66] Zhang (2006) demonstrated increased phagocytosis of Aβ-plaques following treatment with 0.1 μM curcumin in macrophages isolated from patients with Alzheimer's disease.[66] Fiala et al. (2007) further demonstrated increased expression of two important genes involved in pathogen recognition and phagocytosis, MGAT-3 and TLR-4, in macrophages isolated from patients with Alzheimer's disease following treatment with curcuminoids.[50] Curcumin was also shown to exert protective effects on cell viability. Pathak (2008) observed a significantly higher proportion of viable immune-challenged viable cells treated with curcumin and cadmium compared to control.[67]

Ginger (*Zingiber officinale*) has also been shown to significantly increase pathogen uptake and degradation in several studies.[60,68]Dugenci et al. (2003) observed significant increases in phagocytosis and extracellular respiratory burst in leukocytes isolated from rainbow trout treated with 1.0% aqueous ginger extract for three weeks.[60] In another study, a fractionated portion of ginger, 1-dehydrogingerdione, significantly increased phagocytosis in raw 264.7 macrophages.[68] Additionally, although supportive data is limited, metabolites in ginger may mediate differentiation of immune cells.[33,69]

Treatment with non volatile components of ginger including oleoresin, gingerol, shogaol and zingeron, increased the distribution of CD4+/CD8+ T-cell surface molecules in human lymphocytes in culture.[33] However, other studies have indicated an inhibitory role for ginger on the proliferation of T lymphocytes, and T helper cells in mice.[69] Zhou (2006) also observed decreases in secretion of IL-1α in macrophages following treatment with ginger.[69]

1.3.2 Immunomodulation by non-root spices

Cinnamon (*Cinnamomum zeylanicum*) which is native to Southern Asia and South America, has been studied for its potential health benefits most predominantly relating to prevention of type 2 diabetes through improved maintenance of blood glucose concentrations.[70,71]However, cinnamon is also a rich source of phytochemicals including phenolics, coumarins, and tannins which may confer antioxidant, anti-inflammatory, antimicrobial and antiviral activity.[71] Cinnamon has been shown to exert antibacterial activity against a broad range of pathogens including *Helicobacter pylori, Streptococcus oralis, Streptococcus anginosus, Streptococcus intermedius, Streptococcus sanguis, Enterobacter aerogenes* and *Micrococcus roseus.*[70] Cinnamon has also exhibited anti-fungal activity against *Aspergillus, Candida, Cryptococcus*, and *Histoplasma.*[72] Additionally, cinnamon has been implicated as an anti-viral agent.[70,73] In mice infected with influenza and treated with cinnamon, the overall survival rate was observed to be 4 times higher than in control mice.[73] However, the current state of knowledge of the effect of cinnamon on functions of the innate immune system specifically, is limited. Pepper (*Piper nigrum*) of the *Piperaceae* family has been used in traditional Indian and Chinese medicine for many years as an antimicrobial agent. *P. nigrum* was shown to effectively

inhibit the growth of *Pseudomonas aeruginosa, Salmonella typhi*, and *Staphylococcus aureus*.[74,75] Extract of pepper was also shown to exhibit immune stimulating activity in a study by Miwa et al. (1990).[6] In that study, treatment with aqueous pepper extract resulted in activation of macrophages in culture which was associated with increases in nitric oxide production and IL-1 secretion. Pathak (2008) also observed increased cell viability following treatment with pepper.[67]

1.3.3 Immunomodulation by *Lamiaceae* herbs

Herbs belonging to the *Lamiaceae* family, including rosemary (*Rosmarinus officinalis)* oregano (*Origanum vulgare*), basil (*Ocimum basilicum*), sage (*Salvia officinalis*) and thyme (*Thymus vulgaris*) are important culinary components of diets across the world and are also rich in bioactive phytochemicals with health promoting properties.[7,76-78] *Lamiaceae* herbs constitute natural sources of a variety of phenolic compounds such as flavonoids and phenolic acids that have been shown to exert antioxidant, anti-inflammatory, antimicrobial, antimutagenic and anticancer protection.[79,80] They have also been implicated for the prevention and management of chronic diseases of oxidative origin including cardiovascular disease, diabetes mellitus and cancer.[80]

Rosemary (*Rosmarinis officinalis*) is rich in antioxidant phenolic compounds and phenolic acids such as rosmarinic acid, caffeic acid, chlorogenic acid, and carnosolic acid as well as the terpenes, borneol, camphor, carophylene, cineol, humulene, linalool and thujone.[81,82,83] It is also a primary dietary source of flavonoids, tannins and essential oils. Traditionally, rosemary was used as an antispasmodic, anti-inflammatory and antidepressant. It was also implicated in the treatment of allergies and disorders of the

respiratory tract including asthma.[81,84,85] Additionally, rosemary was used therapeutically for the management of cataracts, cardiovascular disease and to promote reproductive health and fertility.[81] As an antimicrobial agent, rosemary is effective against a broad range of gram positive and gram negative bacteria including *Psuedomonas aeruginosa, Staphylococcus aureus*, *Stapylococcus epidermidis, Escherichia coli*, *Listeria monocytogenes, Listeria ivanovii, Listeria grayi, Listeria innocua.*[81,86-88] Juhas et al. (2009) recently demonstrated anti-inflammatory activity by essential oil constituents in rosemary. They observed significant reductions in TNF-α, IL-1β, IL-4, IL-5 in lymphocytes and monocytes of mice with trinitrobenzene sulfonic acid (TNBS) induced colitis treated with essential oils of rosemary.[89] However, there is a lack of further data investigating the impact of rosemary on physiological parameters of the innate immune response, especially through *in-vivo* analysis.

Thyme (*Thymus vulgaris*) is rich in antioxidant molecules which include phenolic compounds, flavonoids, essential oils, tannins and catechins.[28,90] Some of the most predominant phytochemicals in thyme include terpineol, anethole, apigenin, rosmarinic acid, myristic acid, palmitic acid, kaempferol, ferulic acid, caffeic acid, thymol, anethol, carvacrol, eugenol.[28,90] Thyme has traditionally been used as a natural remedy for a variety of applications including as an antibacterial and antitussive agent in cough drops and mouthwashes.[11,91] Current literature supports the antibacterial properties of thyme even against antibiotic resistant strains.[11] These properties of thyme are believed to be due, in part, to the presence of caffeic acid which has exhibited not only antibacterial activity but also antifungal and antiviral activity in multiple studies.[92-96] Additionally, the diverse range of antioxidant molecules present in thyme confers a high free radical

quenching ability.[90] Recently, the immunomodulatory effects of thyme have been investigated. Elhabazi et al. (2005) observed a proliferative effect of thyme on polymorphonuclear leukocytes as well as total lymphocytes, TCD4+, TCD8+ and natural killer cells (NK).[97] Moreover, essential oil components of thyme have been shown to exert both anti-inflammatory and immune stimulating activity in various studies.[98,99] Treatment with carvacrol resulted in suppression of cyclooxygenase-2 (COX-2) expression in macrophage-like U937 cells.[99] This effect was hypothesized to be a result of an agonistic effect of carvacrol on PPARα and PPARγ.[99] In another study, thymol promoted superoxide anion generation in neutrophils isolated from guinea pigs.[98]

Also of the *Lamiaceae* family, sage (*Salvia officinalis*) contains a variety of bioactive phytochemicals including phenolic compounds, flavonoids, essential oils, catechins and tannins that may confer important health benefits.[90] Predominant components include apigenin, β-sitosterol, caffeic acid, campestrol, camphene, carnosol, ferulic acid, gallic acid, rosmarinic acid, palmitic as well as the essential oils, thymol and uvaol.[90] Sage has traditionally been used to enhance cognitive performance and memory but more recently for the therapeutic management of neurodegenerative diseases such as Alzheimer's disease.[100-102] Apeginin, a flavonoid in sage, has specifically been shown to reduce amyloid-β plaques in neurons of patients with Alzheimer's disease.[100,101] The abundance of phenolic compounds in sage including rosmarinc acid, genkwanin and luteolin, for example, contribute to its high antioxidant capacity.[90] Additionally, components of sage such as α-pinene have been observed to exhibit anti-inflammatory activity by inhibiting eicosinoid synthesis via suppression of cyclo-oxygease-2 (COX-2) expression.[101,103]

Oregano (*Origanum vulgare*), has been used historically to improve circulation as an emmenagogue, for infections of the oral cavity, as a carminative for digestive health, and for the treatment of inflammatory disorders such as arthritis.[91,104] Its phytochemical profile includes a variety of antioxidant molecules including rosmarinic acid, camphene, carvacrol, gamma-terpinene, myrcene, terpinen and thymol, among others, which contribute to its high capacity to stabilize intracellular radicals.[90,82] The essential oils of oregano also exhibit antibacterial, antifungal and anthelmintic activity.[105] A recent study by Bukovska (2007) indicated immunomodulatory activity by a combination of oregano and thyme. Specifically, they observed significant decreases in the pro-inflammatory cytokines, IL-1β and IL-6 mRNA in colonic tissue of mice with colitis.[91]

Basil (*Basilicum ocimum)*, is also rich in plant secondary metabolites that may confer health benefits. It is a concentrated source of polyphenolic compounds and essential oils.[106-108] The high concentration of phenolic compounds in basil most likely contributes to the high antioxidant capacity of the herb.[10,108] Moreover, treatment with basil has been shown in recent studies to modulate the intracellular oxidant tone not only as an antioxidant itself, but by increasing the activity of detoxifying enzymes such as cytochrome p450 and glutathione *S*-transferase.[10] These effects may further contribute to the anti-mutagen and anti-carcinogen properties commonly associated with basil.[10]

1.4 Use of invertebrate models to study innate immunity

The use of invertebrate organisms as models to study the innate immune system has become increasingly popular.[109,110] The innate immune system is evolutionarily conserved among species and a significant amount of comparative data exists between

the immune systems of invertebrate and vertebrate species.[109,111] Thus, structural and functional parallels exist between the components of the innate immune system of invertebrate organisms, and those in mammalian organisms.[109,111] The use of *Lumbricus terrestris* has, therefore, emerged as an ideal model organism for studying the immune system and most recently to assess biomarkers for chemotoxicity *in vivo*.[109 112-115] Currently, the United States Environmental Protection Agency (EPA) uses *Lumbricus terrestris* as a model organism to detect potential ecological threats caused by chemical or metal toxicity at hazardous waste sites (2001).[116] Furthermore, the use of *L. terrestris* is economical as the cost of maintenance is minimal, socially uncontroversial, and experimental manipulation can be direct and rapid.[111] Additionally, the availability of expressed sequence tags for various genes in the earthworm genome has allowed scientists to use molecular tools to study transcriptional effects of various treatments.[117] We have recently adopted this model organism and further developed standardized protocols for growth medium, treatment application and extraction of immune cells, seminal vesicles and muscles to study their function.[118]

1.5 Methods and materials

3.5.1 Growth conditions and priming of *Lumbricus terrestris*[118]

Earthworms of the species *Lumbricus terrestris* were selected based on the presence of a fully developed clitellum indicative of sexual maturation. Worms were then washed in distilled water to ensure the skin was free from soil and debris. The earthworms were then transferred to petri plates containing *Lumbricus* Growth Medium (LGM) comprised of 1.25% agar, 0.31% Gerber single grain oatmeal (Nestle, S.A Vevey,

Switzerland) and incubated at 10°C for 48 hours to clear the digestive tract of soil. After worms were primed, the animals were selected for treatment. Prior to weighing, worms were gently massaged along the posterior length of the body to clear the gut of digestive contents.

3.5.2 Treatment of *L. terrestris*[118]

Worms with a consistent weight (± 1.5-2.0g), within a range of 4.5 to 6.5 g were selected for experimentation. Treatment plates contained concentrations of 0.1% w/v or 0.5% w/v of herb or spice in standard LGM. Spices tested include turmeric, ginger, cinnamon and pepper. Herbs used include oregano, basil, sage, thyme and rosemary. For each extract tested, thirty six earthworms were evenly divided into six groups (n=6) and allowed to feed *ad libitum* for 2, 4 and 6 days at 18-20°C in the dark. Treatment plates were renewed on the fourth day of treatment.

3.5.3 Ethanol extrusion of coelomocytes

Coelomocytes were isolated by ethanol extrusion.[119] Fecal contamination during extrusion was avoided by gently massaging one fourth of the posterior length of the worm to expel intestinal contents of the worm. Briefly, worms were rinsed in 20 mL Ca-LBSS (1.5 mM NaCl, 4.8 mM KCl, 1.1 mM MgSO4, 0.4 mM KH2PO4, 0.3 mM Na2HPO4, 4.2 mM NaHCO3, 3.8 mM CaCl2, and adjusted to pH 7.3 with HCl) in a beaker to remove contaminants. Cleansed worms were placed in a glass petri dish containing 3 mL extrusion medium (5% ethanol in saline, 23.5 mg/mL EDTA, 10 mg/mL guaiacol glycerol ether, and adjusted to pH 7.3 with HCl) for a total of 3 minutes at room temperature. Whole coelomic fluid was transferred from the petri dish to a 15 mL falcon

tube and worms were discarded. Nitric oxide analysis was performed using whole coelomic fluid and is described in detail below. Finally, 10 mL Ca-LBSS was transferred to the falcon tube followed by centrifugation at 150 X g for 15 minutes at 4°C. The pellet was then resuspended in 0.425 mL Ca-LBSS and stored on ice for further analysis.[120]

3.5.4 Total coelomocyte count assay

Total coelomocyte count was determined using an Improved Neubauer 1/400 sq. mm hemocytometer.[121] Briefly, 10 µl of resuspended coelomocytes in Ca-LBSS was transferred to the hemacytometer. Total coelomocytes were counted on two of the large outer squares and total coelomocyte count was estimated using the following formula: number of cells counted X 2 X 2500 X dilution factor = total coelomocytes.

3.5.5 Coelomocyte viability assay

Coelomocyte viability was determined by trypan blue exclusion as previously described elsewhere.[121] Briefly, resuspended coelomocytes were mixed at a ratio of 1:1 with 0.04% trypan blue (0.004 g/ 10 mM PBS) and vortexed. 20 µl of this mixture was transferred to a glass microscope slide with a cover slip. Live coelomocytes were determined by dye exclusion and reported as percent live coelomocytes per 50 total cells counted.

3.5.6 Differential coelomocyte count assay

Differential coelomocyte count was determined using a modified Wright-Giemsa blood smear technique and visualized using bright field microscopy (Volu-Sol, Inc).[122] To a clean microscope slide, 20 µl resuspended coelomocytes was smeared and heat fixed. The slides were stained by direct immersion in 15 mL Wright-Giemsa stain for a

total of 3.5 minutes. Slides were then washed briefly with 5 mL of 10 mM phosphate buffer saline (PBS) followed by brief rinsing with 5 mL DW. Slides were then gently dried by briefly holding over a low flame. Coelomocytes were analyzed for the neutrophil to basophil ratio via bright field microscopy. Total neutrophils and basophils were determined per 50 total coelomocytes counted and reported as a percentage of total coelomocytes. Neutrophils were detected by the presence of a prominent pink nucleus and pale pink cytoplasm upon staining. Basophils were visualized and determined by the presence of a dark purple, highly granular cytoplasm.

3.5.7 Phagocytosis assay

Phagocytosis was stimulated using a modified assay previously described by Adamowicz et al.[123] Briefly, 100 µl resuspended coelomocytes were incubated for 24 hours in 100 µl of a yeast/Congo red solution prepared as follows: 1 g yeast was added to 3 mL 0.87% Congo red in 10 mM PBS. 10 mL DW was then added and the solution was autoclaved. Finally, the yeast solution was diluted to 10^{-2} with Ca-LBSS. After 24 hours, the reaction mixture was centrifuged at 150 X g for 15 minutes at 4°C and the pellet was resuspended in 200 µl Ca-LBSS. 20 µl was transferred to a microscope slide for analysis. Phagocytosis was determined visually by the presence of engulfed Congo red stained yeast particles within the coelomocytes. Phagocytic activity was calculated as the number of phagocytic cells per 50 total cells counted.

3.5.8 Respiratory burst activity assay

Respiratory burst activity was indirectly determined as the absorbance of diformazan at 570 nm, formed by oxidation of Nitroblue Tetrazolium (NBT) during an

NBT reduction assay.[124] In brief, 100 µl resuspended coelomocytes was transferred to a microcentrifuge tube. Phagocytosis was stimulated by addition of 100 µl of a yeast solution prepared as follows (1 g yeast in 3 mL 10 mM Ca-LBSS incubated for 15 minutes at room temperature. 10 mL DW was then added and the solution was autoclaved. Finally, the yeast solution was diluted to 10^{-2} with Ca-LBSS). 50 µl NBT (1.5 mg/mL in Ca-LBSS) was transferred to the reaction mixture, which then incubated for 24 hours at 10°C, followed by centrifugation at 13,000 rpm for 10 minutes at 4°C. To the pellet, 120 µl of 2 M KOH and 140 µl DMSO was added to extract the pigment. Tubes were then re-centrifuged at 13,000 rpm for 10 minutes at 4°C to precipitate debris. 200 µl of the supernatant was measured spectrophotometrically at 570 nm in a 96-well microplate (Bioteck EL 808; Houston, TX).

1.5.9 Nitric oxide production assay

Nitric oxide production (NOx) was indirectly measured using a modified Griess diazotization assay for the detection of total nitrites/nitrates.[125] Briefly, 100 µl of whole coelomic fluid was transferred to a microplate followed by addition of 100 µl of vanadium chloride (0.08 g/10 mL 0.1 M HCl) and 100 µl Griess reagent. Alternatively, 50 µl sulfanilamide and 50 µl N-(1-Naphthyl) ethylendiamine dihydrochloride (NEDD) can be substituted for Griess reagent in the reaction. The microplate incubated for 30 minutes at 37°C and absorbance was measured at 540 nm using the Bioteck EL 808 (Houston, TX). The concentration of nitric oxide was determined by calculating the % change based on a linear standard curve: Conc (umol/L) = (A540-0.0344)/0.0057).

1.5.10 Statistical Analysis

Statistical significance was determined using a Student's one tailed t-test. Treatments were compared to control worms feeding on LGM only. Statistical significance was indicated by p values of <0.05.

1.6 Results

1.6.1 Effect of spices on total coelomocyte count

Total cell count (TCC) was estimated using an Improved Neubauer 1/400 sq. mm hemocytometer, as previously described. For control worms feeding on *Lumbricus* Growth Medium (LGM), comprised of 0.31% (w/v) Gerber oatmeal in 1.25% (w/v) agar, TCC was 2.31×10^6, 2.49×10^6 and 2.49×10^6 on day 2, 4 and 6, respectively. Treatment with turmeric over six days at concentrations of 0.1% (w/v) and 0.5% (w/v) resulted in significant increases in TCC compared to the control (Table 1). For worms fed 0.1% (w/v) turmeric, TCC was 4.89×10^6 (p=0.000), 2.98×10^6 (p=0.472) and 1.14×10^7 (p=0.017) for day 2, 4 and 6, respectively. Worms feeding on LGM infused with 0.5% (w/v) turmeric TCC was 3.88×10^6 (p=0.015), 7.29×10^6 (p=0.0028) and 7.67×10^6 (p=0.0059) on day 2, 4 and 6, respectively. The overall effect of treatment with turmeric was assessed to be significantly more effective at 0.5% (w/v) (p=0.015) compared to 0.1% (w/v) as indicated by determination of the area under the curve (AUC) for both concentrations (Figure 1).

Following treatment with ginger at 0.1% (w/v) and 0.5% (w/v), TCC significantly increased in all experimental groups compared to the control (Table 1). Greatest increases in TCC occurred after two days of treatment with 0.1% (w/v) ginger. TCC after treatment

with 0.1% (w/v) ginger was 8.12 x 10^6 (p=0.001), 6.93 x 10^6 (p=0.026), and 5.76 x 10^6 (p=0.031) for day 2, 4 and 6, respectively. For worms treated with 0.5% (w/v) ginger, TCC was 8.0 x 10^6, (p=0.034), 6.81 x 10^6 (p=0.000), 7.39 x 10^6 (p=0.000) for days 2, 4 and 6, respectively. Between the two concentrations of ginger, treatment with 0.5% (w/v) ginger resulted in the greatest net effect on TCC, although a significant difference was not detected (p=0.056) (Figure 1).

Upon consumption of LGM infused with 0.1% (w/v) cinnamon, TCC increased compared to control on day 4 and 6 of treatment with cell counts of 5.46 x 10^6 and 7.41 x 10^6, respectively (Table 1). However, TCC was lower than the control following treatment with 0.5% (w/v) cinnamon on day 6 and significantly lower on day 2 (p=0.004). Overall, treatment with 0.1% (w/v) cinnamon resulted in significantly higher TCC compared to 0.5% (w/v) (p=0.002) (Figure 2).

Following treatment with pepper at 0.1% (w/v), TCC increased over the control on day 2, 4 and 6 with counts of 2.53 X 10^6, 3.07 X 10^6, and 6.68 X 10^6, respectively (Table 1). Significant decreases in TCC, however, occurred on day 2 at 0.5% (w/v) pepper (p=0.001). Between the two concentrations of pepper, treatment with 0.1% (w/v) resulted in significantly more total cells than 0.5% (p=0.002) as evidenced by AUC values (Figure 1).

Table 1. Effect of spices on total coelomocyte count (TCC) in *L. terrestris*. TCC was estimated using an Improved Neubauer 1/400 sq. mm hemocytometer and expressed as total cells per millileter. *Indicates significantly higher than control. **Indicates significantly lower than control. $p<0.05$. n=6.

Spice	Total Coelomocyte Count n=6						
	Conc. *(w v)*	Day 2 *(cells ml)*	*P value*	Day 4 *(cells ml)*	*P value*	Day 6 *(cells ml)*	*P value*
Turmeric	0.10%	4.89×10^{6}	0.000*	2.98×10^{6}	0.472	1.14×10^{7}	0.01?*
	0.50%	3.88×10^{6}	0.015*	7.29×10^{6}	0.000*	7.67×10^{6}	0.005*
Ginger	0.10%	8.12×10^{6}	0.001*	6.93×10^{6}	0.026*	5.76×10^{6}	0.031*
	0.50%	8.00×10^{6}	0.034*	6.81×10^{6}	0.000*	7.39×10^{6}	0.000*
Cinnamon	0.10%	2.71×10^{6}	0.320	5.46×10^{6}	0.012*	7.41×10^{6}	0.029*
	0.50%	9.33×10^{5}	0.004**	2.73×10^{6}	0.488	2.18×10^{6}	0.403
Pepper	0.10%	2.53×10^{6}	0.121	3.07×10^{6}	0.460	6.68×10^{6}	0.084
	0.50%	3.36×10^{5}	0.001**	2.13×10^{6}	0.49?	1.50×10^{5}	---
Control	---	2.31×10^{6}	---	2.49×10^{6}	---	2.49×10^{6}	---

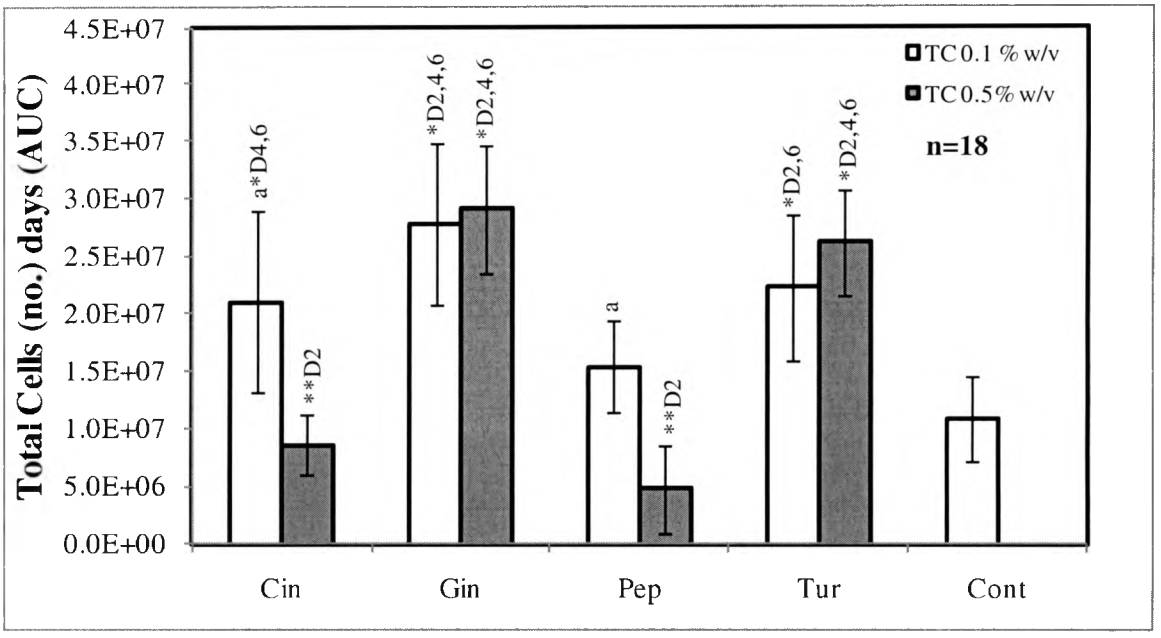

Figure 1. Overall effect of spices on total coelomocyte count (TCC) in *L. terrestris*. Overall TCC for 0.1% (w/v) and 0.5% (w/v) of each spice, calculated as area under the curve (AUC) and reported as total number of coelomocytes over the duration of the experiment. The data is represented as means ± SEM. a- indicates the treatment concentration which was significantly different ($p<0.05$). *- indicates significant difference from the control ($p<0.05$). D2= day 2; D4= day 4; D6= day6 – indicates days when treatment was significantly different from control ($p<0.05$). n=18.

1.6.2 Effect of spices on coelomocyte viability

Coelomocyte viability (CV) was determined by trypan blue exclusion assay as described above and reported as the percentage of unstained cells per 50 coelomocytes counted. For control worms feeding on LGM only, CV was 46.7%, 47.3% and 50.0% on day 2, 4 and 6, respectively (Table 2). However, CV significantly increased following treatment with turmeric at 0.1% (w/v) and 0.5% (w/v) on all days excluding day 2 at 0.1% (w/v). LGM prepared with 0.1% (w/v) turmeric resulted in 53.3% (p=0.120), 73.3% (p=0.000), and 67.3% (p=0.002) CV on day 2, 4 and 6, respectively. For worms fed 0.5% (w/v) turmeric, CV was 60.3% on day 2 (p=0.0004), 63% day 4 (p=0.0064), and 60.0% day 6 (p=0.0066) (Table 2). Assessment of the area under the curve for both

concentrations of turmeric indicated 0.1% (w/v) to be significantly more effective at increasing CV than 0.5% (w/v) (p=0.030) (Figure 2).

Treatment with ginger resulted in significantly higher percentages of viable coelomocytes in all treatment groups at both concentrations compared to the control (Table 2). For worms feeding on 0.1% (w/v) ginger, CV was 63.7% (p=0.000), 65.3% (p=0.023), and 61.7% (p=0.004) on day 2, day 4, and day 6, respectively. CV following treatment with 0.5% (w/v) ginger, was 60.3% (p=0.008), 68.7% (p=0.000) and 62.7% (p=0.000) on day 2, day 4 and day 6, respectively. Analysis of AUC values indicated viability of coelomocytes to be higher in worms consuming 0.5% (w/v) ginger compared worms consuming 0.1% (w/v) (p=0.148) (Figure 2).

CV significantly decreased compared to the control in response to treatment with cinnamon in all experimental groups (Table 2). On day 2 at both concentrations of cinnamon, viability of coelomocytes decreased by approximately 38% compared to the control. Specifically, for 0.1% (w/v) cinnamon in LGM, 28.3% (p=0.000), 39.0% (p=0.005) and 32.3% (p=0.000) of coelomocytes were viable on day 2, 4, and 6, respectively. Viability of cells after treatment with 0.5% (w/v) cinnamon in LGM was 29.0% (p=0.018), 41.7% (p=0.011) and 37.0% (p=0.011) on day 2, day 4, and day 6, respectively. The overall effect of cinnamon on CV over the duration of the experiment was significantly higher following treatment with 0.5% (w/v) cinnamon (p=0.033) (Figure 2).

Among all of the spices tested, treatment with pepper resulted in the greatest decreases in CV. Significantly less coelomocytes were viable on day 2 at 0.1% (w/v)

(p=0.000) and 0.5% (w/v) (p=0.029) as well as on day 4 at 0.1% (w/v) (p=0.000) (Table 2). Compared to control, ~24% fewer coelomocytes were viable on day 2 at 0.1% (w/v) pepper and 40% fewer on day 6 at 0.5% (w/v) pepper. Between the two concentrations, treatment with LGM prepared with 0.1% (w/v) pepper resulted in higher CV overall as indicated by AUC values (p=0.038) (Figure 2).

Table 2. Effect of spices on coelomocyte viability (CV) in *L. terrestris*. CV, measured by trypan blue exclusion, and reported as percent live coelomocytes per 50 total cells counted. *Indicates significantly higher than control. **Indicates significantly lower than control. p<0.05. n=6.

Spice	Coelomocyte Viability n=6						
	Conc. (w/v)	Day 2 (%)	*P Value*	Day 4 (%)	*P Value*	Day 6 (%)	*P Value*
Turmeric	0.10%	53.3	0.120	73.3	0.000*	67.3	0.002*
	0.50%	60.3	0.000*	63.0	0.006*	60.0	0.006*
Ginger	0.10%	63.7	0.000*	65.3	0.023*	61.7	0.004*
	0.50%	60.3	0.008*	68.7	0.000*	62.7	0.000*
Cinnamon	0.10%	28.3	0.000**	39.0	0.005**	32.3	0.000**
	0.50%	29.0	0.018**	41.7	0.011**	37.0	0.011**
Pepper	0.10%	33.3	0.000**	35.0	0.000**	45.3	0.5
	0.50%	35.3	0.029**	37.0	0.019**	30.0	---
Control	---	46.7	---	47.3	---	50.0	---

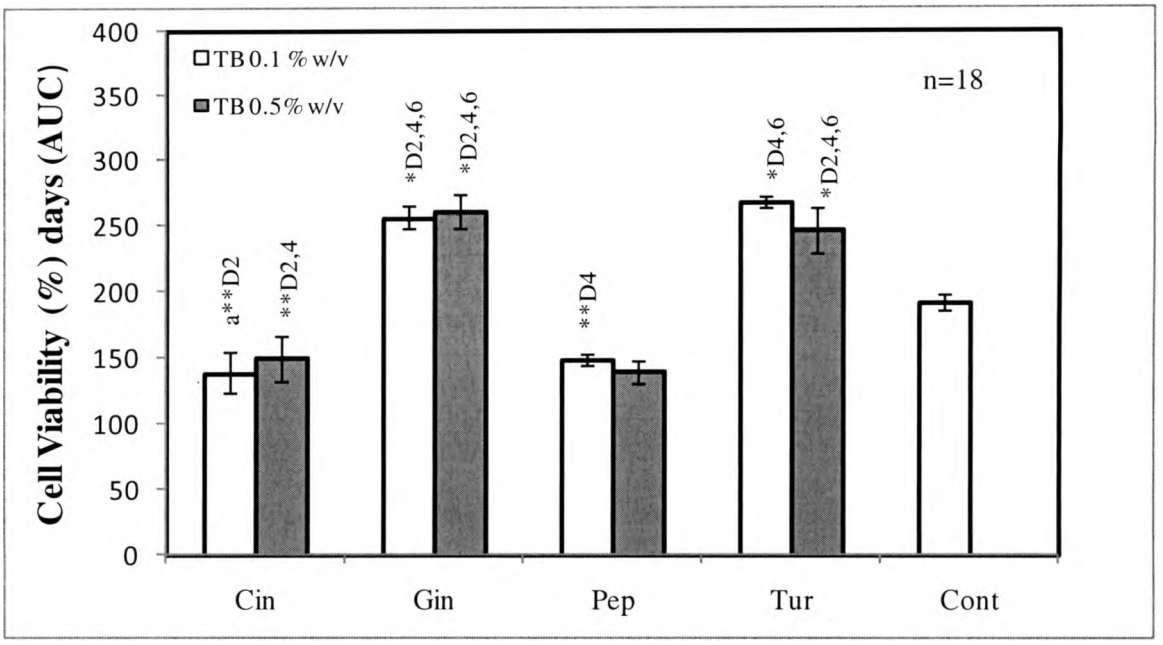

Figure 2. Overall effect of spices on coelomocyte viability in *L. terrestris*. Overall CV at 0.1% (w/v) and 0.5% (w/v) of each spice, calculated as area under the curve, and reported as % viable cells. The data is represented as means ± SEM. a- indicates the treatment concentration which was significantly different ($p<0.05$). *- indicates significant difference from the control ($p<0.05$). D2= day 2; D4= day 4; D6= day6 – indicates days when treatment was significantly different from control ($p<0.05$). n=18.

1.6.3 Effect of spices on relative neutrophil count

Assessment of the distribution of coelomocyte type per 50 total cells counted was carried out by differential cell count technique using Wright's Giemsa stain and visualized under light microscopy. For control worms feeding on LGM only, the relative neutrophil count (RNC) was 37.7%, 36.9% and 34.7% on day 2, 4 and 6, respectively (Table 3). The RNC was significantly higher following treatment with LGM supplemented with turmeric at 0.1% (w/v) and 0.5% (w/v) excluding on day 2 at 0.1% (w/v) (Table 3). The greatest increases in the RNC occurred on day 2 with 0.5% (w/v) turmeric in which a 47.7% increase over the control was observed. Following treatment with 0.1% (w/v) turmeric, 44.7% (p=0.133), 50.3% (p=0.003), and 50.3% (p=0.000) of

coelomocytes counted were identified as neutrophils on day 2, 4 and 6, respectively. In response to treatment with 0.5% (w/v) turmeric RNC were 55.7% (p=0.0018), 49.35% (p=0.0036) and 42.7% (p=0.0319) on day 2, 4 and 6, respectively, which were all significantly higher than control (Table 3). The net effect on RNC between concentrations of turmeric was assessed to be greater following treatment with 0.5% (w/v) as evidenced by AUC values (p=0.368) (Figure 3).

When worms were fed ginger at 0.1% (w/v) and 0.5% (w/v), significant increases in the RNC was observed. With the exception of day 2 at 0.5% (w/v) ginger, the percentage of neutrophils was significantly increased on all days at both concentrations (Table 3). The RNC following treatment with 0.1% (w/v) ginger was 42% (p=0.0176), 46% (p=0.0011) and 39.7% (p=0.0026) on day 2, 4 and 6, respectively. In comparison, 41% (p=0.070), 44% (p=0.0004), and 44% (p=0.0004) of total cells counted were identified as neutrophils on day 2, 4 and 6, respectively, after treatment with 0.5% (w/v) ginger. Although minimal, treatment with 0.1% (w/v) ginger was slightly more effective at increasing the RNC (p=0.65) (Figure 3).

The neutrophil to basophil ratio was not increased upon treatment with cinnamon in any of the treatment groups (Table 3). In fact, the RNC significantly decreased compared to control on day 2 at both concentrations as well as on day 4 at 0.5% (w/v) cinnamon. For worms treated with 0.1% (w/v) cinnamon, the RNC was 23.0% (p=0.000), 34.3% (p=0.090), and 32.3% (p=0.159) on day 2, 4 and 6, respectively. For 0.5% (w/v), the coelomocyte distribution was 29.2% (p=0.001), 27.35% (p=0.008) and 23.6% (p=0.067) neutrophils on day 2, 4 and 6, respectively. Treatment with 0.1% (w/v)

cinnamon resulted in significantly higher proportions of neutrophils over the course of the experiment compared to 0.5% (w/v) of the spice (p=0.017) (Figure 3).

In response to treatment with pepper, the RNC was increased on day 2 and 6 at 0.1% (w/v) of the spice with neutrophil percentages of 40.7% (p=0.404) and 36.0% (p=0.085), respectively (Table 3). However, the RNC was significantly decreased on day 4 at 0.1% (w/v) pepper (p=0.030) and 0.5% (w/v) pepper (p=0.008). Assessment of the overall effect of pepper on the distribution of neutrophils between concentrations indicated 0.1% (w/v) pepper to be significantly more effective (p=0.005) (Figure 3).

Table 3. Effect of spices on relative neutrophil count (RNC) in *L. terrestris*. The distribution of cell type, determined by differential staining using Wright's stain, and expressed as the percent neutrophils per 50 total cells counted. *Indicates significantly higher than control values. **Indicates significantly lower than control values. p<0.05. n=6.

Spice	Relative Neutrophil Count n=6						
	Conc. (w/v)	Day 2 (%)	*P Value*	Day 4 (%)	*P Value*	Day 6 (%)	*P Value*
Turmeric	0.10%	44.7	0.133	50.3	0.003*	50.3	0.000*
	0.50%	55.7	0.001*	49.3	0.031*	42.7	0.036*
Ginger	0.10%	42.0	0.017*	46.0	0.001*	39.7	0.002*
	0.50%	41.0	0.070*	44.0	0.000*	44.0	0.000*
Cinnamon	0.10%	23.0	0.000**	34.3	0.09	32.3	0.159
	0.50%	29.2	0.001**	27.3	0.008**	23.6	0.067
Pepper	0.10%	40.7	0.404	30.0	0.030**	36.0	0.062
	0.50%	26.5	0.085	14.0	0.008**	28.0	---
Control	---	37.7	---	36.9	---	34.7	---

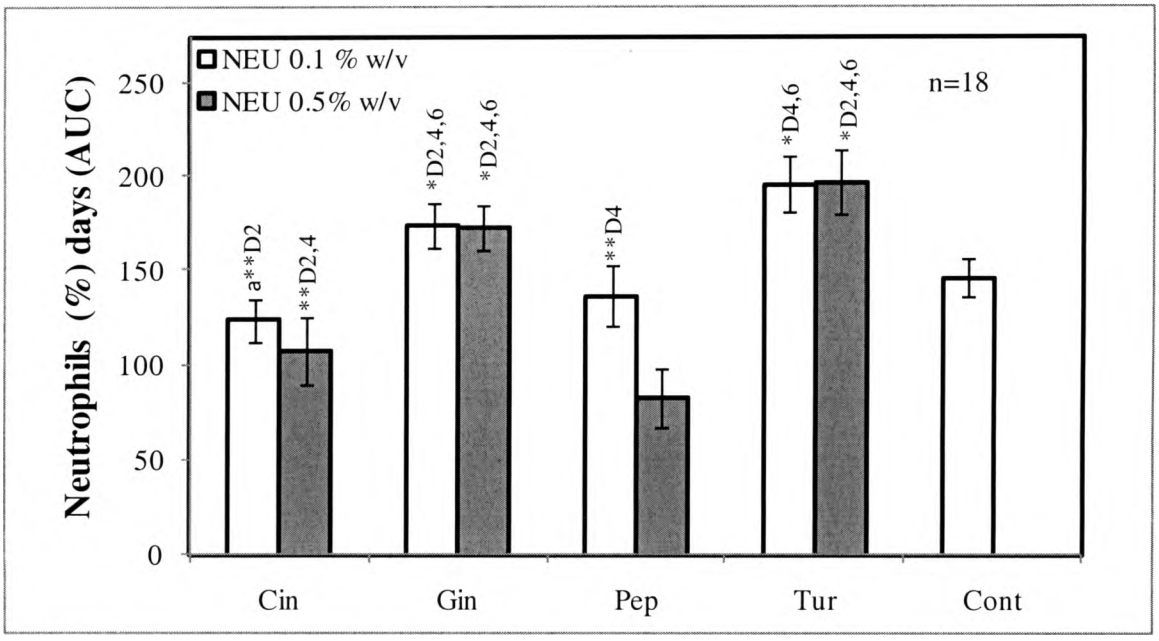

Figure 3. Overall effect of spices on relative neutrophil count (RNC) in *L. terrestris*. Overall RNC for 0.1% (w/v) and 0.5% (w/v) of spice calculated as area under the curve (AUC) and expressed as percent neutrophils. The data is represented as means ± SEM. a- indicates the treatment concentration which was significantly different ($p<0.05$). *- indicates significant difference from the control ($p<0.05$). D2= day 2; D4= day 4; D6= day6 – indicates days when treatment was significantly different from control ($p<0.05$). n=18.

1.6.4 Effect of spices on phagocytic activity

Phagocytic activity (PA) was determined by counting the number of coelomocytes containing at least one engulfed Congo-red stained *Saccharomyces cereviseae* per 50 cells counted, as described above. Basal PA for control worms was relatively consistent over the duration of the experiment with 53.1%, 56.4% and 56.1% of total cells identified as actively phagocytosing coelomocytes on day 2, 4 and 6, respectively (Table 4). Worms treated with turmeric at 0.1% (w/v) and 0.5% (w/v) exhibited higher PA on all days (Table 4). For 0.1% (w/v) turmeric, PA was 66.3% (p=0.000), 71.7% (p=0.000), and 70.3% (p=0.001). At 0.5% (w/v) turmeric, 66.3%

(p=0.017), 70.7% (p=0.000) and 78.7% (p=0.000) of total cells counted had at least one engulfed yeast particle on day 2, 4, and 6, respectively. Determination of the area under the curve for both concentrations indicated that 0.5% (w/v) turmeric resulted in greater increases in PA of coelomocytes (w/v) (Figure 4).

Upon treatment with ginger, PA significantly increased in all experimental groups (Table 4). PA for worms consuming 0.1% (w/v) ginger in LGM was 68.7% (p=0.005), 66.3% (p=0.000), and 58.0% (p=0.034) for day 2, 4 and 6, respectively. For worms feeding on 0.5% (w/v) ginger, PA was 71.3% (p=0.002), 74.3% (p=0.000), and 65.0% (p=0.001) on day 2, 4 and 6, respectively (Table 4). Overall, 0.5% (w/v) ginger was significantly more effective at increasing PA compared to 0.1% (w/v) (p=0.026) (Figure 4).

Treatment with cinnamon at 0.1% (w/v) and 0.5% (w/v) resulted in diminished PA of coelomocytes throughout the duration of the six day experiment (Table 4). Significant decreases in activity occurred on day 2 at both concentrations as well as on day 6 at 0.5% (w/v) cinnamon. PA was 28.0% (p=0.001), 51.7% (p=0.365) and 55.3% (p=0.222) after treatment with 0.1% (w/v) cinnamon on day 2, 4 and 6, respectively. Treatment with 0.5% (w/v) cinnamon resulted in 39.0% (p=0.003), 51.7% (p=0.090) and 47.7% (p=0.015) of cells identified as actively phagocytosing coelomocytes on day 2, 4 and 6, respectively. AUC analysis indicated slightly higher PA overall following treatment with 0.5% (w/v) cinnamon (p=0.141) (Figure 4).

PA was significantly increased compared to control after treatment with pepper at 0.1% (w/v) on day 2 (p=0.014) in which 55% of coelomocytes were identified as actively

phagocytosing *S. cerevisiae* (Table 4). However, PA decreased on day 2 in worms consuming 0.5% (w/v) pepper (p=0.125) as only 21% of coelomocytes counted were determined to have at least one engulfed *S. cereviseae*. Additionally, significant decreases in PA occurred on day 4 at 0.1% (w/v) pepper (p=0.037) and 0.5% (w/v) pepper (p=0.005) with PA values of 36% and 21.6%, respectively. Overall, treatment with 0.1% (w/v) pepper resulted in significantly higher PA compared to treatment with 0.5% (w/v) (p=0.004) (Figure 4).

Table 4. Effect of spices on phagocytic activity (PA) of coelomocytes in *L. terrestris*. PA of coelomocytes, determined by a counting the number of cells containing one more *S. cereviseae* particles per 50 total cells and reported as percent phagocytic cells. *Indicates significantly higher than control. **Indicates significantly lower than control. p<0.05. n=6.

Spice	Phagocytic Activity n=6						
	Conc. (w/v)	Day 2 (%)	*P* Value	Day 4 (%)	*P* Value	Day 6 (%)	*P* Value
Turmeric	0.10%	66.3	0.000*	71.7	0.000*	70.3	0.001*
	0.50%	66.3	0.017*	70.7	0.000*	78.7	0.000*
Ginger	0.10%	68.7	0.005*	66.3	0.000*	58.0	0.034*
	0.50%	71.3	0.002*	74.3	0.000*	65.0	0.001*
Cinnamon	0.10%	28.0	0.001**	51.7	0.365	55.3	0.222
	0.50%	39.0	0.003**	51.7	0.09	47.7	0.015**
Pepper	0.10%	55.0	0.014*	36.0	0.037**	60.7	---
	0.50%	21.0	0.125	21.6	0.005**	40.0	---
Control	---	53.1	---	56.4	---	56.1	---

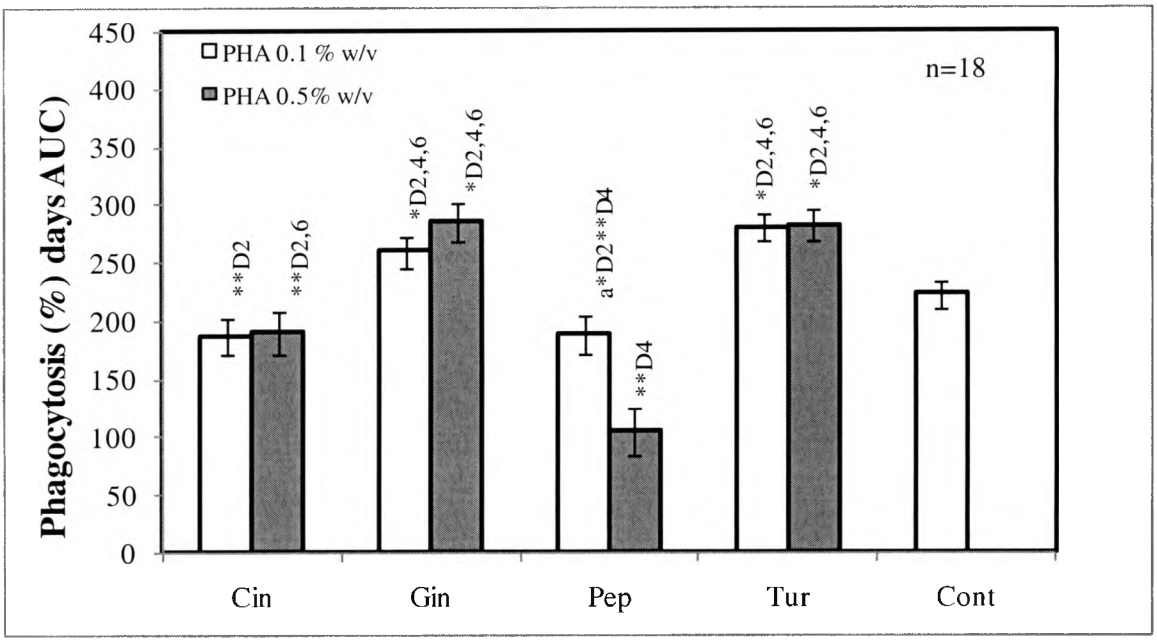

Figure 4. Overall effect of spices on total phagocytic activity (PA) in *L. terrestris*. Overall phagocytic activity at 0.1% (w/v) and 0.5% (w/v) of each spice calculated as area under the curve (AUC) and expressed as percent total activity over the duration of the experiment. The data is represented as means ± SEM. a- indicates the treatment concentration which was significantly different ($p<0.05$).*- indicates significant difference from the control ($p<0.05$). D2= day 2; D4= day 4; D6= day6 – indicates days when treatment was significantly different from control ($p<0.05$). n=18.

1.6.5 Effect of spices on respiratory burst activity

Respiratory burst (RB) in coelomocytes was indirectly measured as the absorbance of diformazan at 570 nm formed by oxidation of Nitroblue Tetrazolium (NBT) reduction assay as previously described above. For control worms, the A_{570} of diformazan was 1.05 AU, 1.02 AU, and 1.00 AU on day 2, 4 and 6, respectively (Table 5). Upon treatment with turmeric at 0.1% (w/v) and 0.5% (w/v), RB activity significantly increased on all days excluding day 4 at 0.1% (w/v) (Table 5). For worms consuming 0.1% (w/v) turmeric, the A_{570} of diformazan was 2.2 AU (p=0.035), 1.2 AU (p=0.051) and 1.6 AU (p=0.007) for day 2, 4 and 6, respectively. For worms feeding on 0.5% (w/v)

turmeric, the A_{570} of diformazan was 1.4 AU (p=0.002), 1.3 AU (p=0.036) and 1.8 AU (p=0.000). Between concentrations of turmeric, treatment with 0.1% (w/v) resulted in greater overall increases in RB activity as indicated by AUC values (p=0.053) (Figure 5).

When worms were treated with LGM prepared with ginger at 0.1% (w/v) and 0.5% (w/v), RB activity increased on all days compared to the control (Table 5). Significant increases occurred on the day 4 of treatment with 0.1% (w/v) ginger (p=0.000), as well as on day 6 of treatment with ginger at 0.1% (w/v) (p=0.001) and 0.5% (w/v) (p=0.000). The greatest increases in RB occurred on day 6 as the A_{570} of diformazan was 1.4 AU for both 0.1% (w/v) and 0.5% (w/v) ginger. Assessment of the total effects of ginger on RB indicated 0.1% (w/v) ginger to be slightly more effective (Figure 5).

RB significantly decreased following treatment with cinnamon in all treatment groups compared to control (Table 5). For 0.1% (w/v) cinnamon, the A_{570} of diformazan was 0.9 AU (p=0.009), 0.5 AU (p=0.000) and 0.9 AU (p=0.005) for day 2, 4 and 6 respectively. When 0.5% (w/v) cinnamon was consumed by the worms, the A_{570} of diformazan was 0.7 AU (p=0.011), 0.3 AU (p=0.000) and 0.7 AU (p=0.027) on day 2, 4 and 6, respectively. AUC values indicated RB activity of coelomocytes to be significantly greater overall in response to treatment with 0.1% (w/v) cinnamon compared to 0.5% (w/v) cinnamon (p=0.007) (Figure 5).

Treatment with pepper also resulted in significantly decreased RB activity throughout the duration of the experiment at both concentrations (Table 5). For 0.1% (w/v) pepper, the A_{570} of diformazan was 0.4 AU (p=0.0007), 0.3 AU (p=0.000), and -0.3

AU (p=0.016) on day 2, 4 and 6, respectively. At the higher concentration of 0.5% (w/v) pepper, RB activity was even lower and the A_{570} of diformazan was -0.1 AU (p=0.000), 0.2 AU (p=0.000) and -0.1 AU, on days 2, 4 and 6, respectively. Overall, worms feeding on LGM supplemented with 0.1% (w/v) pepper had higher RB activity than those treated with 0.5% (w/v) pepper over the six day experiment (p=0.002) (Figure 5).

Table 5. Effect of spices on respiratory burst activity (RB) of coelomocytes in *L. terrestris*. RB activity, indirectly measured as absorbance of diformazan formed during NBT reduction assay, and expressed in absorbance units (AU) at 570 nm. *Indicates significantly higher than control. **Indicates significantly lower than control. p<0.05. n=6.

Spice	Respiratory Burst Activity n=6						
	Conc. (w/v)	Day 2 (AU)	*P* Value	Day 4 (AU)	*P* Value	Day 6 (AU)	*P* Value
Turmeric	0.10%	2.20	0.035*	1.20	0.051	1.60	0.007*
	0.50%	1.40	0.002*	1.30	0.036*	1.80	0.000*
Ginger	0.10%	1.20	0.083	1.20	0.000*	1.40	0.001*
	0.50%	1.30	0.066	1.10	0.238	1.40	0.000*
Cinnamon	0.10%	0.90	0.009**	0.50	0.000**	0.90	0.005**
	0.50%	0.70	0.011**	0.30	0.000**	0.70	0.027**
Pepper	0.10%	0.40	0.000**	0.30	0.000**	-0.30	0.016**
	0.50%	-0.10	0.000**	0.20	0.000**	-0.10	---
Control	---	1.05	---	1.02	---	1.00	---

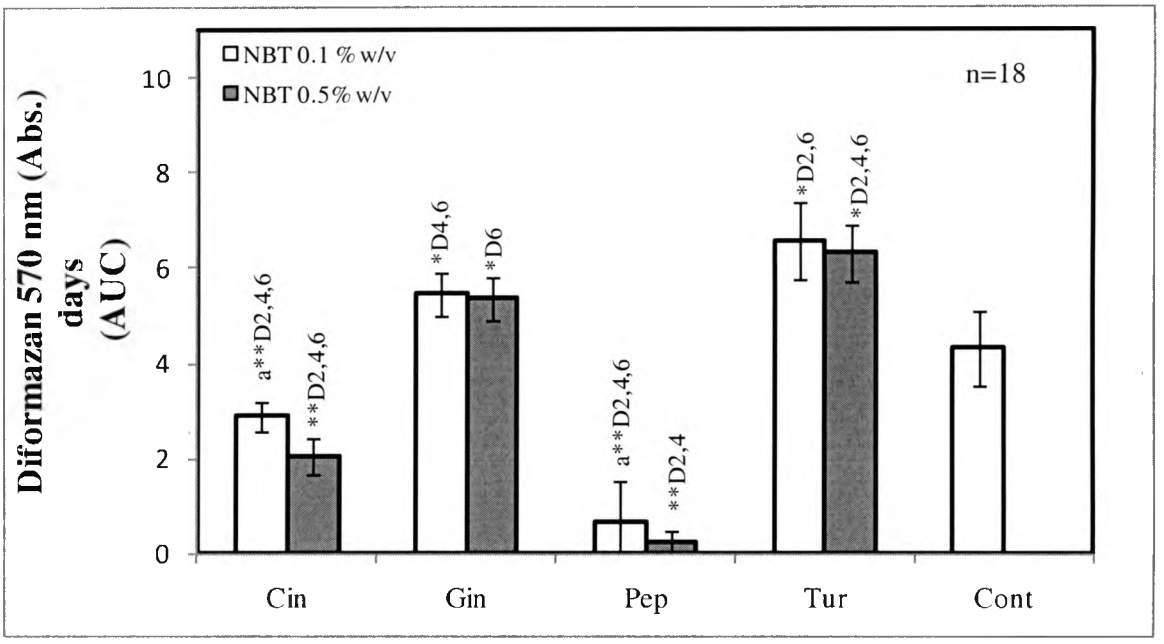

Figure 5. Overall effect of spices on respiratory burst activity (RB) in *L. terrestris*. Overall RB activity for 0.1% (w/v) and 0.5% (w/v) of each spice, calculated as area under the curve (AUC) and expressed as total absorbance of diformazan at 570 nm over the duration of the experiment. a- indicates the treatment concentration which was significantly different ($p<0.05$).*- indicates significant difference from the control ($p<0.05$). D2= day 2; D4= day 4; D6= day6 – indicates days when treatment was significantly different from control ($p<0.05$). n=18.

1.6.6 Effect of spices on nitric oxide production

Nitric oxide production (NOx) was indirectly quantified as total nitrites/nitrates via Griess assay as described above. NOx was 27.7 μM, 28.8 μM and 27.1 μM for control worms feeding on LGM alone on day 2, 4 and 6, respectively (Table 6). Following treatment with turmeric, NOx was significantly higher than control on day 2 at both concentrations with values of 57.7 μM (p=0.000) and 34.6 μM (p=0.002) at 0.1% (w/v) and 0.5% (w/v), respectively (Table 6). Additionally, NOx was significantly higher than control on day 6 at 0.1% (w/v) turmeric with a value of 35.2 μM (p=0.009).

Evaluation of the overall effect of treatment with turmeric on NOx production indicated 0.1% (w/v) turmeric to be more effective compared to 0.5% (w/v) (Figure 6).

Following treatment with ginger, an initial significant increase in NOx was observed in worms consuming LGM prepared with 0.1% (w/v) ginger (Table 6). In this group, 76.1 μM nitric oxide was detected, which was ~175% higher than the control. NOx production was 21.3 μM on day 4 of treatment with 0.1% (w/v) ginger and 36.1 μM on the day 6 treatment with 0.1% (w/v) ginger, which was significantly higher than the control (p=0.034). For worms treated with 0.5% (w/v) ginger, a significant increase in NOx was observed on day 6 in which 38.9 μM NOx was detected (p=0.021). On day 2 and 4 of the treatment, nitric oxide was 37.0 μM, and 25.7 μM, respectively. The net effect of ginger on NOx was determined to be significantly greater in worms treated with 0.1% (w/v) ginger than in worms treated with 0.5% (w/v)(p=0.012) (Figure 6).

NOx production was reduced following treatment with cinnamon at 0.1% (w/v) and 0.5% (w/v) and significantly decreased on the fourth day at both concentrations (Table 6). On day 4, NOx was 11.9 μM (p=0.001) and 7.1 μM (p=0.001) for 0.1% (w/v) and 0.5% (w/v) cinnamon, respectively. However, day 6 of the treatment with 0.1% (w/v) cinnamon 50.3 μM NOx was detected, which amounted to an increase of 85.6% over the control. Overall, worms consuming LGM prepared with 0.1% cinnamon had significantly higher levels of NOx than those consuming LGM with 0.5% (w/v) cinnamon (p=0.002) (Figure 6).

Total NOx decreased in all experimental groups consuming LGM supplemented with 0.1% (w/v) and 0.5% (w/v) pepper (Table 6). For 0.1% (w/v) pepper, NOx was 14.9

μM, 12.9 μM and 1.8 μM on day 2, 4 and 6, respectively. Assessment of the AUC for both concentrations of pepper indicated total NOx to be significantly lower following treatment with 0.5% (w/v) compared 0.1% (w/v) (p=0.000) (Figure 6).

Table 6. Effect of spices on nitric oxide production (NOx) in coelomic fluid of *L. terrestris*. NOx, measured indirectly as total nitrites/nitrates via Griess assay, and reported as μM NOx.*Indicates significantly higher than control. **Indicates significantly lower than control. p<0.05. n=6.

Spice	Nitric Oxide Production n=6						
	Conc. *(w/v)*	Day 2 *(µM)*	*P Value*	Day 4 *(µM)*	*P Value*	Day 6 *(µM)*	*P Value*
Turmeric	0.10%	57.7	0.000*	26.5	0.443	35.2	0.009*
	0.50%	34.6	0.002*	----	----	----	----
Ginger	0.10%	76.1	0.004*	21.3	0.157	36.1	0.034*
	0.50%	37	0.234	25.7	0.391	38.9	0.021*
Cinnamon	0.10%	12.5	0.088	11.9	0.001**	50.3	0.162
	0.50%	6.8	0.284	7.1	0.001**	11.7	0.061
Pepper	0.10%	14.9	0.000**	12.9	0.364	1.8	0.169
	0.50%	0.9	0.058	7.3	----	-30.47	---
Control	---	27.7	---	28.8	---	27.1	---

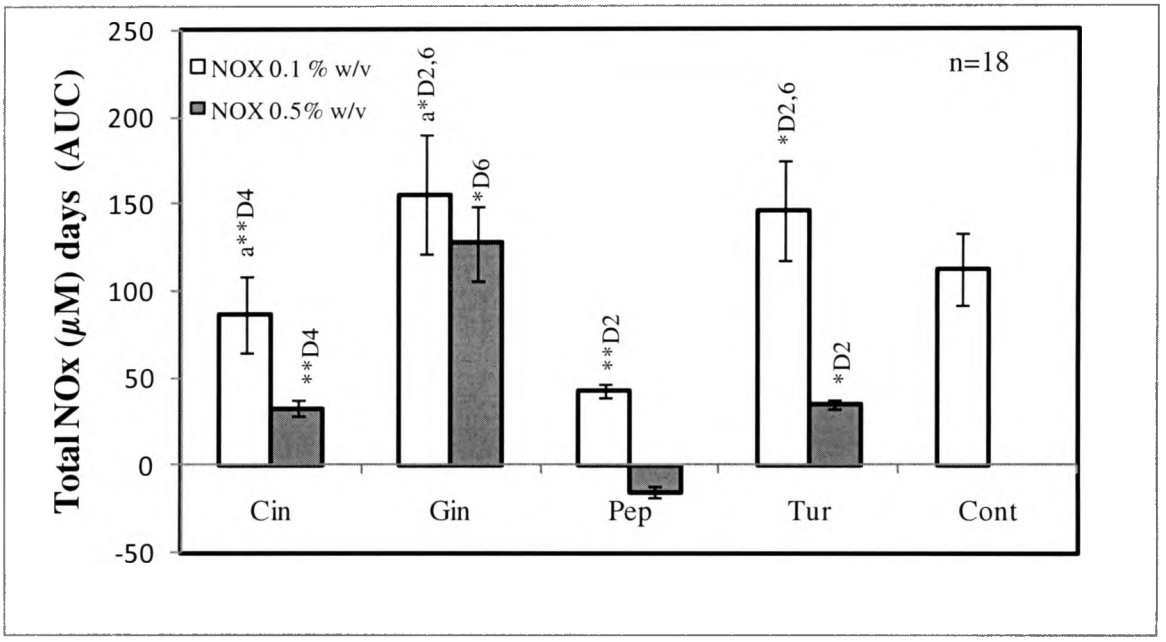

Figure 6. Overall effect of spices on nitric oxide (NOx) production in *L. terrestris*. Total nitric oxide production (NOx) for 0.1% (w/v) and 0.5% (w/v) of each spice, calculated as AUC and reported as μM of total NOx over the duration of the experiment. The data is represented as means $\pm$ SEM. a- indicates the treatment concentration which was significantly different ($p<0.05$).*- indicates significant difference from the control ($p<0.05$). D2= day 2; D4= day 4; D6= day6 – indicates days when treatment was significantly different from control ($p<0.05$). n=18.

1.6.7 Effect of herbs on total coelomocyte count

Total coelomocyte count (TCC) was estimated using an Improved Neubauer 1/400 sq. mm hemocytometer, as previously described. For control worms feeding on *Lumbricus* Growth Medium (LGM), comprised of 0.3% (w/v) Gerber oatmeal in 1.25% agar, TCC was 2.31 x 10^6, 2.49 x 10^6 and 2.49 x 10^6 on day 2, 4 and 6, respectively (Table 7). Significant increases in TCC was observed following treatment with LGM supplemented with 0.1% (w/v) and 0.5% (w/v) oregano throughout the duration of the six day experiment (Table 7). For worms consuming 0.1% (w/v) oregano, TCC was 3.78 x 10^6 (p=0.026), 6.88 x 10^6 (p=0.002), and 9.95 x 10^7 (p=0.020) on day 2, 4 and 6, respectively. Following treatment with 0.5% (w/v) oregano, TCC was 5.96 x 10^6

(p=0.005), 4.42 x 10^6 (p=0.000) and 3.22 x 10^6 (p=0.029) for day 2, 4 and 6, respectively. Analysis of the area under the curve (AUC) for both experimental concentrations of oregano indicated TCC to be significantly higher following treatment with 0.1% (w/v) (p=0.006) (Figure 7).

Upon treatment with sage, TCC significantly increased on all days excluding on day 4 at 0.5% (w/v). TCC 5.73 x 10^6 (p=0.020), 7.21 x 10^6 (p=0.003) and 5.33 x 10^6 (p=0.005) in worms treated with 0.1% (w/v) sage on day 2, 4 and 6, respectively (Table 7). For worms consuming 0.5% (w/v) sage, TCC was 3.85 x 10^6 (p=0.006), 3.02 x 10^6 (p=0.143), and 5.29 x 10^6 (p=0.009) on day 2, 4 and 6, respectively. The overall effect of sage on TCC was significantly higher in response to treatment with 0.1% (w/v) (p=0.004) compared to 0.5% (w/v) as indicated by AUC values (Figure 7).

Over the six day treatment with basil, TCC significantly increased in a progressive manner with highest cell counts occurring on day 6 (Table 7). For 0.1% (w/v) basil, TCC was 4.23 x 10^6 (p=0.020), 4.64 x 10^6 (p=0.006) and 5.19 x 10^6 (p=0.035) for day 2, 4 and 6, respectively. Treatment with 0.5% (w/v), resulted in TCC of 3.28 x 10^5 (p=0.028), 3.83 x 10^6 (p=0.011) and 4.07 x 10^6 (p=0.001) on day 2, 4 and 6, respectively. Increases in TCC of ~108% and ~63.5% over control values were observed on day 6 at 0.1% (w/v) and 0.5% (w/v) basil, respectively. Over the duration of the experiment, treatment with 0.1% (w/v) basil resulted in higher TCC overall compared to worms treated with 0.5% (w/v) (p=0.011) (Figure 7).

Worms consuming rosemary supplemented LGM had significantly higher TCC values compared to the control in all experimental groups (Table 7). For 0.1% (w/v)

rosemary, TCC was 3.91 x 10^6 (p=0.009), 6.28 x 10^6 (p=0.000) and 6.43 x 10^6 (p=0.006) on day 2, 4 and 6, respectively. Treatment with rosemary at 0.5% (w/v) resulted in TCC of 5.88 x 10^6 (p=0.009), 3.65 x 10^6 (p= 0.101) and 5.6 x 10^6 (p= 0.037) on day 2, 4 and 6, respectively. AUC values indicated significantly greater TCC following treatment with 0.1% (w/v) rosemary overall compared to 0.5% (w/v) (p=0.012) (Figure 7).

TCC went up in worms treated with 0.1% (w/v) thyme on day 2 and day 4 of the treatment, compared to the control (Table 7). On those days, TCC was 3.28 x 10^6 (p=0.145), and 5.13 x 10^6 (p=0.012), respectively. However, on day 6 of treatment with 0.1% (w/v) thyme, TCC was 2.3 x 10^6 (p=0.204), which was lower than the control. Treatment with 0.5% (w/v) thyme resulted in higher TCC on days 4 and 6 but significantly lower TCC on day 2 (Table 7). On days 2, 4 and 6, TCC was 4.33 x 10^5 (p=0.029), 3.34 x 10^6 (p=0.335) and 2.84 x 10^6 (p=0.163), respectively. Analysis of the AUC indicated that overall, worms consuming 0.1% thyme had significantly higher TCC than those in the 0.5% (w/v) experimental group over the course of the treatment (p=0.015) (Figure 7).

Table 7. Effect of herbs on total cell count (TCC) in *L. terrestris*. TCC was estimated using a Improved Neubauer 1/400 sq. mm hemacytometer and expressed as cell concentration per milliliter. *Indicates significantly higher than control. **Indicates significantly lower than control. $p<0.05$. n=6.

Herb	Total Coelomocyte Count n=6						
	Conc. *(w/v)*	Day 2 *(cells ml)*	*P value*	Day 4 *(cells ml)*	*P value*	Day 6 *(cells ml)*	*P value*
Oregano	0.10%	3.78×10^6	0.026*	6.88×10^6	0.002*	9.95×10^7	0.020*
	0.50%	5.96×10^6	0.005*	4.42×10^6	0.000*	3.22×10^6	0.029*
Sage	0.10%	5.73×10^6	0.020*	7.21×10^6	0.003*	5.33×10^6	0.005*
	0.50%	3.85×10^6	0.006*	3.02×10^6	0.143	5.29×10^6	0.009*
Basil	0.10%	4.23×10^6	0.020*	4.64×10^6	0.006*	5.19×10^6	0.035*
	0.50%	3.28×10^5	0.028*	3.83×10^6	0.011*	4.07×10^6	0.001*
Rosemary	0.10%	3.91×10^6	0.009*	6.28×10^6	0.000*	6.43×10^6	0.006*
	0.50%	5.88×10^6	0.009*	3.65×10^6	0.101	5.6×10^6	0.037*
Thyme	0.10%	3.28×10^6	0.145	5.13×10^6	0.012*	2.3×10^6	0.204
	0.50%	4.33×10^5	0.029*	3.34×10^6	0.335	2.84×10^6	0.163
Control	---	2.31×10^6	---	2.49×10^6	---	2.49×10^6	---

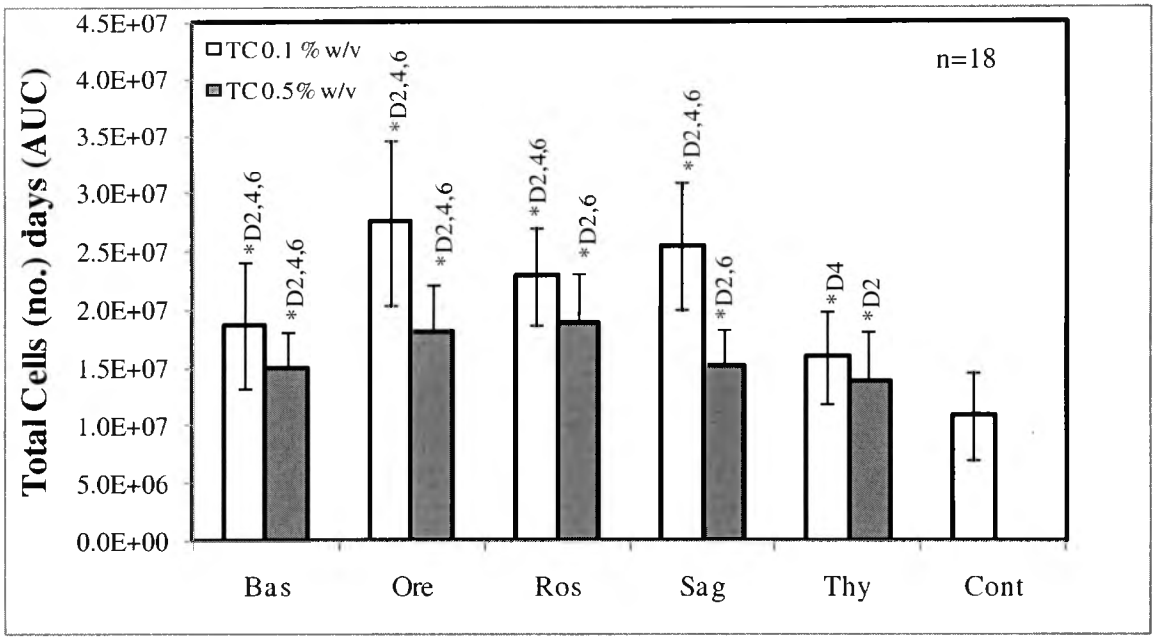

Figure 7. Overall effect of herbs on total coelomocyte count (TCC) in *L. terrestris*. Overall TCC for 0.1% (w/v) and 0.5% (w/v) of each herb, calculated as area under the curve (AUC) and reported as total number of coelomocytes over the duration of the experiment. The data is represented as means ± SEM. a- indicates the treatment concentration which was significantly different ($p<0.05$).*- indicates significant difference from the control ($p<0.05$). D2= day 2; D4= day 4; D6= day6 – indicates days when treatment was significantly different from control ($p<0.05$). n=18.

1.6.8 Effect of herbs on coelomocyte viability

Coelomocyte viability (CV) was determined by trypan blue exclusion assay as described above and reported as the percentage of unstained cells per 50 coelomocytes counted. For control worms feeding on LGM only, CV was 46.7%, 47.3% and 50.0% on day 2, 4 and 6, respectively (Table 8). When worms were treated with LGM prepared with oregano at 0.1% (w/v) and 0.5% (w/v), CV significantly increased on day 2, 4 and 6 (Table 8). Greatest increases in CV occurred on day 6 of treatment with oregano at 0.1% (w/v) and 0.5% (w/v) of the herb as viability was observed to be 67.0% (p=0.000) and 73.0% (p=0.000), respectively. CV on day 2 and 4 in worms feeding on 0.1% (w/v) oregano was 55.7% (p=0.007) and 69.3% (p=0.001), respectively. Treatment with

oregano at 0.5% (w/v) resulted in CV values of 71.3% (p=0.000) and 65.0% (p=0.000) on day 2 and 4, respectively. The overall effect of oregano on CV was determined to be higher following treatment with oregano at 0.5% (w/v) compared to 0.1% (w/v) as indicated by AUC values (p=0.051) (Figure 8).

Upon treatment with sage, CV significantly increased in all experimental groups throughout the duration of the treatment compared to control, with the exception of day 4 at 0.5% (w/v) (Table 8). For worms consuming LGM with 0.1% (w/v) sage, CV was 62.0% (p=0.003), 68.3% (p=0.000) and 59.0% (p=0.001) on day 2, 4 and 6, respectively. CV for worms treated with 0.5% (w/v) sage in LGM was 66.0% (p=0.004), 58.0% (p=0.040) and 62.3% (p=0.002) on day 2, 4 and 6, respectively. Overall, a significantly greater number of coelomocytes were identified as viable following treatment with 0.1% (w/v) sage than in 0.5% (w/v) sage (p=0.044) (Figure 8).

Increases in CV were also observed following treatment with basil throughout the duration of the experiment (Table 8). Significantly more viable coelomocytes were identified on day 4 following consumption of LGM with 0.5% (w/v) basil in which viability was 60.3% (p=0.001). CV after treatment with 0.1% basil was 45.7% (p=0.195), 51.7% (p=0.086) and 50.7% (p=0.500) on day 2, 4 and 6, respectively. On day 2 and 6 of treatment with 0.5% (w/v), 47.0%, (p=0.090), and 47.3% (p=0.444) of cells were determined to be viable, respectively. Overall, CV was determined to be significantly higher following treatment with basil at 0.5% (w/v) compared to 0.1% (w/v) as indicated by AUC values (p=0.033) (Figure 8).

CV increased in response to treatment with rosemary on the fourth day with both concentrations and on the sixth day with 0.1% (w/v) of the herb (Table 8). Significantly more cells were viable on day 4 at 0.5% rosemary with 58.7% (p=0.001) of cells identified as viable. CV significantly decreased on day 2 at both concentrations and on day 6 at 0.5% (w/v) rosemary. Between the two experimental concentrations, treatment with 0.5% (w/v) rosemary resulted in a significantly higher number viable cells when compared to 0.1% (w/v) (p=0.036) (Figure 8).

Following treatment with LGM supplemented with thyme, CV increased on day 4 at 0.1% (w/v) and significantly increased on day 4 at 0.5% (w/v) of the herb (Table 8). However, CV was significantly reduced on day 2 of the treatment with 0.1% (w/v) thyme as well as on the day 6 at both concentrations. CV was 33.3% (p=0.0.38), 55.7% (p=0.052) and 38.3% (p=0.001) in worms consuming 0.1% (w/v) thyme on day 2, 4 and 6, respectively. For worms treated with 0.5% (w/v) thyme, CV was 46.3% (p=0.335), 51.3% (p=0.029) and 38.3% (p=0.014) on day 2, 4 and 6, respectively. There was not a significant difference in the effect of thyme on CV between concentrations although CV was slightly higher overall following treatment with 0.5% (w/v) thyme (p=0.106) (Figure 8).

Table 8. Effect of herbs on coelomocyte viability (CV) in *L. terrestris*. CV, as measured by trypan blue exclusion, reported as percent live coelomocytes per 50 cells counted. *Indicates significantly higher than control. **Indicates significantly lower than control. $p<0.05$. n=6.

Herb	Coelomocyte Viability n=6						
	Conc. (w/v)	Day 2 (%)	*P* Value	Day 4 (%)	*P* Value	Day 6 (%)	*P* Value
Oregano	0.10%	55.7	0.007*	69.3	0.001*	67.0	0.000*
	0.50%	71.3	0.015*	65.0	0.00*	73.0	0.005*
Sage	0.10%	62.0	0.003*	68.3	0.000*	59.0	0.001*
	0.50%	66.0	0.004*	58.0	0.040*	62.3	0.002*
Basil	0.10%	45.7	0.195	51.7	0.086	50.7	0.5
	0.50%	47.0	0.09	60.3	0.001*	47.3	0.444
Rosemary	0.10%	43.0	0.003**	48.3	0.447	50.7	0.5
	0.50%	44.3	0.000**	58.7	0.001*	42.3	0.013**
Thyme	0.10%	33.3	0.038**	55.7	0.052	38.3	0.001**
	0.50%	46.3	0.335	51.3	0.029*	38.3	0.014**
Control	---	46.7	---	47.3	---	50.0	---

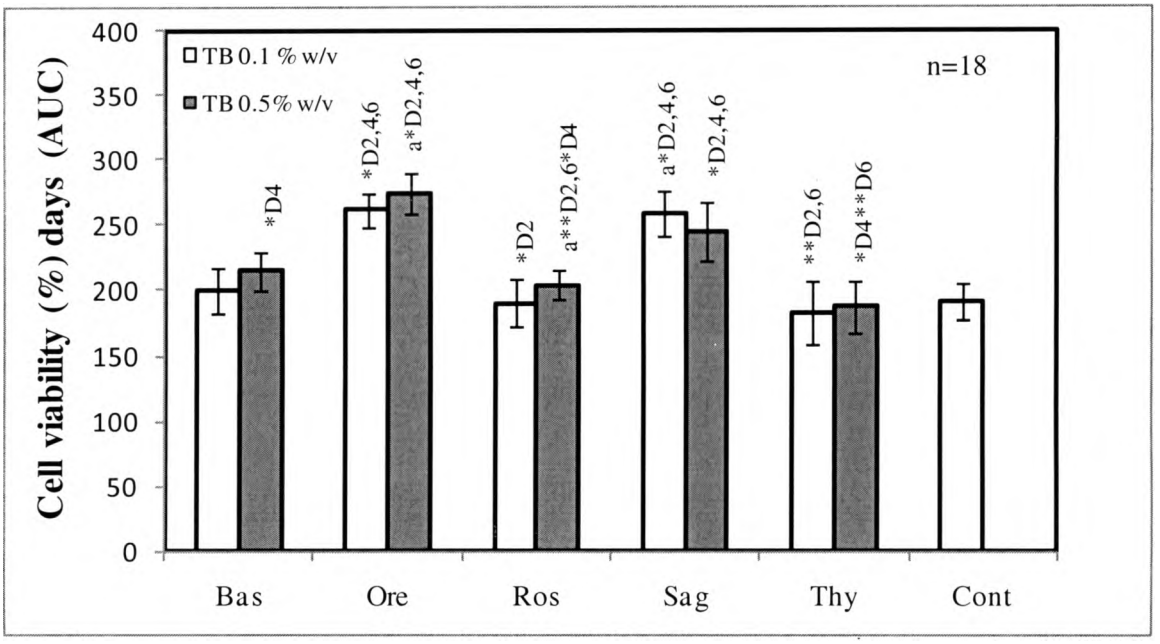

Figure 8. Overall effect of herbs on coelomocyte viability (CV) in *L. terrestris*. Net CV of 0.1% (w/v) and 0.5% (w/v) of each herb, determined by calculation of area under the curve, and reported as percent viable cells over the duration of the experiment. The data is represented as means ± SEM. a- indicates the treatment concentration which was significantly different ($p<0.05$).*- indicates significant difference from the control ($p<0.05$). D2= day 2; D4= day 4; D6= day6– indicates days when treatment was significantly different from control ($p<0.05$). n=18.

1.6.9 Effect of herbs on relative neutrophil count

The distribution of coelomocyte type per 50 total cells counted was carried out by differential cell count technique using Wright's Giemsa stain and visualized under light microscopy at 200X. For control worms feeding on LGM only, the relative neutrophil count (RNC) was 37.7%, 36.9% and 34.7% on day 2, 4 and 6, respectively (Table 9). In response to treatment with oregano in LGM, the RNC increased in all experimental groups excluding day 6 at 0.5% (w/v) of the herb (Table 9). Additionally, a significant increase in the RNC was observed on day 2 with 0.1% (w/v) oregano as 45.3% (p=0.0029) of cells counted were identified as neutrophils. Overall, the RNC was

significantly higher in worms fed 0.1% (w/v) oregano than in those consuming 0.5% (w/v) as indicated by AUC values (p=0.017) (Figure 9).

Treatment with sage resulted in significant increases in RNC on all treatment days at both experimental concentrations (Table 9). The RNC in worms treated with 0.1% (w/v) sage was 49.7% (p=0.000), 53.3% (p=0.001) and 38.0% (p=0.031) on day 2, 4 and 6, respectively. For worms feeding on 0.5% (w/v) sage, the RNC was 40.3% (p=0.033), 45.3% (p=0.000) and 43.7% (p=0.000) on day 2, 4 and 6, respectively. A greater proportion of coelomocytes determined to be neutrophils was observed in worms treated with 0.1% (w/v) sage compared to 0.5% (w/v) overall (p=0.017) (Figure 9).

When worms were fed LGM supplemented with 0.1% (w/v) basil, the RNC increased significantly on all treatment days (Table 9, Figure 9). Specifically, the RNC for worms feeding on 0.1% (w/v) basil was 43.0% (p=0.031), 38.3% (p=0.007) and 42.3% (p=0.017), on day 2, 4, and 6, respectively. For 0.5% (w/v) basil, 36.3%, 39.7% and 36.0% of total coelomocytes were determined to be neutrophils on day 2, 4 and day 6, respectively. Determination of the AUC for both concentrations of basil indicated that the RNC was significantly higher in response to treatment with 0.1% (w/v) of the herb over the duration of the experiment (p=0.037) (Figure 9).

Treatment with rosemary resulted in a significant increase in the RNC on day 4 at 0.5% (w/v) (Table 9). However, in all remaining experimental groups, the RNC decreased with significant decreases occurring on day 2 at 0.1% (w/v) and 0.5% (w/v) as well as on day 6 at 0.5% (w/v) of the herb. For worms feeding on 0.1% (w/v) rosemary, the RNC per sample analyzed was 35.3% (p=0.017), 34.0% (p=0.008) and 38.0%

(p=0.106) on day 2, 4 and 6, respectively. For worms treated with 0.5% (w/v) rosemary, the RNC was 36.3% (p=0.351), 40.3% (p=0.006) and 30.0% (p=0.003) for day 2, 4 and 6, respectively. A significant difference between the two concentrations of rosemary on the distribution of cells was not detected although the proportion of neutrophils was slightly higher in worms treated with 0.5% (w/v) than in those treated with 0.1% (w/v) (p=0.063) (Figure 9).

Following the six day treatment with thyme, the RNC significantly decreased on day 2 at both experimental concentrations as well on day 4 at 0.5% (w/v) (Table 9). The percentage of total cells determined to be neutrophils following consumption of 0.1% (w/v) thyme was 31.2% (p=0.018), 44.0% (p=0.063), and 31.6% (p=0.365) on day 2, 4 and 6, respectively. For worms treated with 0.5% (w/v) thyme, the RNC was 32.0% (p=0.003), 30.7% (p=0.028) and 29.0% (p=0.128) on day 2, 4 and 6, respectively. Overall, treatment with 0.1% (w/v) thyme resulted in a significantly greater proportion of neutrophils compared to treatment with 0.5% (w/v) thyme as determined by AUC values (p=0.014) (Figure 9).

Table 9. Effect of herbs on relative neutrophil count (RNC) in *L. terrestris*. The RNC, determined by differential staining using Wright's stain, and expressed as the percent neutrophils per 50 total cells counted. *Indicates significantly higher than control. **Indicates significantly lower than control. $p<0.05$. n=6.

Herb	Relative Neutrophil Count n=6						
	Conc. (w/v)	Day 2 (%)	*P* Value	Day 4 (%)	*P* Value	Day 6 (%)	*P* Value
Oregano	0.10%	45.3	0.002*	45.3	0.102	39.7	0.063
	0.50%	39.7	0.107	41.0	0.102	30.7	0.366
Sage	0.10%	49.7	0.000*	53.3	0.001*	38.0	0.031*
	0.50%	40.3	0.033*	45.3	0.000*	43.7	0.000*
Basil	0.10%	43.0	0.031*	38.3	0.007*	42.3	0.017*
	0.50%	36.3	0.162	39.7	0.358	36.0	0.107
Rosemary	0.10%	35.3	0.017**	34.0	0.008**	38.0	0.106
	0.50%	36.3	0.351	40.3	0.006*	30.0	0.003**
Thyme	0.10%	31.2	0.018**	44.0	0.063	31.6	0.365
	0.50%	32.0	0.003**	30.7	0.028**	29.0	0.128
Control	---	37.7	---	36.9	---	34.7	---

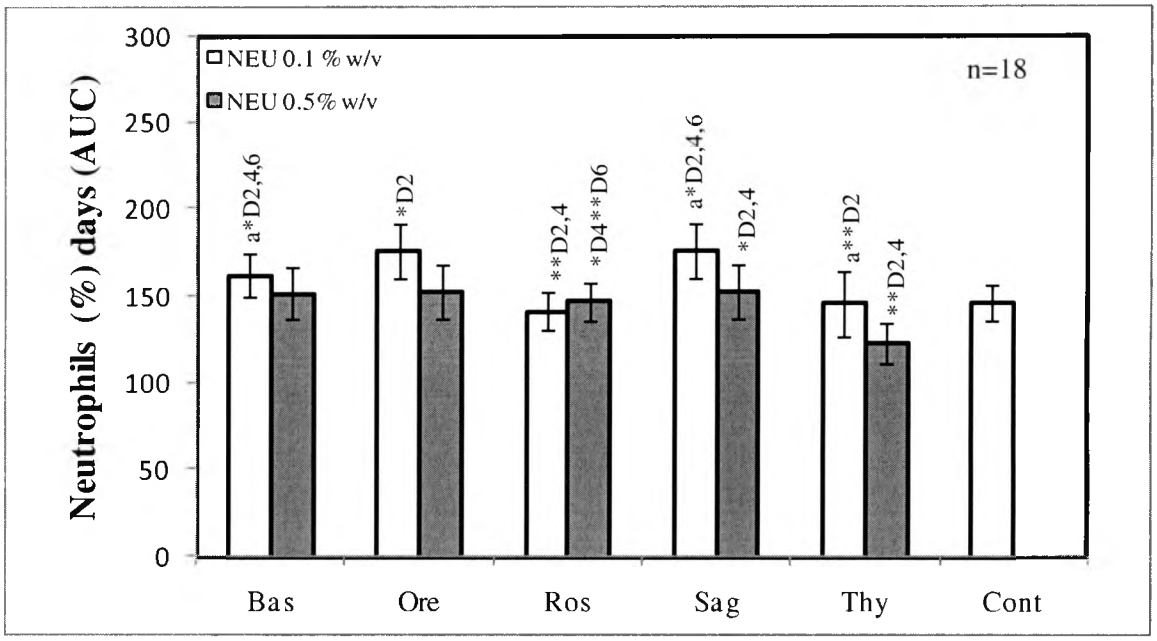

Figure 9. Overall effect of spices on relative neutrophil count (RNC) in *L. terrestris*. Overall RNC 0.1% (w/v) and 0.5% (w/v) of herb calculated as area under the curve (AUC) and expressed as percent neutrophils. The data is represented as means + SEM. a- indicates the treatment concentration which was significantly different ($p<0.05$).*- indicates significant difference from the control ($p<0.05$). D2= day 2; D4= day 4; D6= day6 – indicates days when treatment was significantly different from control ($p<0.05$). n=18.

1.6.10 Effect of herbs on phagocytic activity

Phagocytic activity (PA) was determined by counting the number of coelomocytes containing at least one engulfed *S. cereviseae* stained with Congo as described above. For control worms feeding on LGM along, basal PA was 53.1%, 56.4% and 56.1% on day 2, 4 and 6, respectively (Table 10). Significant increases in PA occurred in response to treatment with LGM supplemented with oregano at 0.1% (w/v) and 0.5% (w/v) throughout the duration of the six day experiment (Table 10). The percentage of actively phagocytosing coelomocytes in worms fed 0.1% (w/v) oregano was 67.0% (p=0.004), 63.3% (p=0.001) and 66.0% (p=0.000) on day 2, 4 and 6, respectively. For worms consuming 0.5% (w/v) oregano, PA was 63.0% (p=0.001),

66.7% (p=0.021) and 67.3% (p=0.004) for day 2, 4 and 6, respectively. Overall, the percentage of actively phagocytosing coelomocytes was higher after treatment with 0.5% (w/v) oregano when compared to 0.1% (w/v) although the variation between the two concentrations was not significant (p=0.163) (Figure 10).

Upon treatment with sage, PA increased in all experimental groups with increases ranging from ~18.6% to ~23.6% above that of the control on day 2 at 0.5% (w/v) and day 4 at 0.1% (w/v), respectively (Table 10). For worms fed 0.1% (w/v) sage, 64.7% (p=0.001), 69.7% (p=0.007), and 69.7% (p=0.004) of cells counted were actively phagocytosing yeast on day 2, 4 and 6, respectively. PA was 63.0% (p=0.001), 68.3% (p=0.004) and 68.3% (p=0.002) upon treatment with 0.5% (w/v) sage on day 2, 4 and 6, respectively. The greatest overall increase in PA occurred following treatment with 0.1% (w/v) sage, as evidenced by AUC values (p=0.119) (Figure 10).

Treatment of basil at concentrations of 0.1% (w/v) and 0.5% (w/v) resulted in significant increases in the percentage of phagocytic cells in all experimental groups (Table 10). PA was 62.3% (p=0.002), 62.3% (p=0.008) and 62.7% (p=0.000) in worms consuming LGM with 0.1% (w/v) basil on day 2, 4 and 6, respectively. In worms treated with 0.5% (w/v) basil, PA was 61.3% (p=0.002), 61.3% (p=0.000) and 67.7% (p=0.031) on day 2, 4 and 6, respectively. PA of coelomocytes was slightly higher overall in worms consuming 0.5% (w/v) basil than in those consuming 0.1% (w/v) basil (p=0.313) (Figure 10).

When worms were fed rosemary over six days, significant increases in PA were observed on each day at both concentrations excluding on day 4 at 0.5% (w/v) (Table

10). In worms treated with 0.1% (w/v) rosemary, PA was 60.7% (p=0.006), 62.3% (p=0.005) and 61.7% (p=0.011) on day 2, 4 and 6, respectively. PA for worms treated with 0.5% (w/v) rosemary was 60.0% (p=0.011), 55.7% (p=0.195) and 63.3% (p=0.007) on day 2, 4 and 6, respectively. AUC values indicated 0.1% (w/v) rosemary to be significantly more effective on increasing PA compared to 0.5% (w/v) (p=0.048) (Figure 10).

Following treatment with thyme, PA of coelomocytes significantly decreased on day 2 at 0.1% (w/v) and on day 6 at both experimental concentrations (Table 10). PA was 44.3% (p=0.001) on day 2 at 0.1% (w/v) thyme, 48.4% (p=0.006) on day 6 at 0.1% (w/v) thyme and 35.6% (p=0.021) on day 6 at 0.5% (w/v) thyme, which were all significantly lower than the control. Conversely, PA significantly increased on day 2 at 0.5% (w/v) thyme as well as on day 4 at both concentrations. For day 2 at 0.5% (w/v) thyme, the percentage of actively phagocytosing cells was 60.0% (p=0.009). On day 4, PA was 60.3% (p=0.002) and 60.3% (p=0.006) following treatment with 0.1% (w/v) and 0.5% (w/v) thyme, respectively, which were both significantly higher than the control. AUC analysis indicated that overall, worms treated with 0.5% (w/v) thyme exhibited higher percentages of actively phagocytosing coelomocytes compared to 0.1% (w/v) of the herb (p=0.187) (Figure 10).

Table 10. Effect of herbs on phagocytic activity (PA) of coelomocytes in *L. terrestris*. PA of coelomocytes, determined by a counting the number of cells containing one more *S. cereviseae* particles per 50 total cells and reported as percent phagocytic cells. *Indicates significantly higher than control. **Indicates significantly lower than control. $p<0.05$. n=6.

Herb	Phagocytic Activity n=6						
	Conc. *(w/v)*	Day 2 *(%)*	*P Value*	Day 4 *(%)*	*P Value*	Day 6 *(%)*	*P Value*
Oregano	0.10%	67.0	0.004*	63.3	0.001*	66.0	0.000*
	0.50%	63.0	0.014*	66.7	0.021*	67.3	0.004*
Sage	0.10%	64.7	0.001*	69.7	0.007*	69.7	0.004*
	0.50%	63.0	0.001*	68.3	0.004*	68.3	0.002*
Basil	0.10%	62.3	0.002*	62.3	0.008*	62.7	0.000*
	0.50%	61.3	0.002*	61.3	0.000*	67.7	0.031*
Rosemary	0.10%	60.7	0.006*	62.3	0.005*	61.7	0.011*
	0.50%	60.0	0.011*	55.7	0.195	63.3	0.007*
Thyme	0.10%	44.3	0.001**	60.3	0.002*	48.4	0.006**
	0.50%	60.0	0.009*	60.3	0.006*	35.6	0.021**
Control	---	53.1	---	56.4	---	56.1	---

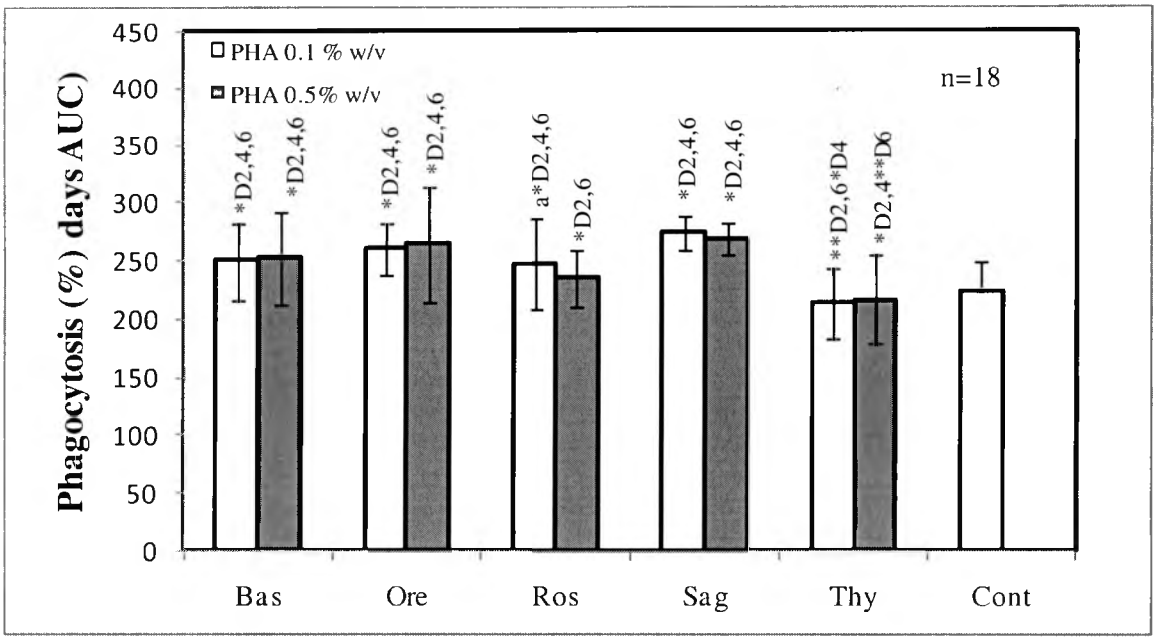

Figure 10. Overall effect of spices on total phagocytic activity (PA) in *L. terrestris*. Overall PA for 0.1% (w/v) and 0.5% (w/v) of herb calculated as area under the curve (AUC) and expressed as percent total activity over the duration of the experiment. The data is represented as means ± SEM. a- indicates the treatment concentration which was significantly different ($p<0.05$).*- indicates significant difference from the control ($p<0.05$). D2= day 2; D4= day 4; D6= day6 – indicates days when treatment was significantly different from control ($p<0.05$). n=18.

1.6.11 Effect of herbs on respiratory burst activity

Respiratory burst (RB) activity of coelomocytes was indirectly measured as the absorbance of diformazan formed during a nitroblue tetrazolium (NBT) reduction assay as previously described above. For worms feeding on LGM alone, the A_{570} of diformazan was 1.05 AU, 1.02 AU, and 1.00 AU for day 2, 4 and 6, respectively (Table 11). Significant increases in RB activity occurred following treatment with oregano in all experimental groups (Table 11). For worms treated with 0.1% (w/v) oregano, the A_{5470} of diformazan was 1.6 AU (p=0.039), 1.6 AU (p=0.035) and 1.7 AU (p=0.020) for day 2, 4 and 6, respectively. When oregano was fed to worms at 0.5% (w/v), the A_{570} of diformazan was 1.6 AU (p=0.027), 2.1 AU (p=0.023), and 1.9 AU (p=0.005) for days 2,

4 and 6, respectively. Increases in RB ranged from ~56.8% on day 4 at 0.1% (w/v) to ~105.8% higher than the control on day 4 at 0.5% (w/v) oregano. Assessment of the overall effect of oregano on RB indicated 0.5% (w/v) to be more effective than 0.1% (w/v) as evidenced by AUC values (p=0.012) (Figure 11).

Upon treatment with sage, RB activity increased in all experimental groups (Table 11). Significant increases occurred in worms fed LGM prepared with 0.1% sage on day 4 and 6 as the A_{570} of diformazan was 1.6 AU (p=0.007), and 1.7 AU (p=0.000), respectively. Additionally, RB significantly increased following treatment with 0.5% sage on day 4 and 6 with A_{570} values of 1.9 AU (p=0.000) and 1.8 AU (p=0.001), respectively. RB was shown to be significantly higher overall in response to treatment with 0.5% (w/v) sage compared to 0.1% (w/v) sage (p=0.024) as evidenced by AUC values (Figure 11).

RB activity of coelomocytes significantly increased on day 2 and 4 after treatment with 0.1% (w/v) and 0.5% (w/v) basil (Table 11). The A_{570} of diformazan was 1.4 AU (p=0.007), 1.3 AU (p=0.000) and 0.9 AU (p=0.111) for worms treated with 0.1% (w/v) basil on day 2, 4 and 6, respectively. Following treatment with 0.5% (w/v) basil, the A_{570} of diformazan was 1.6 AU (p=0.000), 1.3 AU (p=0.001) and 0.9 AU (p=0.007) on day 2, 4 and day 6, respectively. Assessment of the AUC for both concentrations indicated RB activity to be overall greater following treatment with 0.5% (w/v) basil than in worms were fed with 0.1% (w/v) basil although a significant difference between the concentrations was not detected (p=0.062) (Figure 11).

Upon treatment with rosemary, RB increased in all experimental groups with significant increases occurring on the day 4 at 0.1% (w/v) and 0.5% (w/v) (Table 11). The A_{570} of diformazan for worms treated with LGM supplemented with 0.1% (w/v) rosemary, was 1.3 AU (p=0.488), 1.4 AU (p=0.020), 1.3 AU (p=0.104) on day 2, 4 and 6, respectively. Following treatment with 0.5% (w/v) rosemary, the A_{570} of diformazan was 1.8 AU (p=0.055), 1.8 AU (p=0.006) and 1.0 AU (p=0.105). Significantly higher RB activity was observed following treatment with rosemary at 0.5% (w/v) than in those treated with 0.1% (w/v) rosemary (p=0.013) as determined by AUC values (Figure 11).

When worms were fed LGM prepared with thyme at 0.1% (w/v) and 0.5% (w/v), significant increases in RB activity were observed (Table 11). RB activity was highest on day 2 as the A_{570} of diformazan was of 2.3 (p=0.007) AU for worms feeding on 0.1% (w/v) and 0.5% (w/v) thyme. On the subsequent treatment days the A_{570} of diformazan was of 2.1 AU (p=0.000) and 2.0 AU (p=0.023) in worms treated with 0.1% (w/v) thyme on day 4 and 6, respectively. For worms consuming 0.5% (w/v) thyme, the A_{570} of diformazan was 2.1 AU (p=0.001) and 1.8 AU (p=0.028) on day 4 and 6, respectively. The overall effectiveness of treatment thyme was assessed to be greater with 0.1% (w/v) thyme compared to 0.5% (w/v) of the herb as indicated by AUC values (p=0.321) (Figure 11).

Table 11. Effect of herbs on respiratory burst activity (RB) of coelomocytes in *L. terrestris*. RB activity, indirectly measured as absorbance of diformazan formed during NBT reduction assay, and expressed in absorbance units (AU) at 570 nm. *Indicates significantly higher than control. **Indicates significantly lower than control. $p<0.05$. n=6.

Herb	Respiratory Burst Activity n=6						
	Conc. (w/v)	Day 2 (AU)	*P* Value	Day 4 (AU)	*P* Value	Day 6 (AU)	*P* Value
Oregano	0.10%	1.60	0.039*	1.60	0.035*	1.70	0.020*
	0.50%	1.60	0.027*	2.10	0.023*	1.90	0.005*
Sage	0.10%	1.30	0.169	1.60	0.007*	1.70	0.000*
	0.50%	1.30	0.277	1.90	0.000*	1.80	0.001*
Basil	0.10%	1.40	0.007*	1.30	0.000*	0.90	0.111
	0.50%	1.60	0.000*	1.30	0.001*	0.90	0.007**
Rosemary	0.10%	1.30	0.488	1.40	0.020*	1.30	0.104
	0.50%	1.80	0.055	1.80	0.000*	1.00	0.105
Thyme	0.10%	2.30	0.007*	2.10	0.006*	2.00	0.023*
	0.50%	2.30	0.007*	2.10	0.000*	1.80	0.028*
Control	---	1.05	---	1.02	0.001*	1.00	---

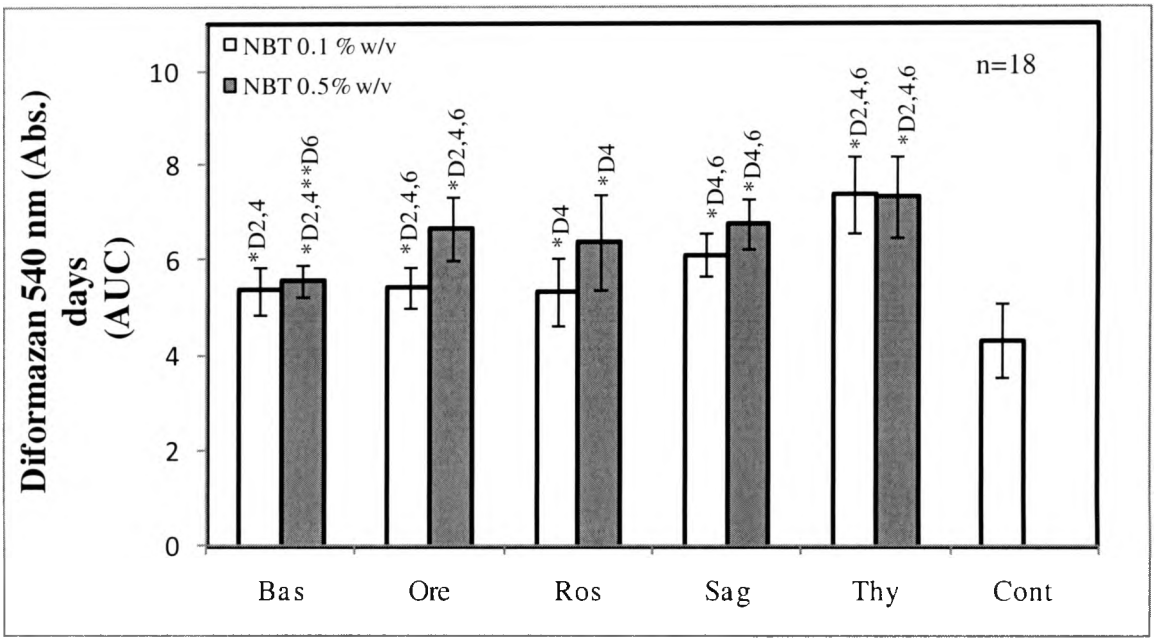

Figure 11. Overall effect of spices on respiratory burst activity (RB) in *L. terrestris*. Overall RB activity for 0.1% (w/v) and 0.5% (w/v) of herb calculated as area under the curve (AUC) and expressed as total absorbance of diformazan at 570 nm over the duration of the experiment. a- indicates the treatment concentration which was significantly different ($p<0.05$).*- indicates significant difference from the control ($p<0.05$). d2= day 2; d4= day 4; d6= day6 – indicates days when treatment was significantly different from control ($p<0.05$). n=18.

1.6.12 Effect of herbs on nitric oxide production

Nitric oxide production (NOx) was indirectly quantified as total nitrites/nitrates via Griess assay as described above. For control worms consuming LGM alone, NOx was 27.7 μM, 28.8 μM and 27.1 μM on day 2, 4 and 6, respectively (Table 12). When worms were treated with oregano at 0.1% (w/v) and 0.5% (w/v) NOx increased on all treatment days excluding day 2 at 0.1% (w/v) (Table 12). On day 2 at 0.5% (w/v), NOx was 34.7 μM (p=0.005), which was significantly higher than control. For worms feeding on 0.1% (w/v) oregano for six days, NOx was 45.0 μM (p=0.036), which was also significantly higher than the control. Conversely, a significant decrease in NOx occurred on day 2 at 0.1% oregano with a calculated value of 8.4 μM (p=0.000). The total effects of oregano

on NOx was determined to be greater following treatment with 0.5% (w/v) oregano when compared to 0.1% (w/v) oregano, as determined by AUC values (p=0.058) (Figure 12).

Following the treatment with sage, NOx increased significantly on day 2 at 0.5% (w/v), day 4 at both concentrations and day 6 at 0.1% (w/v) (Table 12). For worms consuming LGM supplemented with 0.1% (w/v) sage, NOx was calculated to be 25.0 μM (p=0.206), 31.4 μM (p=0.004) and 44.9 μM (p=0.003) on day 2, 4 and 6, respectively. Treatment with sage at 0.5% (w/v) resulted in NOx values of 39.7 μM (p=0.006), 14.1 μM (p=0.012) and 28.2 μM (p=0.173) on day 2, 4 and 6, respectively (Table 12). NOx increased most significantly on day six at 0.1% (w/v) in which ~66% more NOx was detected compared to the control. Treatment with sage at 0.1% (w/v) was assessed to be significantly more effective than treatment with 0.5% (w/v) over the duration of the experiment (p=0.007) (Figure 12).

NOx also increased in response to treatment with basil in all experimental groups with the exception of day 2 at 0.5% (w/v) basil (Table 12). Treatment with 0.1% (w/v) basil resulted in NOx values of 33.0 μM (p=0.002), 35.2 μM (p=0.069), and 32.1 μM (p=0.039) on day 2, 4 and 6, respectively. NOx was 22.2 μM (p=0.255), 32.8 μM (p=0.025), and 30.8 μM (p=0.003) in worms feeding on 0.5% basil for day 2, 4 and 6, respectively. The overall effect of the treatment with basil was determined to be more effective at 0.1% (w/v) basil compared to 0.5% (w/v) (p=0.018) (Figure 12).

Treatment with LGM supplemented with rosemary resulted in significant decreases in NOx on day 2 at 0.1% (w/v) and day 6 at 0.5% (w/v) with values of 26.3 μM (p=0.000) and 19.1 μM (p=0.003), respectively (Table 12). Conversely, NOx increased

significantly on day 4 and 6 at 0.1% (w/v) rosemary with calculated values of 63.0 μM (p=0.014) and 39.3 μM (p=0.005), respectively. Additionally, significant increases in NOx were observed on day 2 and 4 at 0.5% (w/v) rosemary as NOx production was 43.9 μM (p=0.000) and 50.9 μM (p=0.000), respectively. The net production of NOx was significantly higher in worms treated with 0.1% (w/v) rosemary compared to 0.5% (w/v), as indicated by AUC values (p=0.016) (Figure 12).

Upon treatment with thyme, NOx was significantly decreased on day 2 at 0.1% (w/v) and significantly increased compared to the control on day 4 at 0.1% (w/v) thyme (Table 12). Specifically, for worms treated with 0.1% thyme, NOx was 6.8 μM (p=0.000), 43.4 μM (p=0.002), and 27.0 μM (p=0.274) on day 2, 4 and 6, respectively. For worms feeding on 0.5% thyme, NOx was 5.6 μM (p=0.097), 38.1 μM (p=0.064) and 21.9 μM (p=0.389) on day 2, 4 and 6, respectively. Overall, treatment with 0.1% (w/v) thyme produced significantly greater effects on NOx production than treatment with 0.5% (w/v) of the herb (p=0.016) (Figure 12).

Table 12. Effect of herbs on nitric oxide production (NOx) in coelomic fluid of *L. terrestris*. NOx, measured indirectly as total nitrites/nitrates via Griess assay, and reported in μM NOx.*Indicates significantly higher than control. **Indicates significantly lower than control. $p<0.05$. n=6.

Herb	Nitric Oxide Production n=6						
	Conc. (w/v)	Day 2 (μM)	*P Value*	Day 4 (μM)	*P Value*	Day 6 (μM)	*P Value*
Oregano	0.10%	8.4	0.000**	31.0	0.401	45.0	0.036*
	0.50%	34.7	0.005*	28.2	0.369	29.2	0.205
Sage	0.10%	25.0	0.206	31.4	0.004*	44.9	0.003*
	0.50%	39.7	0.006*	14.1	0.012*	28.2	0.173
Basil	0.10%	33.0	0.002*	35.2	0.069	32.1	0.039*
	0.50%	22.2	0.255	32.8	0.025*	30.8	0.003*
Rosemary	0.10%	26.3	0.000**	63.0	0.014*	39.3	0.005*
	0.50%	43.9	0.000*	50.9	0.000*	19.1	0.003**
Thyme	0.10%	6.8	0.000**	43.4	0.002*	27.0	0.274
	0.50%	5.6	0.097	38.1	0.064	21.9	0.389
Control	---	27.7	---	28.8	---	27.1	---

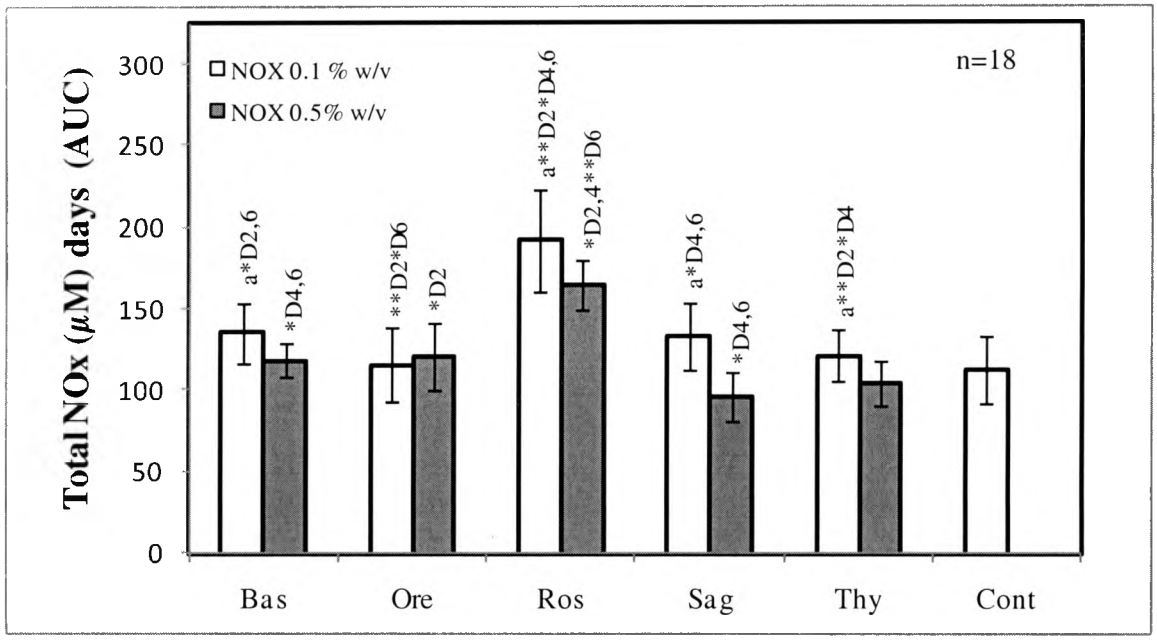

Figure 12. Overall effect of spices on nitric oxide (NOx) production in *L. terrestris*. Total NOx overall for 0.1% (w/v) and 0.5% (w/v) of herb, calculated as AUC and reported as μM of total NOx over the duration of the experiment. The data is represented as means $\pm$ SEM. a- indicates the treatment concentration which was significantly different ($p<0.05$).*- indicates significant difference from the control ($p<0.05$). D2= day 2; D4= day 4; D6= day6 – indicates days when treatment was significantly different from control ($p<0.05$). n=18.

1.7 Discussion

Dietary herbs and spices have traditionally been used to enhance the flavor of food and as medicinal tools for the treatment of disease.[7-12] Epidemiologic data have consistently supported the health promoting benefits associated with dietary herb and spice intake. Many studies have established an inverse relationship between diets high in plant foods, such as herbs and spices, and risk for disease.[22-25,32,76,126] Herbs and spices are naturally rich sources of a diverse range of phytochemicals including the low molecular weight polyphenolics, flavonoids, carotenoids, and anthocyanins. In addition, many herbs and spices also contain high molecular weight polysaccharides which may have a positive effect on human health.[13,26,28] Of the many proposed benefits of herbs and

spices, the ability to modulate innate immune functions and promote health has recently been of interest.[32,33,127] Several studies have indicated, using predominantly *in vitro* models, the ability of herbs and spices to mediate pathogen uptake and degradation by phagocytic leukocytes and macrophages, a property often considered to be hallmark of the innate response.[7,50,51,61,64-66,68]

While many studies have investigated the effect of dietary herbs and spices on the innate immune system, few have studied the molecular mechanism of immunomodulation by natural products, especially through the use of *in vivo* models, which take into account extent of bioavailability and metabolic transformation. Moreover, most studies have only investigated the effect of an isolated bioactive compound on the innate immune response. While this approach may facilitate identification of the biologically active molecule, it does not take into consideration the synergistic effect of various phytochemicals in a natural product which may be contributing to the overall immunomudulatory effect of the natural product. In the present study, we evaluated the effect of several dietary herbs and spices on key parameters of innate immunity such as cell proliferation, myeloid differentiation and cell viability *in vivo* using *L. terrestris*. Additionally, we evaluated the effect of these natural products on functional end points of the innate immune system which include phagocytosis, respiratory burst and nitric oxide production.

1.7.1 Overall effect of spices on innate immune parameters

The overall effect of treatment with turmeric, ginger, cinnamon and pepper was collectively assessed. A decrease in coelomocyte viability was observed overall (Table

13). Additionally, differentiation of cells into neutrophils decreased overall by ~7% compared to control. Concomitant decreases in neutrophil driven innate functions such as phagocytosis and respiratory burst were also observed. However, an increase in nitric oxide production and total coelomocyte count was also observed (Table 13). Overall, total coelomocyte counts increased in worms treated with spices by ~19%. This was associated with a 59.8% increase in nitric oxide production overall. This concurrent increase in total coelomocyte count and nitric oxide is consistent with recent findings which indicate hematopoietic reconstitution and mobilization are significantly mediated by nitric oxide.[128-130] In one such study, significant decreases in circulating levels of progenitor cells and subsequent increased mortality were observed in endothelial nitric oxide synthase (eNOS) knockout mice.[129] Thum (2005) showed that patients treated for coronary heart disease with asymmetric dimethylarginine (ADMA), a known inhibitor of nitric oxide synthase, had decreased numbers of circulating progenitor cells. They further observed an inverse relationship between the concentration of ADMA in plasma and the number of undifferentiated cells in the circulation indicating the role of nitric oxide in the repopulation and mobilization of progenitor cells *in vivo*.[130] However, further evaluation of the potential molecular mechanism by which spices may affect signaling pathways associated with increased hematopoiesis of immune cells mediated by nitric oxide will be undertaken in our future studies.

Table 13. Overall effect of spices on innate immune parameters in *L. terrestris*. Overall effect of each spice, calculated as the average of treatment with 0.1% and 0.5% of the spice, and reported as the percent change compared to control. CV- Coelomocyte viability; TC- Total coelomocyte count; NEU- Neutrophil distribution; RB- Respiratory burst activity; NOX- Total nitric oxide metabolites measures as nitrates/nitrites; PHAGO- Phagocytic activity of coelomocytes.

SPICE	Innate Immune Parameter					
	CV %	TC %	NEU %	RB %	NOX %	PHAGO %
Cinnamon	-33.02	10.97	-26.92	-79.42	-137.18	-17.96
Ginger	25.9	61.75	15.69	20.26	19.6	18.2
Pepper	-32.98	-49.47	-42.04	-1166.2	334.01	-65.78
Turmeric	25.4	54.85	25.56	33.04	22.94	20.85
AVG	-3.67	19.52	-6.92	-298.07	59.84	-11.17

1.7.1.1 Overall effect of root spices on innate immune parameters

Of the four spices tested, turmeric and ginger, of the *Zingiberaceae* family, uniquely modulated innate immune parameters compared to others. When evaluated together, treatment with turmeric and ginger resulted in a ~22% increase in coelomocyte viability and a ~55% increase in the total number of coelomocytes compared to control worms (Table 14). An increase in the total number of coelomocytes, overall viability and further differentiation into neutrophils was accompanied by increased levels of nitric oxide in the coelomic fluid. This may again suggest the probable role of herbs and spices in increasing nitric oxide mediated cell proliferation and differentiation as observed in

previous studies.[128-130] It may also suggest the potential role of nitric oxide in the maintenance and regulation of normal cell cycle.[131,132] Recent advances in nitric oxide signaling have indicated several important myeloid functions are regulated by nitric oxide and the effects are often concentration dependent.[128,131-133] Genaro et al. (1995) observed increased expression of the anti-apoptotic gene, *bcl-2*, in mature splenic B cells cultured with nitric oxide donor molecules.[131] However, the protective effect of nitric oxide was observed to be dose dependent as pro-apoptotic activity at higher levels was observed. The increase in cell viability noted in the present study may therefore be a result of nitric oxide mediated inhibition of premature apoptotic signaling of coelomocytes. Therefore, it is possible that bioactive compounds in root spices may trigger nitric oxide mediated pathways which protect coelomocyte viability.

Additionally, we observed increases in phagocytosis and respiratory burst by ~16% and ~23%, respectively, compared to control (Table 14). This concomitant shift in the distribution of myeloid cells and the increase in phagocytosis and respiratory burst suggest that potentially the frequency in which these innate functions are carried out may be due in part to an increased number of cells capable of executing them. Moreover, respiratory burst in phagocytic leukocytes is a consequence of cytosolic assembly of NADPH oxidases resulting in the rapid release of oxidants such as the superoxide anion (O_2^-), which can dismutate to hydrogen peroxide (H_2O_2) and molecular oxygen.[42,134-138] In addition, enzymes such as myeloperoxidase and nitric oxide synthase in the leukocytes form hypochlorous acid and nitric oxide, respectively, which can react with superoxide to form the hydroxyl radical.[42,136,139] It has been shown that the respiratory burst increases exponentially upon stimulation, an effect that is compounded by an increase in number of

phagocytic leukocytes.[42,138,140-142] It has also been observed that different stimulating agents produce a varied respiratory burst as mediated by oxygen utilization.[42,139] Therefore, it appears that respiratory burst inducing agents differ by the degree to which they trigger phosphorylation and translocation of cytosolic subunits to the cell membrane during NADPH oxidase assembly.[37,42,143-146] When the distribution of neutrophils increased upon treatment with root spices in this study, we observed that respiratory burst activity increased in a near stoichiometrically manner and not exponentially. We believe that the rather muted response in respiratory burst might be due to the following reasons: (A) The decrease in the target to neutrophil ratio potentially reduces the number of phagosomes per cell, resulting in lower cytosomal assembly of NADPH oxidase and therefore lower production of reactive oxygen species (ROS); (B) Differentiation in the presence of root spices may enhance the antioxidant defense responses in the leukocytes, perhaps by affecting antioxidant response element (ARE) mediated expression of antioxidant genes such as glutathione *S*-transferase (GST), NADPH:quinine oxidoreducatase-1 (NQ01) and other phase II enzymes intimately mediated by NRF2 (nuclear factor E2-related factor).[147,148] Several studies have established the ability of synthetic and natural phenolic compounds to increase the expression of ARE controlled genes.[149,150]

Alternatively, studies have shown enhanced phagocytic activity following treatment with *Zingiberaceae* spices associated with increased expression of genes involved in pathogen recognition and uptake.[50-52,66] Fiala et al. (2007) recently observed increased expression of β-1,4-mannosyl-glycoprotein 4-β-N-acetylglucosaminyltransferase (MGAT-3) and toll-like receptors (TLR) in macrophages

isolated from patients with Alzheimer's disease and treated with bisdemethoxycurcumin.[50] MGAT-3 and TLR's are generally down regulated in Alzheimer's disease potentiating the accumulation of amyloid plaques leading to the development of amyloidosis and encephalitis. The importance of MGAT-3 is indicated by significant inhibition of phagocytosis when MGAT-3 is silenced.[50,52] Additionally, the increased expression of MGAT-3 and TLR's by Fiala was also associated with enhanced phagocytosis of amyloid-β plaques. Thus, the observed increased in phagocytosis in the present study may be in part a result of increased expression of genes crucial for recognition and clearance of foreign material thereby enhancing activity.

Additionally many recent studies have shown immune stimulating activity by polysaccharides present in dietary plants such as *Curcuma xanthorrhiza* Roxb.,[64] *Angelica dahurica* root, *Angelica sinensis, Lu hui* (Aloe vera), *Opuntia polyacantha* (prickly pear cactus),[151] *Astragalus membranaceus*,[152] *Platycodon grandiflorum*,[153] among others.[16,154-156] Specifically, plant polysaccharides were shown in several studies to induce leukocyte activation and increase phagocytic activity accompanied by increased release of cytokines involved in propagation and mediation of the immune response.[16,64,151,152] Schepetkin (2008) recently observed activation of differentiated leukocytes treated with polysaccharides extracted from *Opuntia polyacantha*, which occurred with a concomitant and concentration dependent increase in TNF-α and IL-6, and a moderate increase in reactive oxygen species (ROS) indicating a priming effect of macrophages by plant polysaccharides similar to lipopolysaccharides (LPS).[151] Shao (2004) observed similar immune stimulating activity by dietary polysaccharides extracted from the Chinese medicinal plant, *Astragalus membranaceus.* In this study, activation of

macrophages by *Astragalus* polysaccharides (APS) was observed in association with increased IL-1β, TNF-α and activated NFκB. They further demonstrated that stimulation of macrophages by APS was competitively inhibited by LPS indicating activation via TLR-4 dependent pathway and similarity in APS and LPS epitopes.[152] Whether these specific root spices can also mediate these effects using the above described pathways remains to be investigated.

Table 14. Overall effect of root spices on innate immune parameters in *L. terrestris*. Overall effect of each root spice, calculated as the average of treatment with 0.1% and 0.5% of the spice, and reported as the percent change compared to control. CV- coelomocyte viability; TC- Total coelomocyte count; NEU- Neutrophil distribution; RB- Respiratory burst activity; NOX- Total nitric oxide metabolites measures as nitrates/nitrites; PHAGO- Phagocytic activity of coelomocytes.

SPICE	Innate Immune Parameter					
	CV %	TC %	NEU %	RB %	NOX %	PHAGO %
Turmeric	25.4	54.9	25.6	33.0	22.9	20.9
Ginger	25.9	61.8	15.7	20.3	19.6	18.2
AVG	25.7	58.3	20.6	26.7	21.3	19.5

1.7.1.2 Overall effect of non-root spices on innate immune parameters

The overall effects of treatment with the non-root spices, pepper and cinnamon, varied significantly from *Zingiberaceae* spices. Treatment with pepper and cinnamon resulted in an overall immunosuppressive effect indicated by decreases in almost all innate immune parameters examined (Table 15). We observed a ~37% decrease in

coelomocyte viability, a ~23% decrease in total coelomocyte count and a decrease in the percentage of neutrophils by ~38% overall compared to control. This correlated with a 45% decrease in phagocytosis overall and a 62% reduction in respiratory burst activity. However, the true effect of dietary intake of pepper and cinnamon is difficult to extrapolate as intake by *L. terrestris* was markedly lower than with other treatments. This was evidenced by greater weight loss over the course of the treatment. Physically, *L. terrestris* treated with pepper and cinnamon appeared malnourished and the incidence of hemorrhage was frequent. In previous studies, cinnamon and pepper have exhibited anthelmintic and antiparasitic activity which may account for the observations in this study.[157,158] Fractionation of the bioactive compounds in pepper and cinnamon would be necessary to eliminate confounding variables and accurately evaluate the true effect on innate immune markers.

Additionally, we observed nitric oxide levels to be increased by ~95% compared to control. The concurrent increase in nitric oxide metabolites in the coelomic fluid and decrease in differentiation of myeloid cells is consistent with recent findings, as noted above.[128,159,160] Moreover, the significant increase in nitric oxide correlated with decreased cell viability suggests activation of pro-apoptotic signaling pathways initiated by high concentrations of nitric oxide, as previously discussed.[128,159,160] Alternatively, an excessive production of nitric oxide, a potent vasodilator, may account for the frequent incidence of hemorrhage and mortality following treatment with these spices.[128,161]

Table 15. Overall effect of non-root spices on innate immune parameters in *L. terrestris*. Overall effect of each root spice, calculated as the average of treatment with 0.1% and 0.5% of the spice, and reported as the percent change compared to control. CV- Coelomocyte viability; TC- Total coelomocyte count; NEU- Neutrophil distribution; RB- Respiratory burst activity; NOX- Total nitric oxide metabolites measures as nitrates/nitrites; PHAGO- Phagocytic activity of coelomocytes.

SPICE	Innate Immune Parameter					
	CV %	TC %	NEU %	RB %	NOX %	PHAGO %
Cinnamon	-33.0	11.0	-26.9	-79.4	-137.2	-18.0
Pepper	-33.0	-49.5	-42.0	-1166.2	334.0	-65.8
AVG	-33.0	-19.3	-34.5	-622.8	98.4	-41.9

1.7.2 Overall effect of herbs on innate immune parameters

The overall effect of treatment with dietary herbs of the *Lamiaceae* family including oregano, basil, rosemary, sage and thyme, indicated modulation of all of the innate immune parameters examined. In the present study, we observed an overall increase in the total number and the viability of coelomocytes by ~40% and ~12%, respectively, following treatment with *Lamiaceae* herbs (Table 16). The percentage of cells committed as phagocytic neutrophils was increased by 3.25% overall, which was correlated with a ~10% increase in phagocytosis and a ~30% increase in respiratory burst. Nitric oxide production was increased overall by treatment with *Lamiaceae* herbs by approximately 10% compared to the control. As discussed previously, these

observations reiterate the potential role of nitric oxide in hematopoiesis and myeloid cell differentiation.[128-133] Furthermore, these results also suggest that secondary metabolites in *Lamiaceae* herbs may mediate these effects by increasing nitric oxide in the coelomic fluid. Additionally, as discussed above, a stoichiometric increase in respiratory burst with total coelomocyte count, a departure from the often observed exponential increase, may be due to a decrease in NADPH oxidase assembly accompanied by an increased NRF2 mediated expression of ARE regulated antioxidant genes conferring a better cytosolic oxidative buffering power.[147,148] However, within the *Lamiaceae* herbs tested, modulation of the innate parameters by basil and oregano varied significantly than that of rosemary, sage and thyme.

Table 16. Overall effect of herbs on innate immune parameters in *L. terrestris*. Overall effect of each herb, calculated as the average of 0.1% and 0.5% of the herb, and reported as the percent change compared to control. CV- Coelomocyte viability; TC- Total coelomocyte count; NEU- Neutrophil distribution; RB- Respiratory burst activity; NOX- Nitric Total nitric oxide metabolites measures as nitrates/nitrites; PHAGO- Phagocytic activity of coelomocytes.

HERB	Innate Immune Parameter					
	CV %	TC %	NEU %	RB %	NOX %	PHAGO %
Basil	7.6	34.8	6.7	21.4	11.1	11.4
Oregano	28.5	50.1	10.4	28.1	4.7	15.1
Rosemary	2.9	47.4	-1.4	26.1	36.6	7.7
Sage	23.8	43.0	10.4	33.0	-0.8	18.0
Thyme	-3.3	26.6	-9.9	41.5	-0.8	-3.4
AVG	11.9	40.4	3.3	30.0	10.1	9.8

1.7.2.1 Overall effect of basil and oregano on innate immune parameters

Treatment with basil and oregano exhibited immunomudulatory effects similar to that of turmeric and ginger (Table 17). We observed increases in total cell count by ~42% over the control. Viability of coelomocytes was similarly increased by ~18% compared to control. However, unlike *Zingiberaceae* spices, the percentage of cells differentiating into neutrophils was nearly 50% lower following treatment with oregano and basil, although still ~9% higher than control. Phagocytic and respiratory burst activity, were observed to approximate that of *Zingiberaceae* spices with increases of ~13% and ~25%, respectively, over the control. Thus, although the level of circulating neutrophils capable of executing phagocytosis and respiratory burst was approximately 50% less than in worms treated with *Zingiberaceae* spices, the innate functions mediated by these cells were comparable. Interestingly, the increase in total coelomocyte count but lower number of neutrophils upon treatment with basil and oregano was also accompanied by lower detection of nitric oxide metabolites in the coelomic fluid, suggesting that the bioactive compounds in basil and oregano may not be significantly mediating hematopoiesis via nitric oxide. Nevertheless, since lower levels of nitric oxide metabolites were observed in the coelomic fluid with a concomitant decrease in the percentage of neutrophils, these observations may reiterate the role of nitric oxide in myeloid differentiation.[128-133] However, a nearly equal increase in phagocytic function with an almost linear increase in respiratory burst might indicate an enhanced efficiency in the recognition and removal of foreign epitopes potentially via increasing the expression of MGAT-3 and TLR, as previously described.[50,52] Basil and oregano, being high in phenolic phytochemicals may

also promote enhanced antioxidant protection to the phagocytic cells by increasing expression of ARE controlled antioxidant genes as discussed previously.[149,150]

Table 17. Overall effect of basil and oregano on innate immune parameters in *L. terrestris*. Overall effect of each herb, calculated as the average of 0.1% and 0.5% of the herb, and reported as the percent change compared to control. CV- Coelomocyte viability; TC- Total coelomocyte count; NEU- Neutrophil distribution; RB- Respiratory burst activity; NOX- Total nitric oxide metabolites measures as nitrates/nitrites; PHAGO- Phagocytic activity of coelomocytes.

HERB	Innate Immune Parameter					
	CV %	TC %	NEU %	RB %	NOX %	PHAGO %
Basil	7.6	34.8	6.7	21.4	11.1	11.4
Oregano	28.5	50.1	10.4	28.1	4.7	15.1
AVG	18.1	42.5	8.6	24.7	7.9	13.2

1.7.2.2 Overall effect of rosemary on innate immune parameters

When we treated worms with rosemary, modulation of the innate immune system was observed although very different than by basil and oregano (Table 16). Although total coelomocyte counts increased by ~47% over the control, this was not correlated with a significant increase in coelomocyte viability or a shift in the distribution of myeloid cells. In fact, the percentage of neutrophils decreased by ~1.4% compared to control. However, an increase in respiratory burst activity and nitric oxide production was observed by ~26% and ~37%, respectively. Additionally, a moderate increase in

phagocytosis by ~8% over the control was noted (Table 16). Thus, although the percentage of neutrophils in the coelomic fluid was actually slightly lower than the control and phagocytosis was only minimally increased, oxidative degradation via respiratory burst and nitric oxide production remained relatively high. This increase in respiratory burst may be due to a higher target epitope to cell ratio, which may increase the formation of phagosomes per cell and result in increased assembly of NADPH oxidase. Even though rosemary increased the number of nitric oxide in coelomic fluid, it did not increase coelomocyte differentiation, suggesting that in *Lamiaceae* herbs such as basil and oregano, there may be an additional bioactive ingredient which may contribute to a polarized differentiation of myeloid progenitor cells. Rittenhouse et al. (1991) demonstrated a similar effect on respiratory burst activity by two Chinese medicinal herbs.[162] They observed a dose dependent increase in respiratory burst activity in immunosuppressed macrophages following incubation with either *Astragalus membranaceus* or *Ligustrum lucidum.*

1.7.2.3 Overall effect of sage on innate immune parameters

Treatment with sage resulted in increases in virtually all innate immune parameters (Table 16). Total coelomocyte counts were increased by ~43%, viability of coelomocytes was increased by ~23% and the percentage of myeloid cells differentiating into neutrophils was increased by ~10.4% over the control. This increase in neutrophils was associated with increases in neutrophil driven processes including a ~18% increase in phagocytosis and a ~33% increase in respiratory burst (Table 16). However, treatment with sage was not associated with an increase in nitric oxide production, which was actually decreased by 0.8% from the control suggesting the presence of unique

phytochemicals in sage that may promote hematopoiesis and myeloid differentiation independent of nitric oxide. As mentioned previously, plant polysaccharides have been shown to exhibit immunostimulating activity. Moreover, Capek and Hribalova (2004) recently identified unique polysaccharides in sage with immunomodulatory properties.[163]

These findings indicate immunomodulatory activity of dietary herbs and spices varies significantly. Although plants belonging to the same family share similar mechanisms of action, variations in immune modulating activity still exist. In *Zingiberaceae* spices, we observed an overall increase in the number of circulating neutrophils which was correlated with increases in phagocytosis and respiratory burst. In *Lamiaceae* herbs, however, the modest increase in neutrophil distribution was associated with nearly equal phagocytic and respiratory burst activities as worms treated with *Zingiberaceae* spices. Thus, these preliminary results suggest that modulation of the innate immune system via multiple pathways is occurring and may be attributed to differences in phytochemical profiles inherent to each family of herb or spice. It also appears that within the same family, the individual herbs may differ in their biochemical composition which may affect their immunomudulatory properties.

CHAPTER II

EFFECT OF DIETARY HERBS AND SPICES ON CYCLOPHOSPHAMIDE INDUCED IMMUNOSUPPRESSION

2.1 Introduction

As an anticancer drug, cyclophosphamide (CP) is commonly used therapeutically for the treatment of a variety of carcinomas and diseases of the immune system.[164,165] CP is used as an anticancer adjuvant primarily because of its DNA alkylating properties resulting in disruption of the normal cell cycle thereby effectively reducing the risk of reoccurrence.[164,166] Following activation of CP in the liver, it is converted to a number of metabolites including phosphoramide mustard and acrolein (Figure 13).[164,167] Phosphoramide mustard specifically, imparts anti-neoplastic activity by forming DNA adducts at the guanine N-7 position.[167] Because of the non-specific cytoxicity associated with CP, this drug has been implicated for the management of several forms of leukemia, breast cancer, Burkitt's Lymphoma, Hodgkin's disease, Non-Hodgkin's Lymphoma, and Rheumatoid arthritis, among others.[164,167] CP, in conjunction with adoptive immunotherapy treatment, has been shown to suppress tumorigenesis and enhance the antitumor response *in vivo*.[164] However, although an effective anticancer agent, CP is also associated with many negative side effects which are a direct result of its immunotoxic and cytotoxic activity. These include increased susceptibility to infection due to myelosuppression increasing the risk of patient mortality.[168] Specifically, risk for severe infection occurs when neutrophil counts diminish to 1000 cells/mm^3.[169] Furthermore, CP

induced febrile neutropenia may delay chemotherapy regimens if intravenous antibiotics are required.[169]

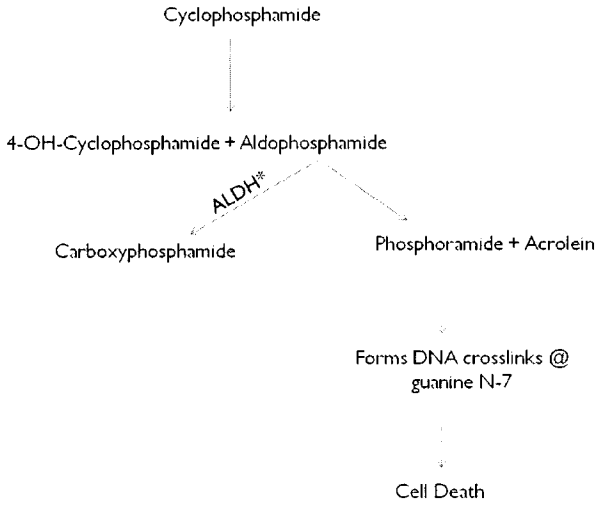

Figure 13. Metabolism of cyclophosphamide.

Therefore, agents capable of countering the myelosuppressive effects of CP may have therapeutic potential in disease management where it is required. Relatively recent research has indicated a protective role of specific dietary herbs and medicinal plants capable of exerting protective effects against CP induced neutropenia.[170-172]

Medicinal plants used in traditional Ayurvedic medicine including *Asparagus racemosus, Tinospora cardifolia* and *Withania somnifera* were shown to significantly increase total leukocyte and neutrophil count in mice co-treated with 100 mg/kg/day CP.[172] Inhibition of myelosuppression by these medicinal plants was also associated with increased levels of serum colony-stimulating factor.[172] Methanolic extracts of *Morus alba* leaves (Mulberry) were also shown to exert myeloprotective effects in mice co-

administered 200 mg/kg CP for ten days.[173] In that study, a 75.71% reduction in total neutrophil count was noted in mice treated with CP only. However, in mice treated with CP and 100 mg/kg *Morus alba* or 1 g/kg *Morus alba*, total neutrophil counts were reduced by 13.71% and 59.14%, respectively, potentially indicating its role in hematopoiesis.[173] In a similar study, treatment with aqueous extract of the medicinal plant *Cassia occidentalis*, was shown to significantly increase total leukocyte counts to normal concentrations in mice co-treated with 50 mg/kg CP.[170] Methanol extracts of *Phyllanthus amarus* at 250 mg/kg and 750 mg/kg significantly increase the total leukocyte counts as well as the population of mature monocytes in mice treated with 25 mg/kg CP for 14 days.[174] Our objective was to further evaluate for the potential immunoprotective capacity of top performing herbs and spices from our previous results against myelosuppression induced by treatment with CP.

2.2 Methods

2.2.1 Cyclophosphamide treatment

Mature, primed worms were selected for experimentation, as previously described in Chapter I (Pg 14-15). Experimental worms were treated with 50 mg/kg/day cyclophosphamide (CP) which was dissolved in 0.9% NaCl and added to LGM agar just prior to pouring. The dose of 50 mg/kg/day CP was chosen because it was it was determined to be approximately the average dose of CP cited in previous literature in which an immunosuppressive effect was achieved.[170-174] Treatment plates contained 0.1% (w/v) or 0.5% (w/v) extract plus 50 mg/kg CP. For each extract tested, thirty six primed worms were divided into 6 groups (n=6) and were allowed to feed *ad libitum* for 2, 4 or 6

days. Worms were maintained at 18-20°C in the dark. Plates were renewed on the fourth day of treatment. Experimental groups included: **Control**- 0 mg CP + 0% extract; **CP**- 50 mg/kg CP + 0.0% extract; **CPT**- 50 mg/kg CP + 0.1% (w/v) Turmeric; **CPG**-50 mg/kg CP + 0.1% or 0.5% (w/v) Ginger; **CPS**- 50 mg/kg CP + 0.1% or 0.5% (w/v) Sage; **CPO**- 50 mg/kg CP + 0.1% or 0.5% (w/v) Oregano. Worms feeding on LGM agar only were established as control. Coelomocytes were harvested via ethanol extrusion, as previously described. Total coelomocyte count, coelomocyte viability, differential coelomocyte count, phagocytic activity, respiratory burst and nitric oxide production were determined, as previously described in Chapter I (Pg 15-18).

2.2.2 Statistical Analysis

Statistical significance was determined using a Student's one tailed t-test. Treatment with CP was compared to control worms feeding on LGM only. Worms treated with CP plus herb or spice was compared to control worms feeding on LGM only and worms feeding on CP only. Statistical significance was indicated by p values of <0.05.

2.3 Results of herb/spice CP study

2.3.1 Effect of herb/spice treatment on total coelomocyte count in CP treated *L. terrestris*

Total coelomocyte count (TCC) was determined using an Improved Neubauer 1/400 sq. mm hemacytometer, as previously described. Control worms were maintained on *Lumbricus* Growth Medium (LGM) comprised of 0.31% (w/v) Gerber oatmeal in 1.25% (w/v) agar. Over the six day treatment, TCC for control worms was 2.31×10^6, 3.03×10^6, and 2.49×10^6 on day 2, day 4, and day 6 of the experiment, respectively

(Table 18). Treatment with cyclophosphamide (50 mg/kg) (CP) resulted in decreased TCC on days 2 and 4 of the treatment. However, TCC was significantly higher than control on day 6 of the treatment with 5.0% (w/w) CP (Table 18). On day 2, day 4, and day 6 of the treatment, TCC was 2.26 x 10^6 (p=0.001), 2.86 x 10^6 (p=0.006) and 2.78 x 10^6 (p=0.033) respectively. Overall, TCC was lower in CP treated worms than control although assessment of the AUC did not indicate a significant difference over the duration of the treatment (p=0.277) (Figure 14).

Compared to worms feeding on CP only, treatment with CP plus 0.1% (w/v) turmeric resulted in higher TCC on all days of the treatment with significant increases occurring on day 2 (p=0.025) and 4 (p=0.015), respectively (Table 18). Interestingly, TCC was also significantly higher than control worms on all days of the experiment. TCC was 2.98 x 10^6 (p=0.001), 3.21 x 10^6 (p=0.006), and 4.86 x 10^6 (p=0.033) on day 2, day 4, and day 6, respectively (Table 18).

In worms treated with 0.1% (w/v) ginger (CPG), TCC was significantly higher than in CP treated worms on all days of the experiment (Table 18). TCC was 3.16 x 10^6 (p=0.049), 5.35 x 10^6 (p=0.000) and 6.71 x 10^6 (p=0.006) on day 2, day 4, and day 6, respectively. Compared to the control, TCC was also significantly higher on day 2 (p=0.003), day 4 (p=0.009) and day 6 (p=0.003) of the treatment (Table 18). In worms treated with 0.5% (w/v) CPG, TCC was lower than in those feeding on only CP on the second day of treatment but significantly higher than CP treated worms on day 4 and day 6. TCC was 1.39 x 10^6 (p=0.164), 3.65 x 10^6 (p=0.000) and 3.94 x 10^6 (p=0.013) on day 2, day 4, and day 6, respectively (Table 18). Similarly, TCC was significantly lower than control on day 2 (p=0.003) but significantly higher on day 4 (p=0.009) and day 6

(p=0.003) in worms feeding on 0.5% (w/v) CPG. Between concentrations, TCC was significantly higher overall following treatment with 0.1% (w/v) CPG than with 0.5% (w/v) CPG as determined by AUC values (p=0.005) (Figure 14).

In worms feeding on CP and 0.1% oregano (CPO), TCC was lower than CP on day 2 but significantly higher on day 4 and 6 (Table 18). TCC over the duration of the experiment was 1.88 x 10^6 (p=0.208), 4.95 x 10^6 (p=0.002) and 4.02 x 10^6 (p=0.003) on day 2, day 4, and day 6, respectively. Likewise, TCC was significantly lower than the control on day 2 (p=0.023) of the experiment but significantly higher on day 4 (p=0.000) and day 6 (p=0.000) of the treatment (Table 18). We did not observe a significant change in TCC in response to treatment with 0.5% (w/v) CPO compared to worms feeding on CP only (Table 18). TCC was 2.17 x 10^6 (p=0.198), 2.90 x 10^6 (p=0.076) and 3.27 x 10^6 (p=0.135) on day 2, day 4, and day 6, respectively. These TCC values were also significantly lower than the control on day 2 (p=0.023) and day 4 (p=0.000). However, TCC was significantly higher than control on day 6 (p=0.031) of the treatment in worms feeding on 0.5% (w/v) CPO. Overall, TCC was significantly higher in worms feeding on 0.1% (w/v) CPO than in those treated with 0.5% (w/v) CPO (p=0.007), as indicated by AUC values (Figure 14).

TCC was significantly higher in response to treatment with CP and 0.1% (w/v) sage (CPS) than in those treated with CP only on day 2 (p=0.008) and 4 (p=0.043) (Table 18). TCC for worms feeding on 0.1% (w/v) CPS was 4.02 x 10^6, 3.00 x 10^6 and 2.58 x 10^6 on day 2, day 4, and day 6, respectively. Compared to the control, TCC was significantly higher following treatment with CP and 0.1% (w/v) sage (CPS) on day 2 (p=0.004) and 6 (p=0.031) of the treatment, but decreased significantly on the fourth day

(p=0.000) (Table 18). Treatment with 0.5% (w/v) CPS resulted in higher TCC on all days compared to worms treated with CP only with significant increases occurring on day 2 (p=0.009) and 4 (p=0.002). On day 2, day 4, and day 6 of the treatment with 0.5% (w/v) CPS, TCC was 3.98 x 10^6, 3.58 x 10^6 and 4.65 x 10^6 respectively. Additionally, these values were all significantly higher than the control (p=0.000, p=0.001, p=0.012 on day 2, 4, and 6, respectively). Between the two concentrations of sage, TCC was significantly higher following treatment with 0.5% (w/v) CPS than with 0.1% (w/v) CPS as evidenced by AUC values (p=0.011) (Figure 14).

Table 18. Effect of herbs/spices on total cell count (TCC) in CP treated *L. terrestris*. TCC was estimated using an Improved Neubauer 1/400 sq. mm hemacytometer and expressed as total cells per milliliter. *Indicates treatment significantly higher than control. **Indicates significantly lower than control. + indicates significantly different from CP. a- concentration of CP is 5.0% (w/w). CPT- 50 mg/kg CP + 0.1% (w/v) turmeric. CPG- 50 mg/kg CP + 0.1% (w/v) ginger. CPO- 50 mg/kg CP + 0.1% (w/v) oregano. CPS- 50 mg/kg CP + 0.1% (w/v) sage. $p<0.05$. n=6.

Treatment	Conc. (w/v)	Total Coelomocyte Count n=6					
		Day 2 (cells/ml)	*P* value	Day 4 (cells/ml)	*P* value	Day 6 (cells ml)	*P* value
CPT	0.10%	2.98×10^6	0.001* 0.025+	3.21×10^6	0.006* 0.015+	4.86×10^6	0.033* 0.099
CPG	0.10%	3.16×10^6	0.003* 0.049+	5.35×10^6	0.009* 0.000+	6.71×10^6	0.003* 0.006+
	0.50%	1.39×10^6	0.003** 0.164	3.65×10^6	0.009* 0.000+	3.94×10^6	0.003* 0.013+
CPO	0.10%	1.88×10^6	0.023** 0.208	4.95×10^6	0.000* 0.002+	4.02×10^6	0.000* 0.003+
	0.50%	2.17×10^6	0.014** 0.198	2.90×10^6	0.000** 0.076	3.27×10^6	0.031* 0.135
CPS	0.10%	4.02×10^6	0.004* 0.008+	3.00×10^6	0.000** 0.043+	2.58×10^6	0.010* 0.198
	0.50%	3.98×10^6	0.000* 0.009+	3.58×10^6	0.001* 0.002+	4.65×10^6	0.012* 0.093
CP		2.26×10^6	0.027**	2.86×10^6	0.059	2.78×10^6	0.007**
Control	---	2.31×10^6	---	3.03×10^6	---	2.49×10^6	

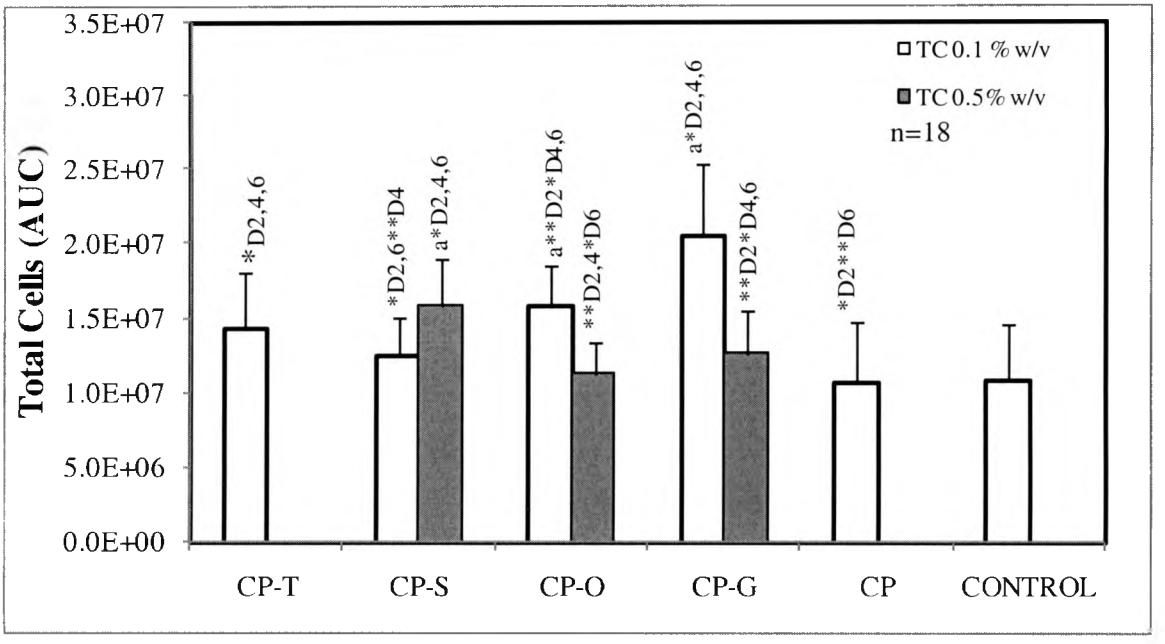

Figure 14. Overall effect of herb/spice on total coelomocyte count (TCC) in CP treated *L. terrestris*. Overall TCC for 0.1% (w/v) and 0.5% (w/v) of each treatment, calculated as area under the curve (AUC) and reported as total number of coelomocytes over the duration of the experiment. The data is represented as means ± SEM. a- indicates the treatment concentration which was significantly different ($p<0.05$). *- indicates significantly higher than control ($p<0.05$). **-indicates significantly lower than control. D2= day 2; D4= day 4; D6= day6 – indicates days when treatment was significantly different from control ($p<0.05$). n=18.

2.3.2 Effect of herb/spice treatment on coelomocyte viability in CP treated *L. terrestris*

Coelomocyte viability (CV) was determined by trypan blue exclusion, as described previously. In control worms, CV was 46.7%, 47.3%, and 50.0% on day 2, day 4, and day 6 of the experiment, respectively (Table 19). Following treatment with cyclophosphamide (CP), CV decreased significantly on day 4 (p=0.003) and day 6 (p=0.008) compared to control worms feeding on LGM only (Table 19). CV in CP treated worms was 38.3% (p=0.397), 29.7% (p=0.003) and 38.3 % (p=0.008) on day 2,

day 4, and day 6, respectively. Overall, CV was significantly lower in worms treated with CP than control over the duration of the treatment, as indicated by AUC values (p=0.007) (Figure 15).

Treatment with 0.1% (w/v) turmeric (CPT) resulted in significantly increased viability of coelomocytes on all days of the experiment when compared to worms treated with CP only (Table 19). CV was 55.7% (p=0.039), 61.7% (p=0.003) and 66.0% (p=0.001) on day 2, day 4, and day 6, respectively. Viability of coelomocytes was also significantly higher than the control on day 2 (p=0.002), day 4 (p=0.018) and day 6 (p=0.005) (Table 19).

Viability of coelomocytes was significantly increased in worms feeding on CP and 0.1% (w/v) ginger (CPG) on all days of the experiment compared to worms feeding on CP only (Table 19). On day 2, day 4, and day 6 of the treatment with 0.1% (w/v) CPG, viability of coelomocytes 50.0% (p=0.033), 44.3% (p=0.001) and 60.0% (p=0.000), respectively. When compared to the control, these values were also significantly higher on day 2 (p=0.000) and 6 (p=0.007) of the treatment. However, significantly fewer coelomocytes were viable on day 4 of the treatment with 0.1% (w/v) CPG than in control (p=0.007). In worms treated with 0.5% (w/v) CPG, CV was higher than in those treated with only CP on all days of the treatment with significant increases occurring on day 4 (p=0.000) and day 6 (p=0.000) of the treatment (Table 19). Viability of coelomocytes was 45.3% (p=0.069), 55.7% and 69.7%. Additionally, on day 4 and day 6, CV was significantly higher than the control in worms feeding on 0.5% (w/v) CPG (p=0.013, p=0.000 for day 4 and 6, respectively). Between concentrations of CPG, CV was

significantly higher following treatment with 0.5% (w/v) CPG, as determined by analysis of the AUC (p=0.019) (Figure 15).

Compared to worms treated with CP only, viability of coelomocytes increased on all days of the treatment with CP and 0.1% (w/v) or 0.5% (w/v) oregano (CPO) (Table 19). CV for worms treated with 0.1% (w/v) CPO was 47.3% (p=0.0157), 51.3% (p=0.000) and 44.7% (p=0.001) on day 2, day 4, and day 6 of the experiment, respectively. When evaluated against the control, viability was also significantly higher on day 2 (p=0.020) and day 4 (p=0.002) of the treatment with 0.1% (w/v) CPO (Table 19). For worms treated with 0.5% (w/v) CPO, viability was 50.7% (p=0.045), 49.0% (p=0.000) and 53.3% (p=0.001) on day 2, day 4, and day 6, respectively, compared to CP (Table 19). Assessment of the AUC indicated CV to be slightly higher in worms treated with 0.5% (w/v) CPO than in those treated with 0.1% (w/v) CPO (p=0.067) over the course of the six day treatment (Figure 15).

More coelomocytes were identified as viable in worms feeding on 0.1% (w/v) CP and sage (CPS) than in those feeding on only CP on day 2 (p=0.052) and day 4 (p=0.011) of the treatment (Table 19). A non-significant decrease in CV was observed on day 6 (p=0.122) in worms treated with 0.1% (w/v) CPS than in those feeding on CP. CV was 51.7%, 52.3% and 35.0% on day 2, 4 and 6, respectively. Additionally, CV was significantly higher than the control on day 2 (p=0.006) and day 4 (p=0.033) following treatment with 0.1% (w/v) CPS (Table 19). In worms treated with 0.5% (w/v) CPS, CV was higher than in those feeding on only CP on all days of the treatment (Table 19). Viability of coelomocytes on day 2, day 4, and day 6, was 42.0% (p=0.341), 54.7% (p=0.000) and 45.7% (p=0.013), respectively (Table 19). However, CV was lower than

the control on day 2 (p=0.357) and 6 (p=0.084) but significantly higher on day 4 (p=0.000) (Table 19). Based on AUC values, CV was higher overall in worms treated with 0.5% (w/v) CPS than in worms treated with 0.1% (w/v) CPS although a significant difference was not detected (p=0.085) (Figure 15).

Table 19. Effect of herbs/spices on coelomocyte viability (CV) in CP treated *L. terrestris*. Cell viability, measured by trypan blue exclusion, and reported as percent live coelomocytes per 50 total cells counted. *Indicates significantly higher than control. **Indicates significantly lower than control. CPT- 50 mg/kg CP + 0.1% (w/v) turmeric.$^{+}$ indicates significantly different from CP. CPG- 50 mg/kg CP + 0.1% (w/v) ginger. CPO- 50 mg/kg CP + 0.1% (w/v) oregano. CPS- 50 mg/kg CP + 0.1% (w/v) sage. p<0.05. n=6.

Treatment	Conc. (w/v)	Coelomocyte Viability n=6					
		Day 2 (%)	*P value*	Day 4 (%)	*P value*	Day 6 (%)	*P value*
CPT	0.10%	55.7	0.002* 0.039^{+}	61.7	0.018* 0.003^{+}	66	0.005* 0.001^{+}
CPG	0.10%	50	0.000* 0.033^{+}	44.3	0.007** 0.001^{+}	60	0.015* 0.000^{+}
	0.50%	45.3	0.011** 0.069	55.7	0.013* 0.000^{+}	69.7	0.000* 0.000^{+}
CPO	0.10%	47.3	0.020* 0.157	51.3	0.002* 0.000^{+}	44.7	0.286 0.001^{+}
	0.50%	50.7	0.002* 0.045^{+}	49	0.000* 0.000^{+}	53.3	0.001* 0.001^{+}
CPS	0.10%	51.7	0.006* 0.052	52.3	0.033* 0.011^{+}	35	0.000** 0.122
	0.50%	42	0.357 0.341	54.7	0.0000* 0.000^{+}	45.7	0.084 0.013^{+}
CP		38.3	0.397	29.7	0.003**	38.3	0.008**
Control	---	46.7	---	47.3	---	50	

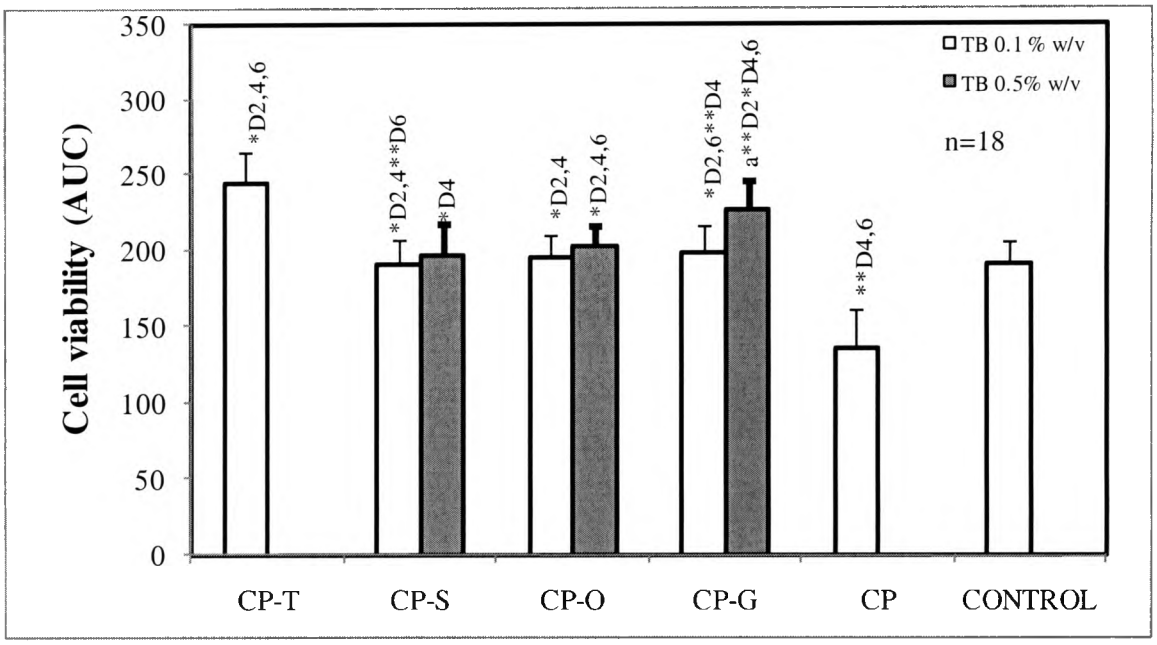

Figure 15. Overall effect of herbs/spices on coelomocyte viability (CV) in CP treated *L. terrestris*. Overall viability at 0.1% (w/v) and each treatment, calculated as of area under the curve, and reported as % viable cells. The data is represented as means ± SEM. a- indicates the treatment concentration which was significantly different ($p<0.05$). *- indicates significantly higher than control ($p<0.05$). **-indicates significantly lower than control. D2= day 2; D4= day 4; D6= day6 – indicates days when treatment was significantly different from control ($p<0.05$). n=18.

2.3.3 Effect of herb/spice treatment on relative neutrophil count in CP treated *L. terrestris*

Differential coelomocyte counts were performed using Wright's Giemsa stain under bright field microscopy, as discussed previously. In control worms feeding on LGM only, the relative neutrophil count (RNC) was 37.7%, 36.9% and 34.7% on day 2, day 4, and day 6, respectively (Table 20). The RNC decreased significantly in worms treated with CP on all days over the duration of the experiment (Table 20). The percentage of neutrophils on day 2, day 4, and day 6 of the treatment was 35.7% (p=0.008), 27.3% (p=0.009), and 34.0% (p=0.021), respectively, which collectively was

significantly lower than control as indicated by AUC values (p=0.015) (Table 20; Figure 16).

Compared to worms feeding on CP, treatment with CP plus 0.1% turmeric (CPT) resulted in significantly higher RNC on all days of the treatment (Table 20). On day 2, day 4, and day 6 of the treatment with CPT, the RNC was 38.3% (p=0.002), 35.7% (p=0.017) and 44.7% (p=0.003). Additionally, compared to the control, RNC was also significantly higher on day 2 (p=0.000) and day 6 (p=0.006) of the treatment (Table 20).

In worms treated with CP and 0.1% (w/v) (CPG), the RNC was higher than in those treated with only CP on day 4 and 6 of the treatment (Table 20). The RNC was 32.7% (p=0.227), 35.7% (p=0.029) and 45.0% (p=0.002) on day 2, day 4 and day 6, respectively. A significant increase compared to the control was also observed on day 6 (p=0.000) of the treatment with 0.1% (w/v) CPG (Table 20). However, the population of neutrophils was lower than the control on day 2 (p=0.384) and 4 (p=0.177) of the treatment with 0.1% (w/v) CPG. Significant increases in RNC values were observed on day 4 and 6 in worms treated with 0.5% (w/v) CPG compared to worms feeding on CP only (Table 20). RNC on day 2, 4 and 6 was 29.2% (p=0.097), 42.7% (p=0.001) and 39.0% (p=0.001), respectively. Compared to the control, RNC was significantly higher on day 4 (p=0.000) and day 6 (p=0.005) of the treatment with 0.5% CPG (Table 20). Overall, treatment with 0.5% (w/v) was assessed to be more effective at increasing the RNC compared to treatment with 0.1% CPG as indicated by AUC values (p=0.083) (Figure 16).

For worms feeding on CP and 0.1% (w/v) oregano, the RNC was significantly higher on all days of the treatment than in worms feeding on CP only (Table 20). At 0.1% (w/v) CPO, RNC was 43.3% (p=0.008), 40.7% (p=0.003) and 44.3% (p=0.007) on day 2, day 4, and day 6, respectively. Additionally, this was significantly higher than the control on day 2 (p=0.001), day 4 (p=0.001) and day 6 (p=0.000) (Table 20). For worms feeding on 0.5% (w/v) CPO, the RNC was higher than worms feeding on CP on day 2 and day 4. On those days the RNC was 41.3% (p=0.137), and 33.3% (p=0.015), respectively (Table 20). However, the RNC was lower than CP only treated worms on day 6 (p=0.331). Compared to the control, the RNC was significantly higher than control on day 2 but lower on day 4 (p=0.380) and significantly lower on day 6 (p=0.008). Assessment of the AUC indicated RNC to be significantly higher in worms treated with 0.1% (w/v) CPO than in those treated with 0.5% (w/v) CPO over the duration of the six day treatment (p=0.013) (Figure 16).

Treatment with CP and 0.1% (w/v) sage (CPS) resulted in a RNC than in worms treated with only CP on all days of the experiment (Table 20). On day 2, day 4, and day 6, the RNC was 41.0% (p=0.002), 48.3% (p=0.001), and 37.0% (p=0.107), respectively. Likewise, these values were also significantly higher than in control worms (p=0.005, p=0.004, p=0.032 for day 2, 4 and 6, respectively) (Table 20). However, treatment with 0.5% (w/v) CPS resulted in lower RNC values than in worms feeding on only CP on day 2 and day 6 of the treatment. The RNC for worms treated with 0.5% (w/v) CPS was 30.0% (p=0.015), 32.3% (p=0.147) and 33.3% (p=0.318) (Table 20). Additionally, this was lower than the control on day 2 (p=0.097), day 4 (p=0.453) and day 6 (p=0.115) of

the treatment. Overall, 0.1% (w/v) CPS was significantly more effective at increasing RNC than 0.5% (w/v) CPS, as determined by analysis of the AUC (Figure 16).

Table 20. Effect of herbs/spices on relative neutrophil count (RNC) in CP treated *L. terrestris*. The distribution of cell type, determined by differential staining using Wright's stain, and expressed as the percent neutrophils per 50 total cells counted. *Indicates significantly higher than control. **Indicates significantly lower than control. + indicates significantly different than CP. CPT- 50 mg/kg CP + 0.1% (w/v) turmeric. CPG- 50 mg/kg CP + 0.1% (w/v) ginger. CPO- 50 mg/kg CP + 0.1% (w/v) oregano. CPS- 50 mg/kg CP + 0.1% (w/v) sage. $p<0.05$. n=6.

Treatment	Conc. (w/v)	Relative Neutrophil Count n=6 Day 2 (%)	*P value*	Day 4 (%)	*P value*	Day 6 (%)	*P value*
CPT	0.10%	38.3	0.000* 0.002+	35.7	0.09 0.017+	44.7	0.006* 0.003+
CPG	0.10%	32.7	0.384 0.227	35.7	0.177 0.029+	45.0	0.000* 0.002+
	0.50%	29.2	0.107 0.097	42.7	0.000* 0.001+	39.0	0.005* 0.001+
CPO	0.10%	43.3	0.001* 0.008+	40.7	0.001* 0.003+	44.3	0.000* 0.007+
	0.50%	41.3	0.017* 0.137	33.3	0.38 0.015+	33.0	0.008** 0.331
CPS	0.10%	41.0	0.005* 0.002+	48.3	0.004* 0.001+	37.0	0.032* 0.107
	0.50%	30.0	0.097 0.015++	32.3	0.453 0.147	33.3	0.115 0.318
CP		35.7	0.008**	27.3	0.009**	34.0	0.021**
Control	---	37.7		36.9		34.7	

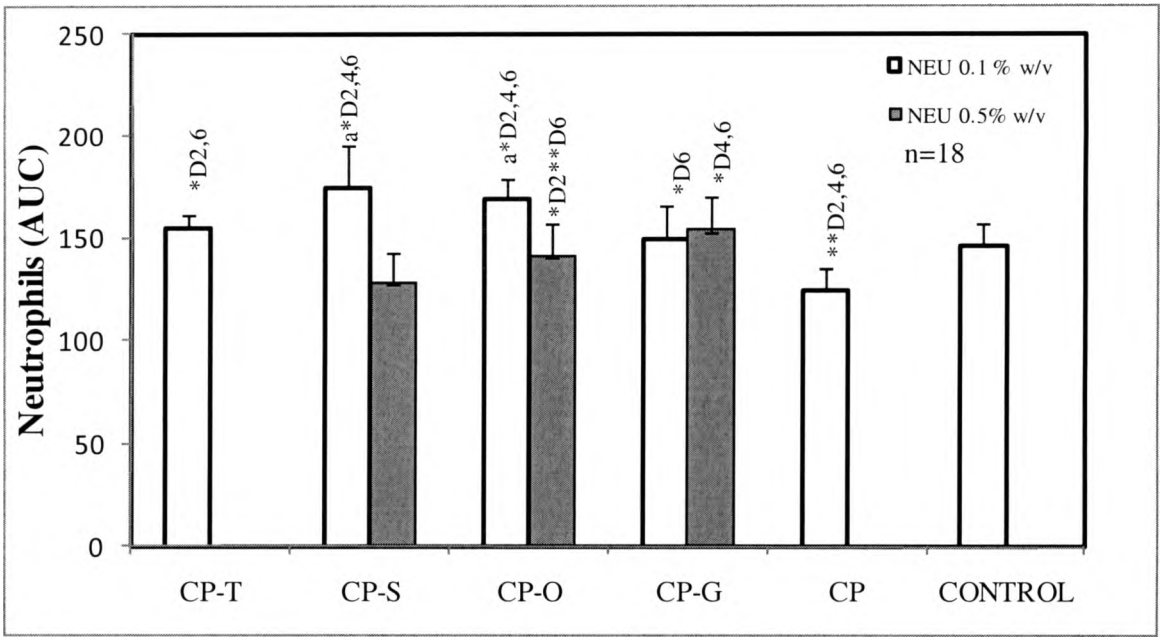

Figure 16. Overall effect of herbs/spices on relative neutrophil count (RNC) in CP treated *L. terrestris*. Overall neutrophil distribution for 0.1% (w/v) and 0.5% (w/v) of treatment calculated as area under the curve (AUC) and expressed as percent neutrophils. The data is represented as means ± SEM. a- indicates the treatment concentration which was significantly different ($p<0.05$). *- indicates significantly higher than control ($p<0.05$). ** indicates significantly lower than control. D2= day 2; D4= day 4; D6= day 6 – indicates days when treatment was significantly different from control ($p<0.05$). n=18.

2.3.4 Effect of herb/spice treatment on phagocytic activity in CP treated *L. terrestris*

Phagocytosis was stimulated by incubating coelomocytes extracted from experimental and control worms with *S. cerevisiae* for 24 hours, as discussed previously. Phagocytic activity of coelomocytes extracted from control worms feeding on LGM only, was 53.1%, 56.4% and 56.1% on day 2, day 4, and day 6, respectively (Table 21). Following treatment with CP, phagocytic activity decreased on day 2 and day 4 of the experiment compared to control (Table 21). At these time points, the percentage of actively phagocytic coelomocytes was 50.3% (p=0.270) and 41.3 (p=0.254), respectively. Phagocytic activity on day 6 was 57.7%, which was significantly higher than control

(p=0.031). AUC values indicate phagocytic activity to be significantly lower in worms treated with CP than control over the duration of the six day experiment (p=0.016) (Figure 17).

Phagocytic activity of coelomocytes in worms treated with CP and 0.1% (w/v) turmeric (CPT) was higher than in worms feeding on only CP on all days of the treatment with significant increases occurring on day 2 and day 4 (Table 21). On day 2, day 4, and day 6, phagocytic activity was 63.7% (p=0.007), 61.7% (p=0.008), and 65.0% (p=0.063). Additionally this was significantly higher than the control on day 2 (p=000), day 4 (p=0.001) and day 6 (p=0.000). Analysis of the AUC revealed phagocytic activity to be significant higher in worms treated with 0.1% (w/v) CPT than control over the duration of the experiment (p=0.019) (Figure 17).

When worms were treated with CP and 0.1% (w/v) ginger (CPG), phagocytic activity increased compared to those treated with only CP on all days of the treatment (Table 21). On day 2, day 4 and day 6, phagocytic activity of coelomocytes was 51.3% (p=0.227), 69.3% (p=0.029), and 66.3% (p=0.002) on day 2, day 4, and day 6, respectively. Phagocytic activity was also significantly higher than the control on day 4 (p=0.005) and day 6 (p=0.000) but significantly lower than the control on day 2 (p=0.001). Following treatment with 0.5% (w/v) CPG phagocytic activity was higher than in CP only treated worms on day 2, 4 and 6. On these days, phagocytic activity was 50.7% (p=0.090), 62.3% (p=0.001) and 62.7.0% (p=0.001), respectively (Table 21). When these values were compared to the control, phagocytic activity was significantly higher on day 4 (p=0.014) and day 6 (p=0.001). AUC values indicate phagocytic activity

of coelomocytes was significantly higher overall following treatment with 0.1% (w/v) CPG than in those treated with 0.5% (w/v) CPG (p=0.033) (Figure 17).

Phagocytic activity of coelomocytes extracted from worms treated with 0.1% (w/v) CP and oregano (CPO) was significantly higher than CP treated worms on all days of the treatment (Table 21). On day 2, day 4, and day 6, phagocytic activity was 59.3% (p=0.008), 53.0% (p=0.003) and 59.0% (p=0.007), respectively. Additionally, phagocytic activity of worms feeding on 0.1% (w/v) CPO was significantly higher than the control on day 2 (p=0.003) and 6 (p=0.004) but significantly lower on day 4 (p=0.007). Similarly, in worms treated with 0.5% (w/v) CPO, phagocytic activity was higher than in those feeding on only CP on day 2 and 4 of the treatment. Phagocytic activity of coelomocytes extracted from these worms was 60.3% (p=0.137), 53.0% (p=0.015), and 57.7% (p=0.331) on day 2, day 4, and day 6, respectively. This was also higher than the control on day 2 (p=0.001) and day 6 (p=0.012) but significantly lower than control on day 4 (p=0.039). AUC values indicate phagocytic activity to be higher following treatment with 0.1% (w/v) CPO than with 0.5% (w/v) CPO (p=1.68) over the duration of the experiment (Figure 17).

Phagocytic activity of coelomocytes extracted from worms feeding on CP supplemented with 0.1% (w/v) sage (CPS) was higher than in CP treated worms on day 4 and 6 of the treatment but lower on day 2 (Table 21). On day 2, 4, and 6 phagocytic activity was 45.7% (p=0.422), 55.0% (p=0.059) and 61.0% (p=0.119), respectively. When compared to the control, phagocytic activity was lower in worms treated with 0.1% CPS on day 2 (p=0.106) and day 4 (p=0.002) but significantly higher on day 6 (p=0.000) (Table 21). Phagocytic activity was also observed to be higher following treatment with

0.5% (w/v) CPS than in worms feeding on only CP on day 4 (p=0.074) and day 6 (p=0.006) but lower on day 2 (p=0.103). On day 2, 4 and 6 of the treatment with 0.5% (w/v) CPS, phagocytic activity was 47.7%, 56.0%, and 64.7%, respectively. These values were significantly lower than the control on day 4 (p=0.000) but significantly higher than the control on day 6 (p=0.008). Overall, more coelomocytes were identified as actively phagocytosing *S. cerevisiae* following treatment with 0.5% (w/v) CPS than with 0.1% (w/v) CPS (p=0.071) over the duration of the experiment (Figure 17).

Table 21. Effect of herbs/spices on phagocytic activity (PA) in CP treated *L. terrestris*. PA of coelomocytes, determined by counting the number of cells containing one or more *S. cerevisiae* particles per 50 total cells and reported as percent phagocytic cells. *Indicates significantly higher than control. **Indicates significantly lower than control. $^{+}$ indicates significantly different than CP. CPT- 50 mg/kg CP + 0.1% (w/v) turmeric. CPG- 50 mg/kg CP + 0.1% (w/v) ginger. CPO- 50 mg/kg CP + 0.1% (w/v) oregano. CPS- 50 mg/kg CP + 0.1% (w/v) sage. p<0.05. n=6.

Treatment	Conc. (w/v)	Phagocytic Activity n=6					
		Day 2 (%)	*P value*	Day 4 (%)	*P value*	Day 6 (%)	*P value*
CPT	0.10%	63.7	0.000* 0.007+	61.7	0.001* 0.008+	65	0.000* 0.063
CPG	0.10%	51.3	0.001** 0.227	69.3	0.005* 0.029+	66.3	0.000* 0.002+
	0.50%	50.7	0.175 0.09	62.3	0.014* 0.001+	62.7	0.001* 0.001+
CPO	0.10%	59.3	0.003* 0.008+	53	0.007** 0.003+	59	0.004* 0.007+
	0.50%	60.3	0.001* 0.137	53	0.039** 0.015+	57.7	0.012* 0.331
CPS	0.10%	45.7	0.106 0.422	55	0.002** 0.059	61	0.000* 0.119
	0.50%	47.7	0.058 0.103	56	0.000** 0.074	64.7	0.008* 0.006+
CP		50.3	0.27	41.3	0.254	57.7	0.031*
Control	---	53.1		56.4		56.1	

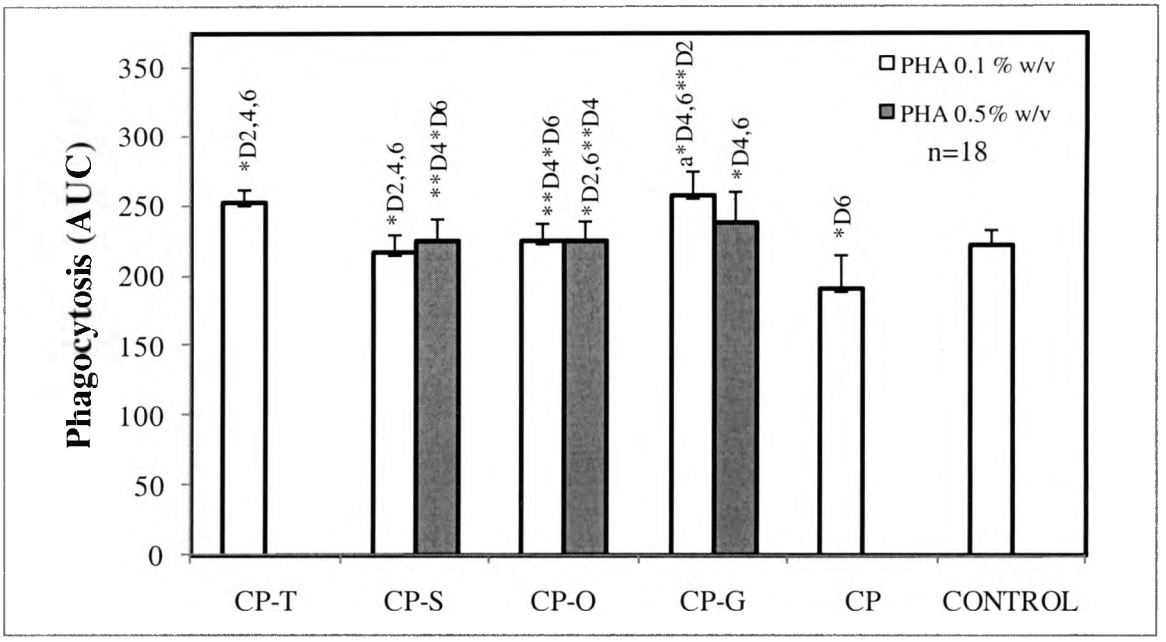

Figure 17. Overall effect of herbs/spices on phagocytic activity (PA) in CP treated *L. terrestris*. Overall PA at 0.1% (w/v) and 0.5% (w/v) of each treatment calculated as area under the curve (AUC) and expressed as percent total activity over the duration of the experiment. The data is represented as means ± SEM. a- indicates the treatment concentration which was significantly different ($p<0.05$).*- indicates significantly higher than control ($p<0.05$). **-indicates significantly lower than control. D2= day 2; D4= day 4; D6= day6 – indicates days when treatment was significantly different from control ($p<0.05$). n=18.

2.3.5 Effect of herb/spice treatment on respiratory burst activity in CP treated *L. terrestris*

Respiratory burst (RB) activity was indirectly measured via Nitroblue Tetrazolium Reduction assay and expressed as the absorbance of diformazan at 570 nm. The relative absorbance of diformazan in control worms maintained on LGM only, was 1.09 AU, 1.07 AU, and 1.05 AU on day 2, day 4, and day 6, respectively. RB activity in worms treated with CP significantly increased on all days throughout the duration of the experiment (Table 22). The relative absorbance of diformazan for CP treated worms was 1.38 AU (p=0.000), 2.00 AU (p=0.000), and 0.99 AU (p=0.003) on day 2, day 4 and day 6, respectively (Table 22). Assessment of the AUC indicates RB activity to be

significantly greater in worms treated with CP compared to control over the duration of the experiment (p=0.005) (Figure 18).

Following treatment with CP and 0.1% (w/v) turmeric (CPT), RB activity was lower than in worms feeding on only CP on day 2 and 4 but significantly higher on day 6 (Table 22). On day 2, day 4, and day 6, the relative absorbance of diformazan was 1.3 AU (p=0.000), 1.9 AU (p=0.055) and 1.9 AU (p=0.000), respectively. However, this was significantly higher than control worms on day 2 (p=0.004), day 4 (p=0.002) and day 6 (p=0.000). AUC values indicate RB activity was higher overall in worms feeding on 0.1% (w/v) CPT than control, as evidenced by AUC values (p=0.005) (Figure 18).

RB activity in worms treated with CP and 0.1% (w/v) was lower than in CP treated worms on days 2 and 4 but significantly higher on day 6 (Table 22). In worms treated with 0.1% (w/v) CPG, the relative absorbance of diformazan was 1.5 AU (p=0.045), 1.8 AU (p=0.273), and 2.3 AU (p=0.011) on day 2, day 4, and day 6, respectively. With respect to control, these values were significantly higher on day 2 (p=0.001), day 4 (p=0.001) and day 6 (p=0.000). For worms feeding on 0.5% (w/v) ginger, RB activity was higher than in CP treated worms on all days of the treatment. The relative absorbance of diformazan 1.5 AU (p=0.180), 2.1 AU (p=0.058) and 1.7 AU (p=0.001) on day2, day 4, and day 6, respectively (Table 22). When compared to the control, RB activity was also significantly higher in worms feeding on 0.5% CPG on day 2 (p=0.000), 4 (p=0.000) and 6 (p=0.000) of the treatment. Analysis of the AUC did not indicate a significant difference overall in RB activity between treatment concentrations of CPG (p=0.352) (Figure 18).

In worms treated with CP and 0.1% (w/v) oregano (CPO), RB activity of coelomocytes decreased significantly compared to those treated with only CP on day 2 and day 4 of the treatment (Table 22). The relative absorbance of diformazan on day 2, day 4, and day 6 was 1.2 AU (p=0.000), 1.8 AU (p=0.007), and 1.7 AU (p=0.000), respectively. However, these values were significantly higher than the control on all days of the treatment (p=0.005, p=0.001, p=0.001 for day 2, 4 and 6, respectively). For worms feeding on 0.5% (w/v) CPO, RB activity was greater on day 2 and 6 but significantly lower on day 4. The relative absorbance of diformazan was 1.6 AU (p=0.048), 1.4 AU (p=0.001) and 1.6 AU (p=0.24) on day 2, day 4, and day 6, respectively (Table 22). Compared to the control, these values were significantly higher on day 2 (p=0.000), day 4 (p=0.002) and day 6 (p=0.000). RB activity was assessed to be greater in worms treated with 0.1% (w/v) CPO than with 0.5% (w/v) CPO as evidenced by AUC values (p=0.025) (Figure 18).

In worms treated with CP and 0.1% (w/v) sage, RB activity was higher than in those treated with only CP on day 2 and significantly higher on day 6 (Table 22). For worms feeding on 0.1% (w/v) CPS, the relative absorbance of diformazan was 1.7 AU (p=0.052), 2.0 AU (p=0.001) and 1.7 AU (p=0.002) on day 2, day 4, and day 6, respectively. With respect to the control, RB was significantly greater on day 2 (p=0.000), day 4 (p=0.000) and day 6 (p=0.001) in worms treated with 0.1% (w/v) CPS. For worms treated with 0.5% (w/v) sage (CPS), RB activity of coelomocytes was higher than in worms feeding on only CP on day 2 and day 6 but significantly lower on day 4 (Table 22). The relative absorbance of diformazan in worms treated with 0.5% (w/v) CPS was 1.5 AU (p=0.001), 1.8 AU (p=0.005) and 1.7 (p=0.000) on day 2, day 4, and day 6,

respectively. However, compared to the control RB activity was significantly higher in worms feeding on 0.5% (w/v) CPS on day 2 (p=0.000), day 4 (p=0.001) and day 6 (p=0.001). Overall, RB activity was determined to be significantly higher in worms treated with 0.1% (w/v) CPS than in those treated with 0.5% (w/v) CPS (p=0.031) as indicated by determination of the AUC (Figure 18).

Table 22. Effect of herbs/spices on respiratory burst activity (RB) in CP treated *L. terrestris*. Respiratory burst activity, indirectly measured as absorbance of diformazan formed during NBT reduction assay, and expressed in absorbance units (AU) at 570 nm. *Indicates significantly higher than control. **Indicates significantly lower than control. + indicates significantly different than CP. CPT- 50 mg/kg CP + 0.1% (w/v) turmeric. CPG- 50 mg/kg CP + 0.1% (w/v) ginger. CPO- 50 mg/kg CP + 0.1% (w/v) oregano. CPS- 50 mg/kg CP + 0.1% (w/v) sage. p<0.05. n=6.

Treatment	Conc. (w/v)	Respiratory Burst Activity n=6					
		Day 2 (AU)	*P value*	Day 4 (AU)	*P value*	Day 6 (AU)	*P value*
CPT	0.10%	1.30	0.004* / 0.000+	1.90	0.002* / 0.055	1.90	0.000* / 0.000+
CPG	0.10%	1.50	0.001* / 0.045+	1.80	0.001* / 0.273	2.30	0.000* / 0.011+
	0.50%	1.50	0.000* / 0.180	2.10	0.000* / 0.058	1.70	0.000* / 0.001+
CPO	0.10%	1.20	0.005* / 0.000+	1.80	0.001* / 0.007+	1.70	0.001* / 0.000+
	0.50%	1.60	0.000* / 0.048+	1.40	0.002* / 0.001+	1.60	0.000* / 0.240
CPS	0.10%	1.70	0.000* / 0.052	2.00	0.000* / 0.001+	1.70	0.001* / 0.002+
	0.50%	1.50	0.000* / 0.001+	1.80	0.001* / 0.005+	1.70	0.001* / 0.000+
CP		1.38	0.000*	2.00	0.000*	0.99	0.003**
Control	—	1.09		1.07		1.05	

n=18

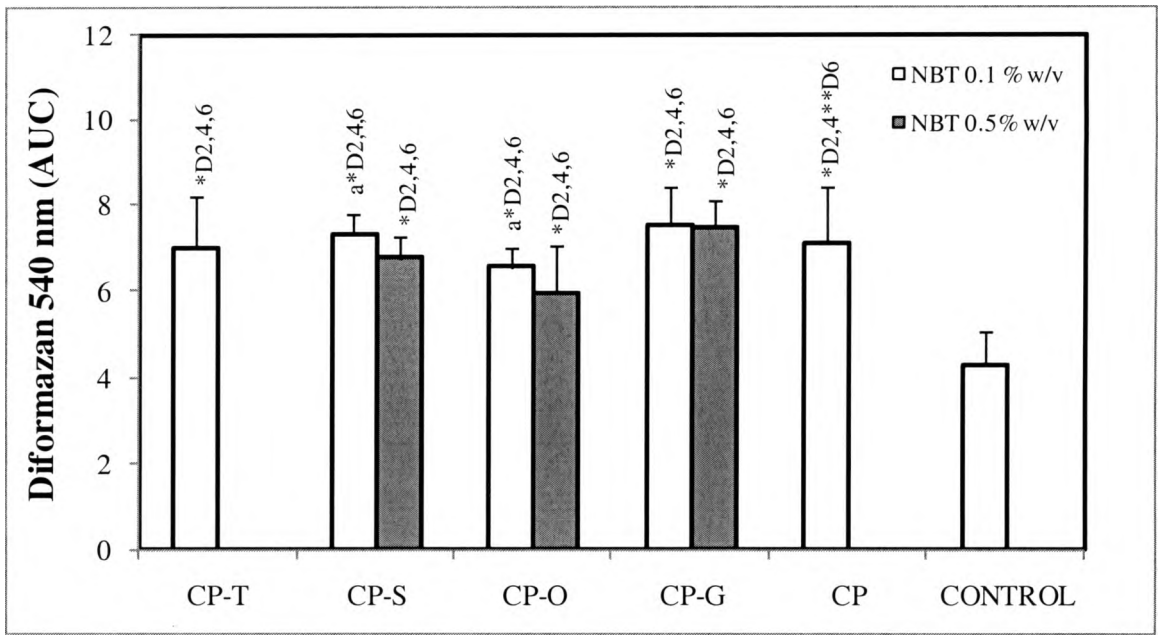

Figure 18. Overall effect of herbs/spices on respiratory burst activity (RB) in CP treated *L. terrestris*. Overall respiratory burst activity for 0.1% (w/v) and 0.5% (w/v) of each treatment, calculated as area under the curve (AUC) and expressed as relative absorbance of diformazan at 570 nm over the duration of the experiment. The data is represented as means ± SEM. a- indicates the treatment concentration which was significantly different ($p<0.05$).*- indicates significantly higher than control ($p<0.05$). **- indicates significantly lower than control. D2= day 2; D4= day 4; D6= day6 – indicates days when treatment was significantly different from control ($p<0.05$). n=18.

2.3.6 Effect of herb/spice treatment on nitric oxide production in CP treated *L. terrestris*

Nitric oxide production was determined using the Griess assay for total nitrites/nitrates (NOx), as described previously. Total NOx for control worms feeding on LGM only was 27.7 µM, 28.8 µM and 27.1 µM on day 2, day 4, and day 6, respectively (Table 23). Following treatment with CP, total NOx was significantly higher than control on day 2 and day 6 of the duration. On these days total NOx was 28.4 µM (p=0.007) and 36.4 µM (p=0.001), respectively (Table 23). However, total NOx on day 4 was 19.3 µM (p=0.021), which was significantly lower than control. Overall, NOx was significantly

lower in worms treated with CP than control over the six duration of the experiment, as evidenced by AUC values (p=0.030) (Figure 19).

When worms were treated with 0.1% (w/v) turmeric (CPT), NOx increased significantly at each time point over the duration of the experiment compared to worms treated with CP only (Table 23). Total NOx in worms treated with 0.1% (w/v) CPT was 32.0 μM (p=0.063), 31.2 μM (p=0.161), and 38.5 μM (p=0.067) on day 2, day 4, and day 6, respectively. These values were also significantly higher than the control on day 2 (p=0.014), day 4 (p=0.000) and day 6 (p=0.000).

Total NOx in worms feeding on 0.1% (w/v) CP and ginger (CPG) was significantly lower than in CP treated worms on day 2 but significantly higher on day 4 and 6 (Table 23). Total NOx was 25.8 μM (p=0.025), 55.0 μM (p=0.004) and 59.9 μM (p=0.031) on day 2, day 4, and day 6, respectively. Similarly, when compared to the control, NOx was also lower on day 2 (p=0.074) but higher on days 4 (p=0.001) and 6 (p=0.006) in worms feeding on 0.1% (w/v) CPG. Total NOx in worms treated with 0.5% (w/v) CPG was higher than worms feeding on only CP on all days of the treatment (Table 23). On day 2, day 4, and day 6 of the treatment, total NOx was 33.2 μM (p=0.145), 53.0 μM (p=0.037), and 48.3 μM (p=0.115) on day 2, day 4, and day 6, respectively. These values were also higher than the control on day 2 (p=0.190), day 4 (p=0.018) and day 6 (p=0.007). Analysis of the AUC indicates total NOx to be higher following treatment with 0.1% (w/v) CPG than in those treated with 0.5% (w/v) CPG (p=0.058) (Figure 19).

In worms treated with CP and 0.1% (w/v) oregano (CPO), NOx was significantly lower than in worms treated with only CP on day 2 but significantly higher on day 4 and

day 6 (Table 23). Total NOx on day 2, day 4, and day 6 was 1.8 μM (p=0.011), 49.5 μM (p=0.012), and 59.8 μM (p=0.004), respectively. With respect to the control, total NOx was lower on day 2 (p=0.088) but significantly higher on day 4 (p=0.000) and day 6 (p=0.000) in worms feeding on 0.1% (w/v) CPO. Likewise, for worms feeding on 0.5% (w/v) CPO, total NOx was lower than in worms feeding on only CP on day 2 but higher on day 4 and day 6. Total NOx in worms treated with 0.5% (w/v) CPO was 5.9 μM (p=0.425), 31.6 μM (p=0.463) and 44.2 μM (p=0.419) on day 2, day 4 and day 6, respectively (Table 23). These values were significantly lower than the control on day 2 (p=0.001) but significantly higher than the control on day 4 (p=0.023) and day 6 (p=0.009). Between concentrations, total NOx was significantly higher overall in worms treated with 0.1% (w/v) CPO, as indicated by AUC (p=0.007) (Figure 19).

Treatment with CP and 0.1% (w/v) or 0.5% (w/v) sage (CPS) resulted in greater total NOx on all days of the treatment compared to worms feeding on CP only (Table 23). For worms treated with 0.1% (w/v) CPS, total NOx was 29.1 μM (p=0.009), 41.6 μM (p=0.029), and 42.6 μM (p=0.392) on day 2, day 4 and day 6, respectively. This was significantly higher than the control on day 2 (p=0.006), day 4 (p=0.002) and day 6 (p=0.000) as well. Total NOx in worms treated with 0.5% (w/v) CPS was 34.1 μM (p=0.018), 45.7 μM (p=0.010) and 36.3 μM (p=0.363), on day 2, day 4, and day 6, respectively which were all greater than worms treated with only CP. Additionally, total NOx in worms treated with 0.5% (w/v) CPS was significantly greater than the control on day 2 (p=0.007), day 4 (p=0.011) and day 6 (p=0.000). AUC values indicate total NOx to be greater in worms treated with 0.5% (w/v) than 0.1% (w/v) sage over the duration of the six day experiment (p=0.056) (Figure 19).

Table 23. Effect of herbs/spices on nitric oxide production (NOx) in CP treated *L. terrestris*. NOx, measured indirectly as total nitrites/nitrates via Griess assay, and reported as μM nitric oxide. *-Indicates significantly higher than control. **Indicates significantly lower than control. CPT- 50 mg/kg CP + 0.1% (w/v) turmeric. $^{+}$ Indicates significantly different than CP. CPG- 50 mg/kg CP + 0.1% (w/v) ginger. CPO- 50 mg/kg CP + 0.1% (w/v) oregano. CPS- 50 mg/kg CP + 0.1% (w/v) sage. $p<0.05$. n=6.

Treatment	Conc. (w/v)	Nitric Oxide Production n=6					
		Day 2 (μM)	*P value*	Day 4 (μM)	*P value*	Day 6 (μM)	*P value*
CPT	0.10%	32	0.014* 0.063	31.2	0.000* 0.161	38.5	0.000* 0.067
CPG	0.10%	25.8	0.074 0.025+	55	0.001* 0.004+	59.9	0.006* 0.031+
	0.50%	33.2	0.19 0.145	53	0.018* 0.037+	48.3	0.007* 0.115
CPO	0.10%	1.8	0.088 0.011+	49.5	0.000* 0.012+	59.8	0.000* 0.004+
	0.50%	5.9	0.001** 0.425	31.6	0.023* 0.463	44.2	0.009* 0.419
CPS	0.10%	29.1	0.006* 0.009+	41.6	0.002* 0.029+	42.6	0.000* 0.392
	0.50%	34.1	0.007* 0.018+	45.7	0.011* 0.010+	36.3	0.000* 0.363
CP		28.4	0.007*	19.3	0.021**	36.4	0.001*
Control	---	27.7	---	28.8	---	27.1	---

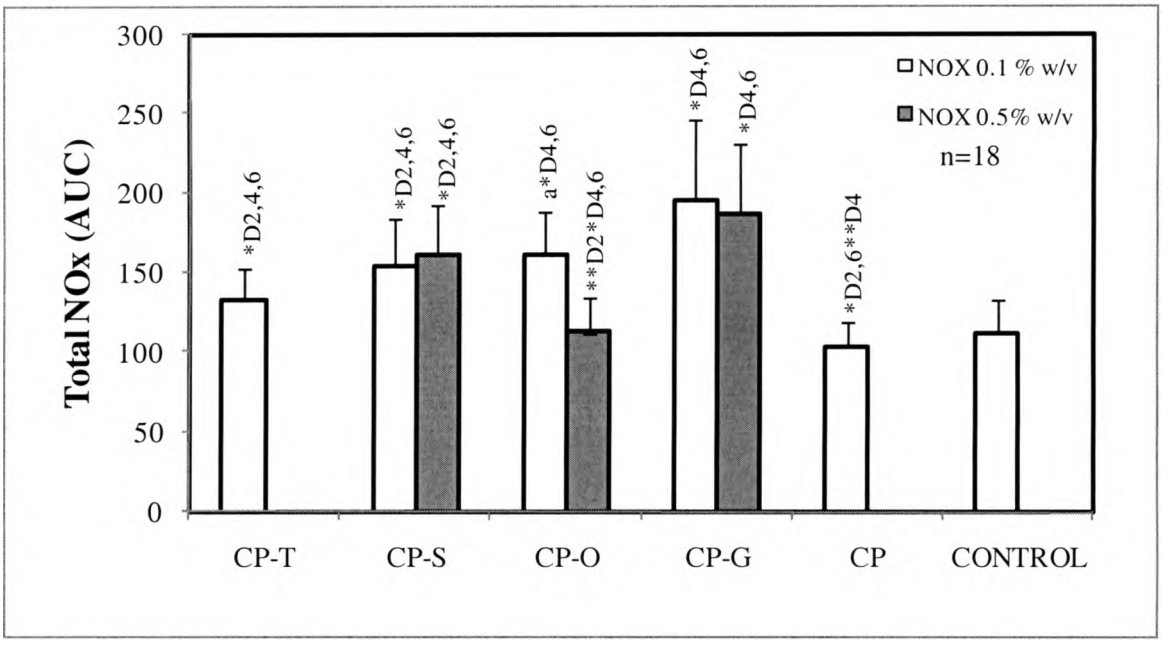

Figure 19. Overall effect of herbs/spices on nitric oxide production (NOx) in CP treated *L. terrestris*. Total nitric oxide production (NOx) for 0.1% (w/v) and 0.5% (w/v) of each treatment, calculated as AUC and reported as μM of total NOx over the duration of the experiment. The data is represented as means $\pm$ SEM. a- indicates the treatment concentration which was significantly different ($p<0.05$).*- indicates significantly higher than control ($p<0.05$). **-indicates significantly lower than control. D2= day 2; D4= day 4; D6= day6 – indicates days when treatment was significantly different from control ($p<0.05$). n=18.

2.4 Discussion cyclophosphamide

Although cyclophosphamide (CP) is widely used because of its effectiveness as a broad spectrum anticancer drug, CP treatment is associated with significant adverse side effects. One of the primary drawbacks of CP based chemotherapy is widespread cytoxicity often resulting in moderate to severe myelosuppression and neutropenia.[164,165] Significant complications from increased susceptibility to infection could therefore interfere with chemotherapy and delay recovery.[168] However, several studies have indicated the potential for plant based products including medicinal and dietary herbs and spices to stimulate leukocyte proliferation and differentiation even during co-treatment

with CP.[164,171,172] Previously, we have reported significant increases in total cell and neutrophil count in the coelomic fluid of *L. terrestris* feeding on dietary herbs and spices. Here, we further evaluated the effectiveness of these herbs and spices in *L. terrestris* co-treated with CP for 2, 4 or 6 days.

The overall effect of the treatments was collectively assessed (Table 24, 25). We observed an overall increase in the total number of coelomocytes in the coelomic fluid of worms co-treated with cyclophosphamide and either turmeric, ginger, sage or oregano by an average of 36.22% compared to worms treated with CP alone (Table 24). This is particularly significant since treatment with CP is associated with widespread cytoxicity and leucopenia which can increase susceptibility to infection.[164,167] Interestingly, the observed increase in total coelomocyte count in worms treated with CP and the different extracts was also 35.0% higher than the control worms feeding on LGM alone (Table 25). Additionally, viability of coelomocytes increased overall in response to all of the treatments tested by an average of 56.25% compared to worms treated with CP only (Table 24). Compared to the control, coelomocyte viability was higher in worms treated with CP and dietary herbs and spices by 11.09% (Table 25). We also observed a very consistent increase in the relative distribution of neutrophils in the coelomic fluid of worms co-treated with CP and herbs and spices of 23.04% compared to worms treated with CP only (Table 24). Moreover, the percentage of neutrophils was 4.68% higher in worms treated with CP and herbs and spices than in the control (Table 25).

It appears that phytochemicals present in the dietary herbs and spices tested may protect against CP induced leucopenia by stimulating hematopoiesis and promoting differentiation of myeloid precursors. These results are consistent with recent findings in

which dietary and medicinal plants including *Asparagus racemosus, Tinospora cardifolia, Withania somnifera. Cassia occidentalis*, and *Morus alba* were shown to increase leukocyte number and neutrophil populations in animals treated with CP.[168,170,171] Other studies have observed increased splenic cell counts in mice co-treated with CP and *E. officinalis*.[162]

We also observed an overall increase of NOx in the coelomic fluid in response to the treatments with CP plus dietary herbs and spices by an average of 49.79% (Table 24). This was also 37.88% higher than the control (Table 25). Nitric oxide is a well known signaling molecule involved in the regulation of number of physiological processes including maintenance and regulation of normal cell cycle, as well as cellular proliferation and differentiation.[126-130] It is possible that bioactive components in the dietary herbs and spices tested may stimulate nitric oxide mediated signaling pathways that contribute to the effects we observed.

Overall, phagocytic activity of coelomocytes was enhanced by 23.75% in CP treated worms supplemented with dietary herbs and spices compared to those treated with CP only (Table 24). This increase in activity was also 6.22% greater than in the control (Table 25). Several factors may contribute to the increased uptake of foreign material which include an increase in the total number of coelomocytes and an increase in the number of neutrophils, the predominate cell type responsible for phagocytosis in *L. terrestris*. Interestingly, we also observed an overall decrease in respiratory burst activity by 2.16% in response to the treatments compared to worms treated with only CP (Table 24). This decrease in respiratory burst activity in association with increased total neutrophil counts may indicate a decreased target epitope to cell ratio resulting in a

decreased production of reactive oxygen species during oxidative degradation consistent with our previous observations. Additionally, the reduction of measurable superoxide produced in coelomocytes challenged with *S. cerevisiae* may indicate increased antioxidant capacity of the cell as a result of treatment with dietary herbs and spices rich in phenolic compounds.

Table 24. Overall effect of cyclophosphamide (CP) on innate immune parameters in *L. terrestris*. Overall effect of treatment with 50 mg/kg/day CP calculated and reported as the percent change compared to control worms. CV- Coelomocyte viability; TC- Total coelomocyte count; NEU- Neutrophil distribution; RB- Respiratory burst activity; NOX- Nitric Total nitric oxide metabolites measures as nitrates/nitrites; PHA- Phagocytic activity of coelomocytes.

	Innate Immune Parameter					
Treatment	CV %	TC %	NEU %	NOX %	PHA %	RB %
CP	-40.7	-0.9	-17.5	-8.6	-16.5	39.4

Table 25. Overall effect of dietary herbs and spices on innate immune parameters in CP treated *L. terrestris*. Overall effect of each treatment, calculated as the average of 0.1% and 0.5% of the herb or spice, and reported as the percent change compared to worms treated with CP only. CV- Coelomocyte viability; TC- Total coelomocyte count; NEU- Neutrophil distribution; RB- Respiratory burst activity; NOX- Nitric Total nitric oxide metabolites measures as nitrates/nitrites; PHA- Phagocytic activity of coelomocytes.

Treatment	Innate Immune Parameter					
	CV %	TC %	NEU %	NOX %	PHA %	RB %
CP-T	80.2	32.6	24.1	28.5	32.2	-1.4
CP-S	42.8	32.1	21.7	53.0	15.7	-0.7
CP-O	45.8	25.8	24.7	32.5	17.6	-11.9
CP-G	56.3	54.4	21.7	85.2	29.6	5.5
AVG	56.3	36.2	23.0	49.8	23.8	-2.2

Table 26. Overall effect of dietary herbs and spices on innate immune parameters in CP treated *L. terrestris* compared to control. Overall effect of each treatment, calculated as the average of 0.1% and 0.5% of the herb or spice, and reported as the percent change compared to control worms feeding on LGM only. CV- Coelomocyte viability; TC- Total coelomocyte count; NEU- Neutrophil distribution; RB- Respiratory burst activity; NOX- Nitric Total nitric oxide metabolites measures as nitrates/nitrites; PHA- Phagocytic activity of coelomocytes.

Treatment	Innate Immune Parameter					
	CV %	TC %	NEU %	NOX %	PHA %	RB %
CP-T	28.1	31.5	5.6	18.3	13.4	62.7
CP-S	1.5	30.9	3.6	40.9	-0.7	63.8
CP-O	3.7	24.6	6.1	22.0	0.9	45.3
CP-G	11.1	53.0	3.5	70.4	11.3	74.0
AVG	11.1	35.0	4.7	37.9	6.2	61.4

CHAPTER III

DIETARY HERB AND SPICE COMBINATION STUDY

3.1 Introduction

The concept of combination therapy using multiple natural products is an important and central component of traditional Chinese and Indian medicine.[175,176] The rationale for using formulations of natural products including medicinal plants as well as dietary herbs and spices is based on potential interactions of these substances which may exhibit additive, synergistic or antagonistic effects.[175,177,178]

Synergism refers to the potential interaction of two or more bioactive compounds present in combination resulting in an overall effect greater than the sum of the individual effects.[175,177-179] Empirical evidence has supported this type of natural product interaction in which enhanced biological effects were observed.[177] For example, Verma et al. observed decreased proliferation of estrogen-positive human breast MCF-7 cells induced by 17-beta estradiol or estrogenic pesticides following treatment with curcumin or genistein.[180] However, complete inhibition of MCF-7 growth was achieved with curcumin and genistein were combined indicating enhanced or synergistic activity.[180] Common dietary extracts such as oregano, cranberry, blueberry and grape seed in combination were also shown to be synergistically effective against *Helicobacter pylori* when compared to the activity of each alone.[179,181]. Similarly, these extracts in combination were also more effective against *Vibrio parahaemolyticus* than the sum of

the individual extracts.[182] Moreover, the antimutagenic capacity of a mixture of cranberry phenolics, ellagic acid and rosmarinic acid was investigated. As a combination these phytochemicals were shown to impart greater antimutagenic capacity against the mutagens, sodium azide and N-methyl-N'-nitro-N-nitrosoguanidine than the individual constituents.[181] These interactions are primarily believed to be a result of multiple mechanisms of action as a result of non-commonality of molecular targets by phytochemicals of different classes.[175,176,178,179]

However, although emerging research has revealed potential interactions of several natural products in combination, there are relatively few studies evaluating these effects *in vivo*. Therefore, in the present study we evaluated the effect of four different combinations of herbs and spices on important innate immune parameters and functional end points in *L. terrestris*.

3.2 Methods

3.2.1 Combination treatment

Mature, primed worms selected for experimentation, as previously described in Chapter I (Pg 14-15) Experimental worms were treated with combinations comprised of the top herb and spice treatments as determined from the initial screening results in Chapter I. Based on our initial observations indicating treatment with herb or spice was most effective at a concentration of 0.1% (w/v), we combined the top treatments in equimolar concentrations to achieve a total concentration of 0.1% (w/v). The following combinations were tested: 0.05% (w/v) turmeric plus 0.05% (w/v) ginger, 0.05% (w/v) turmeric plus 0.05% (w/v) sage, 0.05% (w/v) turmeric plus 0.05% (w/v) oregano, 0.05%

(w/v) sage plus 0.05% (w/v) oregano. For each combination tested, eighteen worms were evenly divided into three groups (n=6) and allowed to feed *ad libitum* for 2, 4 and 6 days at 18-20°C in the dark. Treatment plates were renewed on the fourth day of treatment. Coelomocytes were collected via ethanol extrusion as previously described in Chapter I (Pg 15). Total coelomocyte count, coelomocyte viability, differential coelomocyte count, phagocytic activity, respiratory burst and nitric oxide production were measured as indicated previously in Chapter I (Pg 15-18).

3.2.2 Statistical Analysis

Statistical significance was determined using a Student's one tailed t-test. Treatment combinations were compared to control worms feeding on LGM only. Statistical significance was indicated by p values of <0.05.

3.3 Results of combination study

3.3.1 Effect of herb/spice combinations on total coelomocyte count

Total coelomocyte count (TCC) was estimated using an Improved Neubauer 1/400 sq. mm hemacytometer, as described earlier. Worms feeding on *Lumbricus* Growth Medium (LGM) comprised of 0.31% (w/v) Gerber oatmeal in 1.25% (w/v) agar were established as controls. TCC for control worms was 2.31×10^6, 3.03×10^6, and 2.49×10^6 on day 2, day 4, and day 6 of the experiment (Table 27). Following treatment with LGM and 0.1% (w/v) turmeric and ginger (TU-GI) over six days, TCC increased significantly on day 2 of the treatment, but decreased on day 4 and day 6 (Table 27). TCC was 2.64×10^6 (p=0.148), 1.79×10^6 (p=0.006) and 1.96×10^6 (p=0.132) on day 2, day 4, and day 6,

respectively. Analysis of the AUC indicated TCC was significantly lower in worms treated with 0.1% (w/v) TU-GI than control (p=0.008) (Figure 20).

TCC increased significantly in worms treated with 0.1% (w/v) turmeric and sage (TU-SA) on all days over the duration of the experiment with highest counts occurring on day 2 (Table 27). On day 2, day 4, and day 6, TCC was 4.63 x 10^6 (p=0.000), 4.13 x 10^6 (p=0.003) and 3.71 x 10^6 (p=0.001), respectively. Overall, treatment with 0.1% (w/v) TU-SA resulted in significantly higher TCC than control over the duration of the experiment (p=0.005) (Figure 20).

Following treatment with 0.1% (w/v) turmeric and oregano (TU-OR), TCC was significantly higher than control on day 6 of the experiment (Table 27). On day 6, TCC was 6.83 x 10^6 (p=0.000), an increase of 181% over the control. On day 2 and day 4 of the treatment, TCC was 1.53 x 10^6 (p=0.443) and 2.39 x 10^6 (p=0.118), respectively. AUC values indicate TCC to be significantly lower in worms treated with 0.1% (w/v) TU-OR than control (p=0.005) (Figure 20).

Compared to control, treatment with 0.1% (w/v) sage and oregano (SA-OR) resulted in significantly higher TCC on day 2, but lower TCC on days 4 and 6 of the experiment (Table 27). On day 2, day 4, and day 6, TCC was 2.44 x 10^6 (p=0.008), 2.06 x 10^6 (p=0.058) and 2.43 x 10^6 (p=0.121). Overall, TCC was significantly lower in worms treated with 0.1% (w/v) SA-OR than control over the duration of the experiment as determined by AUC values (p=0.013) (Figure 20).

Table 27. Effect of herb/spice combinations on total coelomocyte count (TCC) in *L. terrestris*. TCC was estimated using an Improved Neubauer 1/400 sq. mm hemocytometer and expressed as total cells per milliliter. *Indicates significantly higher than control. **Indicates significantly lower than control. $p<0.05$. n=6.

Treatment	Total Coelomocyte Count n=6						
	Conc. *(w/v)*	Day 2 *(cells/ml)*	*P value*	Day 4 *(cells/ml)*	*P value*	Day 6 *(cells/ml)*	*P value*
TU-GI	0.10%	2.64×10^6	0.148	1.79×10^6	0.006**	1.96×10^6	0.132
TU-SA	0.10%	4.63×10^6	0.000*	4.13×10^6	0.003*	3.71×10^6	0.001*
TU-OR	0.10%	1.53×10^6	0.443	2.39×10^6	0.118	6.83×10^6	0.000*
SA-OR	0.10%	2.44×10^6	0.008*	2.06×10^6	0.058	2.43×10^6	0.121
Control	---	2.31×10^6	---	3.03×10^6	---	2.49×10^6	---

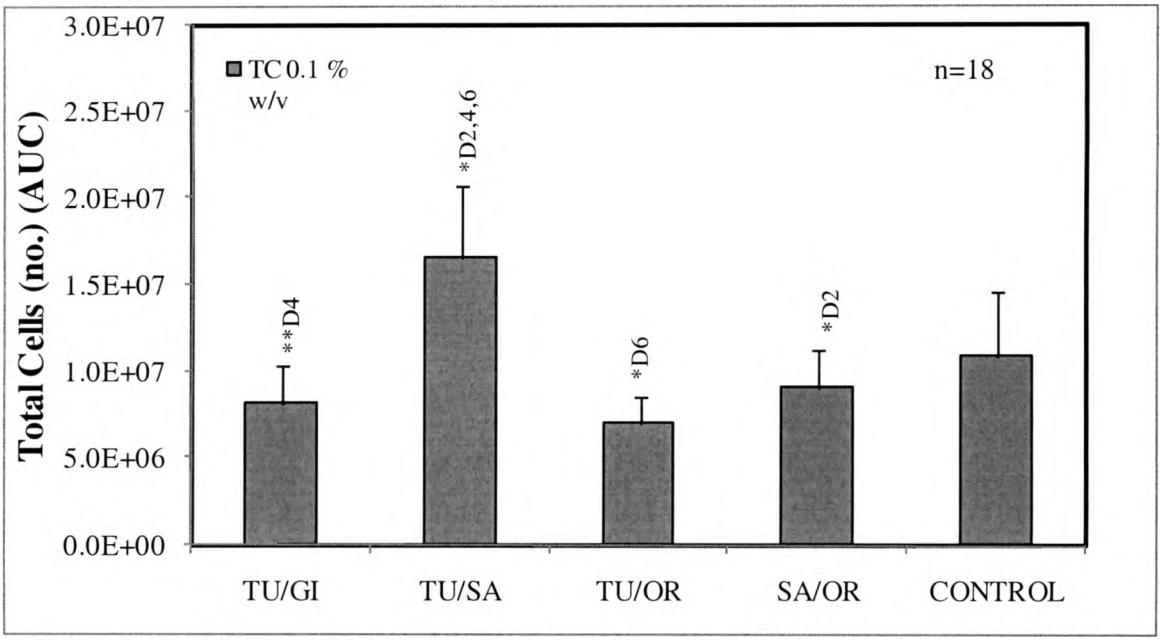

Figure 20. Overall effect of herb/spice combinations on total coelomocyte count (TCC) in *L. terrestris*. Overall TCC for 0.1% (w/v) of each treatment combination, calculated as area under the curve (AUC) and reported as total number of coelomocytes over the duration of the experiment. The data is represented as means ± SEM.*- indicates significantly higher than control ($p<0.05$). **-indicates significantly lower than control. D2= day 2; D4= day 4; D6= day6 – indicates days when treatment was significantly different from control ($p<0.05$). n=18.

3.3.2 Effect of herb/spice combinations on coelomocyte viability

Coelomocyte viability (CV) was analyzed using a trypan blue exclusion assay, as described previously. CV of control worms feeding on LGM only was 46.7%, 47.3%, and 50.0% on day 2, day 4, and day 6 of the experiment, respectively (Table 28). In worms treated with 0.1% (w/v) turmeric and ginger (TU-GI), CV did not change significantly over the duration of the experiment, as indicated by AUC values (p=0.119) (Figure 21). Total CV was 48.0% (p=0.331), 47.7% (p=0.318) and 52.0% (p=0.107) on day 2, day 4 and day 6, respectively (Table 28).

CV increased significantly in worms feeding on 0.1% (w/v) turmeric and sage (TU-SA) on all days over the course of the treatment with highest increases occurring on day 2 (Table 28). CV was 72.7% (p=0.011), 51.7% (p=0.017) and 56.0 % (p=0.001) on day 2, day 4, and day 6, respectively. Assessment of the AUC indicated CV to be significantly greater in worms feeding on 0.1% (w/v) TU-SA than in control worms feeding on LGM only (p=0.013) (Figure 21).

CV in worms treated with 0.1% (w/v) turmeric and oregano (TU-OR) increased on all days of the experiment and significantly increased on day 2 and day 4 compared to control (Table 28). Over the duration of the experiment, CV was 63.3% (p=0.004), 63.7% (p=0.001) and 51.7% (p=0.239) on day 2, day 4, and day 6, respectively, which collectively was significantly higher than control, as determined by analysis of the AUC (p=0.010) (Figure 21).

Likewise, when worms were treated with 0.1% (w/v) sage and oregano (SA-OR), CV was significantly higher than control on day 2 and day 4 of the treatment (Table 28). On these days, CV was 54.3% (p=0.001), 55.7% (p=0.000), respectively. On day 6, 50.3% (p=0.107) of coelomocytes counted were identified as viable cells. Over the duration of the six day experiment, CV was significantly higher following treatment with 0.1% (w/v) SA-OR than control, as indicated by AUC values(p=0.020) (Figure 21).

Table 28. Effect of herb/spice combinations on coelomocyte viability (CV) in *L. terrestris*. CV, measured by trypan blue exclusion, and reported as percent live coelomocytes per 50 total cells counted. *Indicates significantly higher than control. **Indicates significantly lower than control. $p<0.05$. n=6.

Treatment	Coelomocyte Viability n=6						
	Conc. *(w/v)*	Day 2 *(%)*	*P value*	Day 2 *(%)*	*P value*	Day 2 *(%)*	*P value*
TU-GI	0.10%	48.0	0.331	47.7	0.318	52.0	0.107
TU-SA	0.10%	72.7	0.011*	51.7	0.017*	56.0	0.001*
TU-OR	0.10%	63.3	0.004*	63.7	0.001*	51.7	0.239
SA-OR	0.10%	54.3	0.001*	55.7	0.000*	50.3	0.107
Control	---	46.7	---	47.3	---	50.0	---

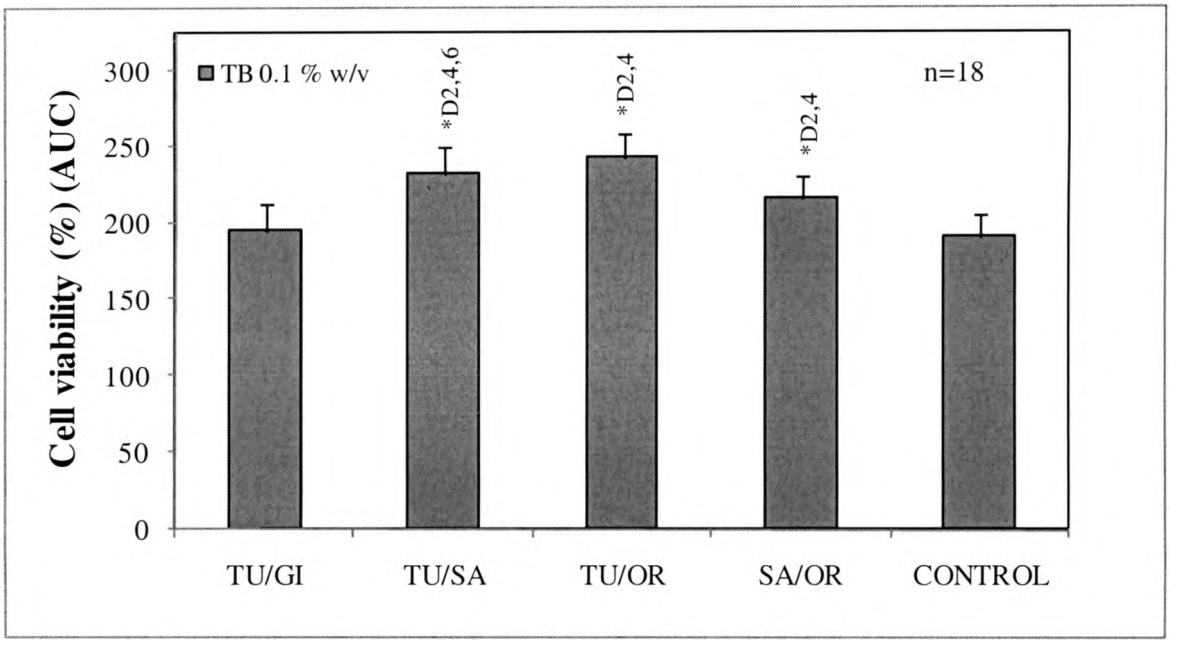

Figure 21. Overall effect of herb/spice combinations on coelomocyte viability (CV) in *L. terrestris*. Net CV of 0.1% (w/v) of each treatment combination, determined by calculation of area under the curve, and reported as percent viable cells over the duration of the experiment. The data is represented as means ± SEM. *- indicates significantly higher than control ($p<0.05$). **-indicates significantly lower than control. D2= day 2; D4= day 4; D6= day6 – indicates days when treatment was significantly different from control ($p<0.05$). n=18.

3.3.3 Effect of herb/spice combinations on relative neutrophil count

Wright's Giemsa staining technique was used to perform differential cell counts of coelomocytes extracted from control and experimental worms, as described previously. Differential cell count of control worms feeding on LGM only revealed 37.7%, 36.9% and 34.7% of total coelomocytes counted were identified as neutrophils on day 2, day 4 and day 6 of the six day experiment, respectively (Table 29). Treatment with 0.1% (w/v) turmeric and ginger (TU-GI) resulted in a significantly relative neutrophil count (RNC) day 2 and day 6 of the treatment (Table 29). The RNC was 40.0% (p=0.006), 37.3% (p=0.195) and 42.7% (p=0.013) on day 2, day 4, and day 6,

respectively. Overall, RNC was significantly greater in worms treated with 0.1% (w/v) TU-GI than in the control as indicated by AUC values (p=0.033) (Figure 22).

Following treatment with 0.1% (w/v) turmeric and sage (TU-SA), the RNC significantly increased on all days of the treatment with highest counts occurring on day 6 (Table 29). On day 2, day 4, and day 6, the RNC was 41.7% (p=0.034), 46.3% (p=0.040) and 50.0% (p=0.002), respectively. AUC values indicated RNC to be higher in worms feeding on 0.1% (w/v) TU-SA than control over the six day experiment (p=0.010) (Figure 22).

Significant increases in the RNC occurred following treatment with 0.1% (w/v) turmeric and oregano (TU-OR) on all days over the duration of the experiment (Table 29). The RNC was 43.3% (p=0.007), 43.7% (p=0.011) and 44.3% (p=0.003) on day 2, day 4, and day 6, respectively. Analysis of the AUC revealed a significantly higher RNC overall in worms treated with 0.1% (w/v) TU-OR than the control (p=0.013) (Figure 22).

For worms feeding on 0.1% (w/v) sage and oregano (SA-OR), the RNC increased significantly on day 2, day 4, and day 6 of the treatment (Table 29). On these days, the RNC was 41.7% (p=0.001), 40.3% (p=0.034), and 36.0% (p=0.045), respectively. Collectively, the RNC was higher following treatment with 0.1% (w/v) SA-OR than the control, as evidenced by AUC values (p=0.061) (Figure 22).

Table 29. Effect of herb/spice combinations on relative neutrophil count (RNC) in *L. terrestris*. The RNC, determined by differential staining using Wright's stain, and expressed as the percent neutrophils per 50 total cells counted. *Indicates significantly higher than control. **Indicates significantly lower than control. $p<0.05$. n=6.

Treatment	Relative Neutrophil Count n=6						
	Conc. *(w/v)*	Day 2 *(%)*	*P value*	Day 2 *(%)*	*P value*	Day 2 *(%)*	*P value*
TU-GI	0.10%	40.0	0.006*	37.3	0.195	42.7	0.013*
TU-SA	0.10%	41.7	0.034*	46.3	0.040*	50.0	0.002*
TU-OR	0.10%	43.3	0.007*	43.7	0.011*	44.3	0.003*
SA-OR	0.10%	41.7	0.001*	40.3	0.034*	36.0	0.045*
Control	---	37.7	---	36.9	---	34.7	---

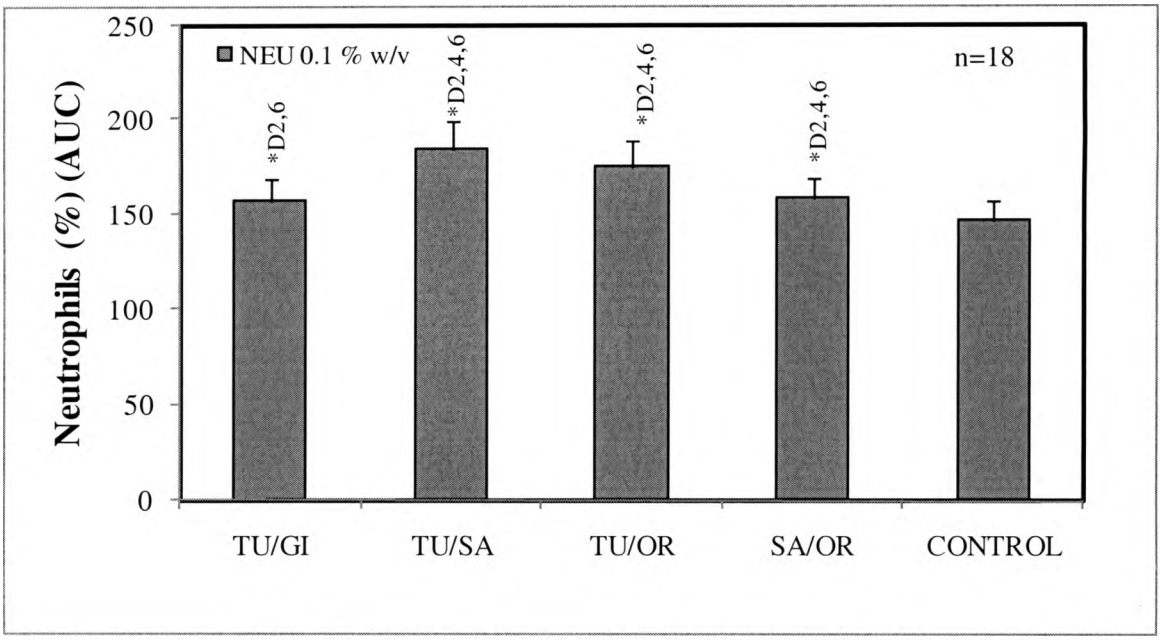

Figure 22. Overall effect of herb/spice combinations on relative neutrophil count (RNC) in *L. terrestris*. Overall RNC for 0.1% (w/v) of each treatment combination calculated as area under the curve (AUC) and expressed as percent neutrophils. The data is represented as means ± SEM. *- indicates significantly higher than control ($p<0.05$). **-indicates significantly lower than control. D2= day 2; D4= day 4; D6= day6 – indicates days when treatment was significantly different from control ($p<0.05$). n=18.

3.3.4 Effect of herb/spice combinations on phagocytic activity

For control worms feeding on LGM only, phagocytic activity (PA) of coelomocytes was 53.1%, 56.4% and 56.1% on day 2, day 4, and day 6 of the experiment, respectively (Table 30). Following treatment with 0.1% (w/v) turmeric and ginger (TU-GI), PA decreased on all days of the treatment with significant decreases occurring on day 6 (Table 30). PA was 50.3% (p=0.384), 52.7% (p=0.195) and 45.7% (p=0.005) on day 2, day 4, and day 6, respectively. Analysis of the AUC indicated PA to be significantly lower in worms treated with 0.1% (w/v) TU-GI than in the control (p=0.025) (Figure 23).

Significant increases in PA of coelomocytes were observed on day 2 and day 4 of the treatment with 0.1% (w/v) turmeric and sage (TU-SA) (Table 30). On these days, 55.3% (p=0.002), and 56.7% (p=0.015) of total coelomocytes counted were identified as actively phagocytosing *S. cerevisiae*. On day 6, PA was 55.3% (p=0.039), which was significantly lower than control. AUC values, however, did not indicate a significant difference in PA overall between worms treated with 0.1% (w/v) TU-SA and those feeding on LGM only (p=0.300) (Figure 23).

In worms treated with 0.1% (w/v) turmeric and oregano (TU-OR), PA of coelomocytes was 53.3% (p=0.208), 66.7% (p=0.006) and 58.0% (p=0.057) on day 2, day 4, and day 6 of the treatment, respectively (Table 30). Overall, treatment with 0.1% (w/v) TU-OR resulted in a significantly higher percentage of coelomocytes identified as actively phagocytosing *S. cerevisiae* than the control over the duration of the experiment (p=0.025) (Figure 23).

Treatment with 0.1% (w/v) sage and oregano (SA-OR) resulted in significant increases in PA on all days of the treatment (Table 30). On day 2, day 4, and day 6, PA of coelomocytes was 54.7% (p=0.007), 59.3% (p=0.017) and 58.0% (p=0.002), respectively. Determination of the AUC indicated PA to be higher following treatment with 0.1% (w/v) SA-OR than control over the duration of the six day experiment (p=0.061) (Figure 23).

Table 30. Effect of herb/spice combinations on phagocytic activity (PA) of coelomocytes in *L. terrestris*. PA of coelomocytes, determined by counting the number of cells containing one or more *S. cerevisiae* particles per 50 total cells and reported as percent phagocytic cells. *Indicates significantly higher than control. **Indicates significantly lower than control. $p<0.05$. n=6.

Treatment	Phagocytic Activity n=6						
	Conc. (w/v)	Day 2 (%)	*P* Value	Day 4 (%)	*P* Value	Day 6 (%)	*P* Value
TU-GI	0.10%	50.3	0.384	52.7	0.195	45.7	0.005**
TU-SA	0.10%	55.3	0.002*	56.7	0.015*	55.3	0.039**
TU-OR	0.10%	53.3	0.208	66.7	0.006*	58	0.057
SA-OR	0.10%	54.7	0.007*	59.3	0.017*	58	0.002*
Control	----	53.1	----	56.4	----	56.1	----

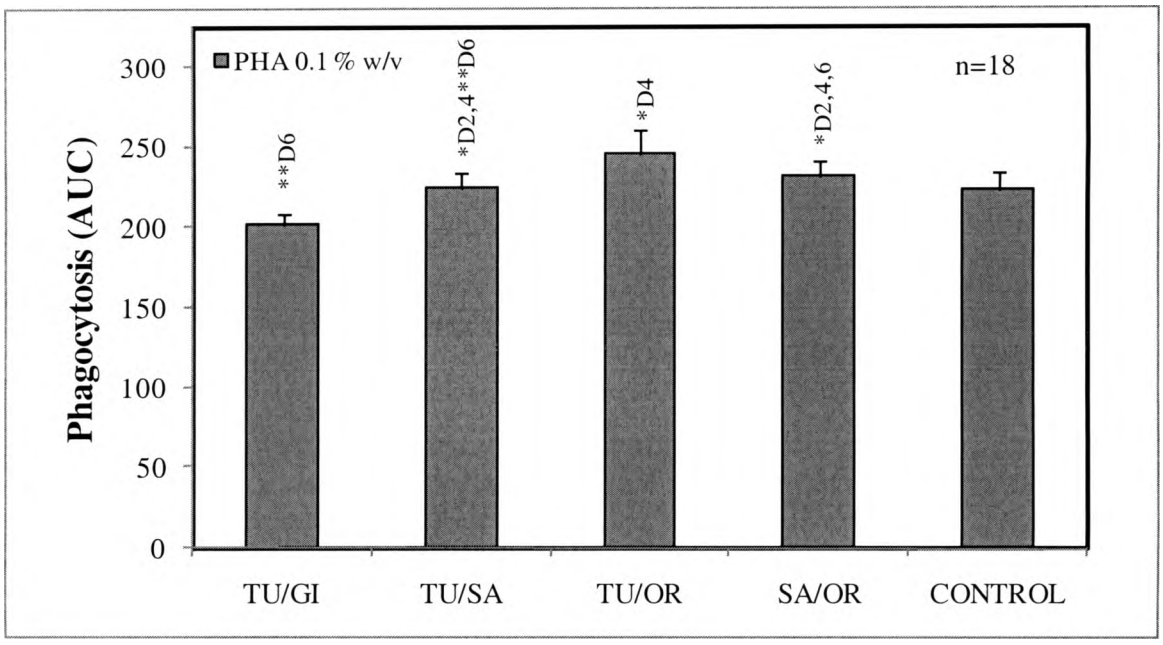

Figure 23. Overall effect of herb/spice combinations on total phagocytic activity (PA) in *L. terrestris*. Overall PA at 0.1% (w/v) of each treatment combination calculated as area under the curve (AUC) and expressed as percent total activity over the duration of the experiment. The data is represented as means ± SEM.*- indicates significantly higher than the control ($p<0.05$). **- indicates significantly lower than the control ($p<0.05$).D2= day 2; D4= day 4; D6= day6 – indicates days when treatment was significantly different from control ($p<0.05$). n=18.

3.3.5 Effect of herb/spice combinations on respiratory burst activity

Respiratory burst (RB) activity of coelomocytes, indirectly measured as the relative absorbance of diformazan, of control worms feeding on LGM only was 1.10 AU, 1.07 AU, and 1.06 AU on day 2, day 4, and day 6 of the experiment (Table 31). Treatment with 0.1% (w/v) turmeric and ginger (TU-GI) resulted in significantly higher RB activity on all days of the treatment (Table 31). On day 2, day 4, and day 6 of the treatment, the relative absorbance of diformazan was 1.86 AU (p=0.001), 2.14 AU (p=0.005), and 2.34 AU (p=0.000), respectively. Over the duration of the experiment, RB activity was significantly higher in worms treated with 0.1% (w/v) TU-GI than the control, as evidenced by AUC values (p=0.003) (Figure 24).

For worms treated with 0.1% (w/v) turmeric and sage (TU-SA) RB activity significantly increased on day 2 and day 4, but significantly decreased on day 6 (Table 31). The relative absorbance of diformazan was 2.20 AU (p=0.001), 2.36 AU (p=0.000) and 0.326 AU (p=0.032) on day 2, day 4, and day 6 of the treatment. AUC values indicate RB activity was significantly higher following treatment with 0.1% (w/v) TU-SA compared to control (p=0.004) (Figure 24).

RB activity significantly increased on all days of the experiment with 0.1% (w/v) turmeric and oregano (TU-OR) (Table 31). Over the duration of the experiment, the relative absorbance of diformazan was 1.26 (p=0.046), 2.79 (p=0.000) and 1.82 (p=0.018) on day 2, day 4, and day 6, respectively. Overall, treatment with 0.1% (w/v) TU-OR resulted in significantly higher RB activity than control as indicated by AUC values (p=0.003) (Figure 24).

In worms treated with 0.1% (w/v) sage and oregano (SA-OR), RB activity increased significantly on day 2 and day 4 of the treatment (Table 31). On those days, the relative absorbance of diformazan was 2.01 (p=0.000) and 2.06 (p=0.001), respectively. On day 6, the relative absorbance of diformazan was 0.270 (p=0.089) which was lower than control. Treatment with 0.1% (w/v) SA-OR resulted in greater RB activity overall throughout the duration of the experiment compared to control (p=0.006) (Figure 24).

Table 31. Effect of herb/spice combinations on respiratory burst (RB) activity in *L. terrestris*. RB activity, indirectly measured as absorbance of diformazan formed during NBT reduction assay, and expressed in absorbance units (AU) at 570 nm. *Indicates significantly higher than control. **Indicates significantly lower than control. $p<0.05$. n=6.

Treatment	Respiratory Burst Activity n=6						
	Conc. (w/v)	Day 2 (AU)	*P* Value	Day 4 (AU)	*P* Value	Day 6 (AU)	*P* Value
TU-GI	0.10%	1.86	0.001*	2.14	0.005*	2.34	0.000*
TU-SA	0.10%	2.2	0.001*	2.36	0.000*	0.326	0.032**
TU-OR	0.10%	1.26	0.046*	2.79	0.000*	1.82	0.018*
SA-OR	0.10%	2.01	0.000*	2.06	0.001*	0.27	0.089
Control	----	1.1	----	1.07	----	1.06	----

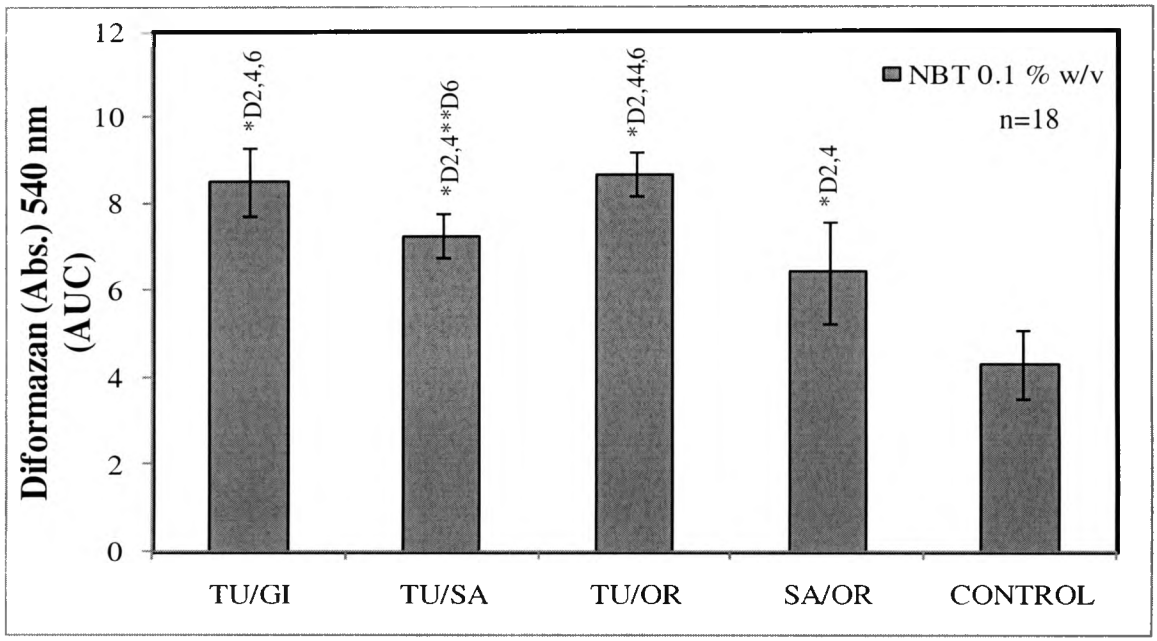

Figure 24. Overall effect of herb/spice combinations on respiratory burst (RB) activity in *L. terrestris*. Overall RB activity for 0.1% (w/v) of each treatment combination calculated as area under the curve (AUC) and expressed as relative absorbance of diformazan at 570 nm over the duration of the experiment. The data is represented as means ± SEM.*- indicates significantly higher than the control ($p<0.05$). **- indicates significantly lower than the control ($p<0.05$).D2= day 2; D4= day 4; D6= day6 – indicates days when treatment was significantly different from control ($p<0.05$). n=18.

3.3.6 Effect of herb/spice combinations on nitric oxide production

Nitric oxide production, indirectly measured as total nitrites/nitrates (NOx) via standard Griess assay, in control worms feeding on LGM only, was 27.7 μM, 28.8 μM, and 27.1 μM on day 2, day 4, and day 6, respectively (Table 32). For worms treated with 0.1% (w/v) turmeric and ginger (TU-GI), total NOx increased significantly on day 2, day 4, and day 6, respectively. On these days, total NOx was 39.7 μM (p=0.042), 40.9 (p=0.016), and 27.9 μM (p=0.048), respectively (Table 32). Over the duration of the experiment, total NOx was significantly higher in worms treated with 0.1% (w/v) TU-GI than control as indicated by assessment of the AUC (p=0.003) (Figure 25).

Total NOx decreased significantly on the second day of treatment with 0.1% (w/v) turmeric and sage (TU-SA) but increased significantly on day 4 and day 6. On day 2, day 4, and day 6, NOx was 18.8 μM (p=0.049), 59.9 μM (p=0.024), and 38.3 μM (p=0.043), respectively (Table 32). AUC values indicate total NOx to be significantly greater following treatment with 0.1 % (w/v) TU-SA than control (p=0.005) (Figure 25).

For worms treated with 0.1% (w/v) turmeric and oregano (TU-OR, NOx increased significantly on day 2 and day 4 of the treatment in which total NOx was 35.2 μM (p=0.043) and 35.4 μM (p=0.045), respectively (Table 32). On day 6, NOx was 20.5 μM (p=0.285), which was lower than control. Assessment of AUC values indicate treatment with 0.1% (w/v) TU-OR resulted in significantly higher total NOx than control (p=0.021) (Figure 25).

Treatment with 0.1% (w/v) sage and oregano (SA-OR) resulted in significant increases in total NOx on all days over the duration of the experiment. NOx was 35.1 μM (p=0.000), 79.5 μM (p=0.005) and 38.7 μM (p=0.025) on day 2, day 4, and day 6, respectively (Table 32), which was collectively significantly greater than the control as determined by assessment of the AUC (p=0.003) (Figure 25).

Table 32. Effect of herb/spice combinations on nitric oxide production (NOx) in *L. terrestris*. NOx production, measured indirectly as total nitrites/nitrates via Griess assay, and reported as μM nitric oxide.*Indicates significantly higher than control. **Indicates significantly lower than control. $p<0.05$. n=6.

Treatment	Nitric Oxide Production n=6						
	Conc. (w/v)	Day 2 (μM)	*P Value*	Day 4 (μM)	*P Value*	Day 6 (μM)	*P Value*
TU-GI	0.10%	39.7	0.042*	40.9	0.016*	27.9	0.048*
TU-SA	0.10%	18.8	0.049**	59.9	0.024*	38.3	0.043*
TU-OR	0.10%	35.2	0.043*	35.4	0.045*	20.5	0.285
SA-OR	0.10%	35.1	0.000*	79.5	0.005*	38.7	0.025*
Control	----	27.7	----	28.8	----	27.1	----

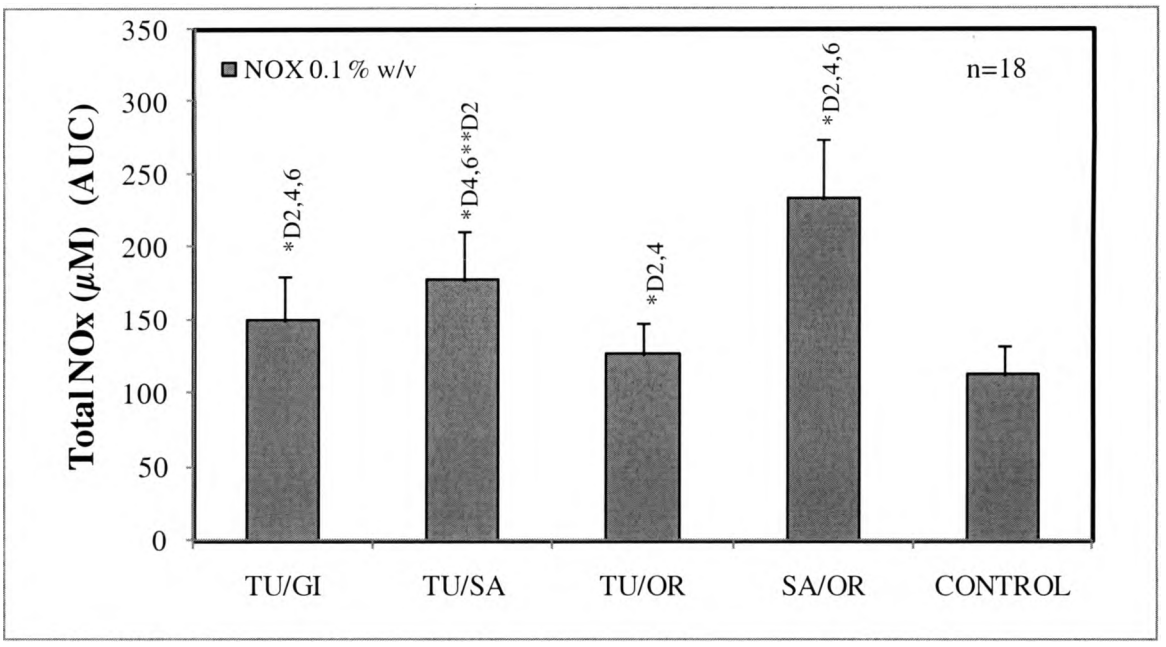

Figure 25. Overall effect of herb/spice combinations on nitric oxide (NOx) production in *L. terrestris*. Total NOx production overall for 0.1% (w/v) of each treatment combination, calculated as AUC and reported as μM of total nitric oxide over the duration of the experiment. The data is represented as means $\pm$ SEM.*- indicates significantly higher than the control ($p<0.05$). **- indicates significantly lower than the control ($p<0.05$). D2= day 2; D4= day 4; D6= day6 – indicates days when treatment was significantly different from control ($p<0.05$). n=18.

3.4 Discussion of combination study

Combinatorial therapy using herbs, spices and medicinal plants has historically played an important role in traditional Eastern medicine.[175,176,183] This concept has been perpetuated by the idea that combining multiple natural products can produce an effect that is greater than the sum of the individual treatments by achieving synergism.[183] However, it is now known that in addition to potential synergistic effects of combination therapy, additive or antagonistic effects may also occur. These interactions in natural product formulations, particularly with respect to synergistic or additive effects, are believed to increase the potency of the individual components thereby lowering the

effective dose required to observe a change.[182] Although observations for the evidence of these interactions effects of natural product combinations have been made, there is still a limited amount of mechanistic data especially through the use of whole system *in-vivo* approaches. We previously reported significant changes in important innate immune parameters and critical end points in *L. terrestris* treated with individual dietary herbs and spices. Here, we evaluated the effect of treatment with combinations of several of the most effective herbs and spices *in vivo* using *L. terrestris*.

3.4.1 Overall effect of combinations on innate immune parameters

The overall effect of herb/spice combinations was collectively assessed (Table 33). Coelomocyte viability of coelomocytes was increased overall by 13.03%. Additionally, a 13% increase an increase in neutrophil population was also observed. These changes were also associated with increased nitric oxide production in the coelomic fluid by 31.03% overall. These findings may suggest a possible role of nitric oxide signaling involved in myeloid cell differentiation as previously discussed.[130] Additionally, nitric oxide mediated anti-apoptotic signaling via increased expression of *bcl-2* may contribute to the observed increases in coelomocyte viability which would be consistent with recent findings by Genaro et al.[131]

Additionally, phagocytic activity and respiratory burst were increased overall compared to control as a result of the combinations by 0.92% and 43.3%, respectively (Table 33). It is likely that the increase in phagocytic cells in the coelomic fluid may be contributing to the increase in phagocytosis and respiratory burst which are mediated predominately by neutrophils. However, many studies have shown respiratory burst

activity to increase exponentially upon stimulation especially following an increase in leukocyte number.[42,138,141,142] In the current study, we observed a moderate increase in respiratory burst activity by 43.3% following challenge with *S. cerevisiae* even though the number of neutrophils in the coelomic fluid increased overall by 13% compared to the control (Table 33). We believe that this muted effect may be a result of a decrease target antigen to neutrophil ratio resulting in a lower number of phagosomes per cell and decrease production of reactive oxygen species overall.

Interestingly, we observed an overall decrease in the total number of coelomocytes in the coelomic fluid as a result of the combinations (Table 33). However, it is important to note that the changes in total coelomocyte number were quite inconsistent. Although individual herbs and spices contributed to significant increases in total coelomocyte number, as described previously, when in combination the opposite effect was observed.

Additionally, many bioactive compounds in natural products are capable of affecting absorption and bioavailability of a number of compounds including pharmaceutical drugs and other phytochemicals. Piperine of black pepper is known to increase both absorption and bioavailability of curcumin.[184] Typically, bioavailability of curcumin is very low due to extensive metabolism in the intestine and liver. It is thought that piperine increases curcumin bioavailability by inhibiting its metabolic modification through hepatic and intestinal hydroxylation and glucuronidation reactions.[184] Also, it is now well known that phytochemicals in citrus fruits such as grapefruit can significantly increase bioavailability of many drugs which can easily result in toxicity.[185] This is predominantly a result of inhibition of the phase I detoxifying enzyme, cytochrome P450

3A4 (CYP 3A4) in the intestine and liver by nearly 50%.[185] Consequently, drugs metabolized by CYP 3A4 such as antiarrhythmics, statins, calcium channel blockers and immunosuppressants may reach dangerously high plasma concentrations.[185]

Therefore, because interactions between phytochemicals in natural products are known to exhibit differential effects which may be synergistic or additive,[185] it is possible that the bioavailability of phytochemicals in the combinations may be increased resulting in toxic levels thereby contributing to the decrease in total cell count overall. However, because we examined only 0.1% (w/v) herb/spice combination, our understanding of the true effect of these formulations is limited. Therefore, potentially much lower concentrations of these combinations may be required to achieve beneficial effects.

Table 33. Overall effect of herb/spice combinations on innate immune parameters in ***L. terrestris***. Overall effect of each combination calculated as the percent change compared to control. CV- Coelomocyte viability; TC- Total coelomocyte count; NEU- Neutrophil distribution; RB- Respiratory burst activity; NOX- Total nitric oxide metabolites measures as nitrates/nitrites; PHA- Phagocytic activity of coelomocytes. $p<0.05$. n=6.

	Innate Immune Parameter					
Treatment	TC %	CV %	NEU %	NOX %	PHA %	RB %
TU/GI	-32.6	2.1	[illegible].1	24.8	-10.3	49.3
TU/SA	34.[illegible]	1[illegible].6	20.[illegible]	36.5	0.8	40.7
TU/OR	-55.0	21.1	16.5	11.1	9.2	50.4
SA/OR	-20.6	11.4	[illegible].[illegible]	51.[illegible]	4.0	32.9
AVG	-18.4	13.0	13.0	31.0	0.9	43.3

CHAPTER IV

EFFECT OF DIETARY HERBS AND SPICES ON EXPRESSION OF GENES RELEVANT TO INNATE IMMUNITY IN *CAENORHABDITIS ELEGANS*

4.1 Introduction to *Caenorhabditis elegans* study

Using *Lumbricus terrestris*, we observed significant changes in innate immunological parameters as a result of treatment with dietary spices and herbs. Phenotypic changes included significant increases in the total number, viability and differentiation of myeloid cells into neutrophil like coelomocytes. These changes were also associated with increased nitric oxide production and enhanced pathogen uptake and degradation via phagocytosis and respiratory burst. To better understand on a molecular level the mechanism of action by which dietary herbs and spices modulate innate immunity, we used transgenic *Caenorhabditis elegans* carrying GFP promoter fusions to genes involved in signaling pathways known to regulate these observable effects.

4.1.1 Use of *C. elegans* to study innate immunity

The use of invertebrate organisms as model systems to study biological processes on molecular and cellular levels in higher organisms has recently become a popular and important research tool.[186-190] A relatively simple organism, *Caenorhabditis elegans*, was the first multicellular organism to be fully sequenced and as a model system is being used to study a wide range of diseases including diabetes, cancer and diseases of the central

nervous system.[186,187,190] This is because *C. elegans* possess many characteristics that make it an ideal organism to study a number of biological processes *in vivo*.[187]

C. elegans have a short life cycle of only a few weeks, are genetically tractable and are now known to carry homologs to 60-80% of human genes. Additionally, *C. elegans* are transparent.[191] This property increases ease of *in vivo* experimentation especially through the use of probes such as GFP tagged to genes of interest which can be visualized using fluorescence microscopy.[191] Moreover, *C. elegans* can be maintained on simple, inexpensive media and can also be stored indefinitely at -80°C in a state of diapause known as dauer. These properties enable the use of *C. elegans* as a high throughput model system for drug screening by allowing close examination of conserved biochemical and molecular pathways over the life cycle of the animal.[186,187,190] As such, *C. elegans* are currently being used to study evolutionarily conserved signaling pathways involved in the innate immune response.[189,191,192]

4.1.2 Innate immunity in *C. elegans*

4.1.2.1 Physical components of innate immunity in *C. elegans*

C. elegans are soil dwelling organisms in constant contact with a diverse range of pathogens including *Pseudomonas aeruginosa* and *Serratia marscecens*. However, pathogenic colonization and infection are minimized as a result of a combination of physical barriers and highly conserved innate immune signaling pathways which regulate the expression of antimicrobial peptides and bactericidal proteins.[192,193] Physically, *C. elegans* are protected from pathogenic infection by an impermeable outer layer comprised of cross linked collagen fibers secreted from the hypodermis.[191,194] This layer,

the cuticle, not only serves as a physical barrier against soil dwelling microorganisms but it also helps preserve body structure and is important for the characteristic locomotory movements of the worm.[191,194]

4.1.2.2 Innate immune signaling pathways in *C. elegans*

In response to infection, expression of genes encoding a wide array of antimicrobial peptides and bactericidal proteins are upregulated through activation of highly conserved innate immune signaling cascades (Figure 26). It is now known that innate immunity in *C. elegans* is primarily mediated by a TGF-β pathway, a p38 mitogen activated kinase pathway (p38 MAPK), a DAF-2/insulin like growth factor pathway (IGFR), and the unfolded protein response (UPR) involved in regulation of apoptosis.[192,193] Additionally, a consequence of innate immune defenses involves alteration of the redox environment of intestinal cells often resulting in a state of oxidative stress. Interestingly, oxidative stress induces hormetic changes mediated predominantly by the conserved p38 MAPK and DAF-2/IGFR signaling cascades thereby linking oxidative stress and innate immunity.[195]

4.1.2.3 TGF-β Signaling in *C. elegans*

In mammals, cellular differentiation is highly regulated by conserved TGF-β (transforming growth factor) signaling.[196,197] In *C. elegans*, TGF-β signaling also regulates important aspects of development by controlling body size and length, male tail patterning, polyploidy in intestinal and hypodermal cells as well as dauer formation.[192,198] Additionally, activation of TGF-β signaling is critical during the pathogen response to infection by *Serratia marcescens* and *Pseudomonas aeruginosa.* TGF-β signaling is

initiated by the interaction of a bone morphogenic-like protein, DBL-1, with a heterodimeric DAF-4/SMA-6 TGF-β receptor leading to activation of a SMA-2/SMA-3/SMA-4 complex (Table 25).[192,198] DBL-1 (**D**rosophilia decapentaplegic/**B**MP-**L**ike) signaling controls expression of the bactericidal proteins such as the lysozymes LYS-1, LYS-7 and LYS-8 in addition to caenacin antimicrobial peptides, C-type lectins and saposins.[199] Mutant *dbl-1 C. elegans* therefore display increased vulnerability to infection and mortality by *S. marcescens, P. aeruginosa* and even *E. coli OP50* which is the food source most commonly used in the laboratory.[200]

Natural products, including dietary spices and herbs as well as medicinal plants have been shown to modulate the activity and/or level of antimicrobial peptides regulated by TGF-β signaling production using both *in vitro* and *in vivo* model systems.[201-203] Yin et al. (2009) recently observed increased lysozyme activity in cell isolated from carp treated with *Astragalus radix* root and *Ganoderma lucidum.*[202] In a similar study, extracts of Echinacea *purpurea, Inula helenium, Tussilago farfara, Brassica nigra* and *Chelidoniume majus* significantly enhanced lysozymal activity in bacterially challenged carp.[203] Aly et al. (2008) observed significantly increased survival rate, growth rate and body size in addition to significantly enhanced lysozyme activity in Nile tilapia (*Oreochromis niloticus*) supplemented with Echiniacea (*Echinacea purpurea).*[201] However, the mechanism of action by which these herbs affect lysozyme expression or activity are unclear. Therefore, one of our objectives was to examine the effect of dietary herbs and spices on the expression of the TGF-β ligand, DBL-1 in *C. elegans.*

4.1.2.4 p38 MAPK signaling in *C. elegans*

The p38 mitogen activated protein kinase (p38 MAPK) pathway is highly conserved and is critical during oxidative stress and pathogenic infection especially in response to gram negative bacteria such as *Pseudomonas aeruginosa* and *Serratia marcescens*.[192,195] In *C. elegans* the p38 MAPK cassette, which includes TIR-1/NSY-1/ SEK-1/PMK-1, homologous to the mammalian SARM/ASK1/MAP kinase kinase/ p38 MAPK mediates the innate immune response to pathogenic colonization in the intestines (Figure 25).[204,205] This innate defense mechanism eventually leads to the transcription of downstream target genes encoding bactericidal effector molecules including neuropeptide like proteins (NLP-29) and C-type lectins mediated by the transcription factor ATF-7 (the cAMP-responsive element binding (CREB) activating factor homolog).[189,195] p38 MAPK activation is initiated by activation of the Toll/Interleukin receptor-1 (TIR-1) domain adapter protein, the invertebrate homolog to mammalian SARM, leading to phosphorylation and activation of NSY-1 (ASK-1-MAPKKK ortholog), SEK-1 (MAPKK ortholog) and PMK-1 (p38 MAPK ortholog). Activated PMK-1 has been shown to directly interact with and phosphorylate the transcriptional repressor ATF-7, resulting in its activation and nuclear accumulation.[193,195]

During oxidative stress, intestinal expression of antioxidant and phase II detoxifying enzymes such as glutamyl cysteine synthetase (*gcs-1*) as well as heat shock proteins (*hsp-16.2*) is mediated by the transcription factor SKN-1 (Nrf2-like ortholog) which is also controlled, in part, by PMK-1.[195] In addition to *gcs-1* and *hsp-16.2*, SKN-1 promotes expression of glutathione S- transferase (*gst-4*), NADPH oxidoreductase (*nq01*), superoxide dismutase-3 (*sod-3*) as well as iron sulfur protein-1 (*isp-1*).[206]

Phosphorylation of SKN-1 by PMK-1 is followed by nuclear translocation and accumulation of SKN-1.[207]

p38 MAPK signaling through the TIR-1/NSY-1/SEK-1/PMK-1 cassette is also intimately associated with apoptosis. NSY-1, homolog to mammalian apoptosis signal regulating kinase (ASK-1), especially plays an important role in the regulation of programmed cell death and is typically considered pro-apoptotic.[208] In the cytosol, NSY-1 exists as a heterodimer with the antioxidant enzyme thioredoxin (TRX-1) and is inactive. During increased oxidative stress interaction with reactive oxygen species such as H_2O_2 results in dissociation of NSY-1 from TRX-1 thereby increasing its susceptibility to phosphorylation and subsequent activation.[208]

Dietary components including commonly consumed herbs and spices have been shown to modulate MAPK signaling. For example, Andreadi et al. (2005) recently observed increased nuclear localization of the transcription factor Nrf2 (SKN-1) in human breast cell lines HBL100 and MDA-MB468 treated with 30 μM curcumin.[209] This effect was abrogated in Nrf2-/- cells.[209] However, the physiological mechanism of action by which dietary phytochemicals modulate MAPK signaling *in vivo* is less clear. Therefore, we were interested in examining the effect of dietary phytochemicals on important proteins involved in p38 MAPK signaling in transgenic *C. elegans*.

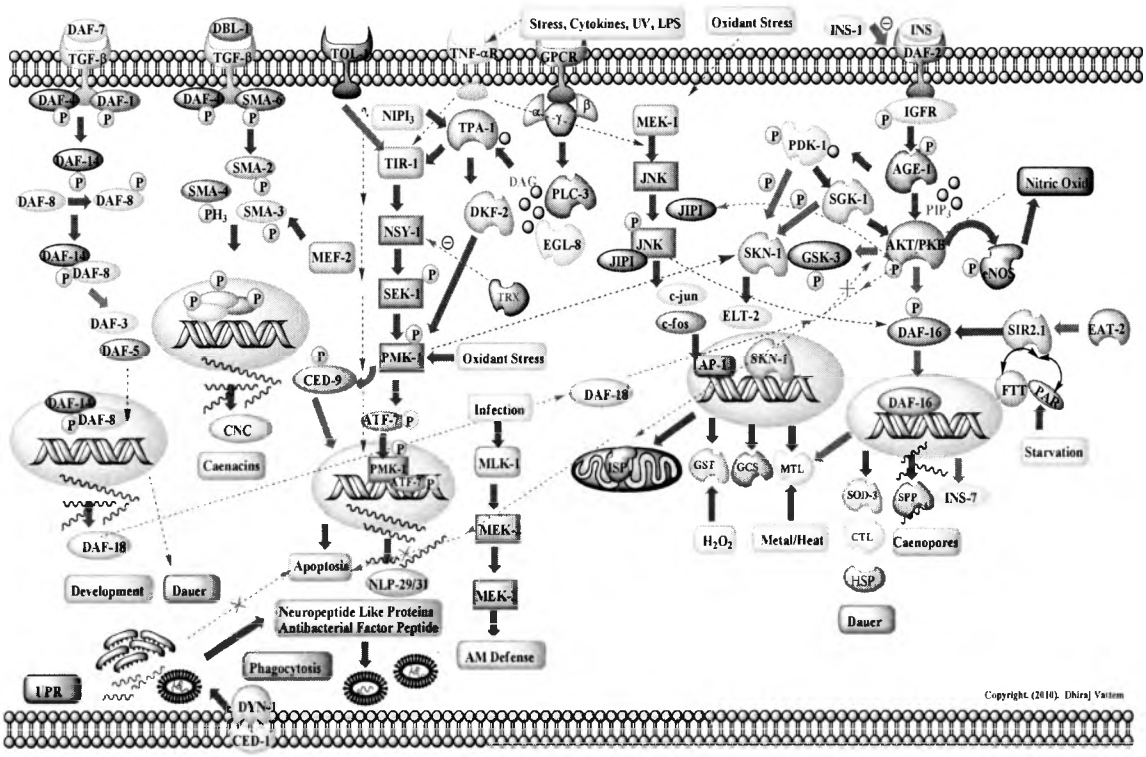

Figure 26. Innate immune signaling pathways in *C. elegans*.

4.1.2.5 DAF-2/IGFR signaling in *C. elegans*

In *C. elegans* dauer formation 2 (DAF-2)/insulin like growth factor 1 (IGFR) signaling pathway is highly involved in the regulation of lifespan, reproduction and metabolism in addition to its role in the pathogen and oxidative stress response.[210-212] *daf-2* mutant *C. elegans* exhibit increased lifespan, increased fat storage, as well as increased resistance to heat and oxidative stress.[210,211] Additionally, depression of DAF-2 activity in *C. elegans* is associated with diapause or dauer formation.[210] The DAF-2/IGFR is a tyrosine kinase that upon activation following binding of insulin like growth factors, results in the phosphorylation and subsequent activation of AGE-1 (a phosphatidylinositol-3-OH (PI3K)) and protein kinase B (AKT) (Figure 25). Protein kinase B regulates important transcription factors that control genes involved in the stress response. One of these targets, the DAF-16/FOXO transcription factor, directly controls

the expression of a number of antioxidant enzymes and proteins involved in response to environmental and oxidative stressors including superoxide dismutase (SOD-3) and heat shock protein (HSP-16.2), among others.[211] Additional genetic targets for DAF-16/FOXO in mammals include those involved in cell growth and apoptosis as well as genes important during the stress and pathogenic response.[210] Phosphorylation of DAF-16 prevents nuclear translocation from the cytosol and thus its ability to function as a transcription factor.[192,210] Therefore, in *daf-2* mutant animals, lifespan is increased and pathogen resistance is enhanced.[213]

Recently, Yu et al. evaluated the effect of herbal combinations used in traditional Chinese medicine on lifespan in *C. elegans*.[214] Two formulations (*Shi Quan Da Bu Tang* and *Huo Luo Xiao Ling Dan*) comprised of multiple root spices and herbs including *Cinnamomum cassia* (cinnamon), *Glyzcyrrhiza uralensis* (licorice), *Panax ginseng,* and *Astragalus membranaceus* root, among others, were shown to significantly increase lifespan in *C. elegans*. Additionally, they observed increased expression of small heat shock proteins (sHSP16) in treated *C. elegans*.[214] In the present study, we evaluated the effect of dietary herbs and spices on *daf-2* expression as well as expression of downstream products of DAF-2/IGFR signaling including *sod-3, hsp-16.2, isp-1* and *gst-4*.

4.1.2.6 Apoptosis and unfolded protein response (UPR) signaling in *C. elegans*

Apoptosis and clearance of apoptotic cells is an important part of a functional innate immune response. This is primarily because controlled uptake and degradation of apoptotic cells prevents the release of inflammatory molecules which can result in tissue

damage.[215] A consequence of activation of innate immune signaling pathways following pathogen recognition involves potential endoplasmic reticulum (ER) stress as a result of increased expression of proteins requiring ER processing. ER stress is linked to induction of a highly conserved unfolded protein response (UPR) mediated by three protein cassettes orchestrated by the *C. elegans* cell death proteins (CED).[191] UPR signaling functions to expand the ER, increase the degradation of misfolded proteins and decreases synthesis of nascent proteins.[191]

Apoptotic signaling in *C. elegans* as a result of infection by pathogens such as *Salmonella enterica* results in activation of an EGL-1/CED-9/CED-4/CED-3 cassette that is homologous to the mammalian BCL-2/APAF-1/CASPASE cascade downstream of PMK-1/p38 MAPK activation.[191] Additionally, two other CED cascades including the CED-1/CED-6/CED-7 and the CED-2/CED-5/CED-10/CED-12 mediate clearance of apoptotic cells. Mammals deficient in proteins critical for apoptotic signaling are considered immunocompromised and are therefore susceptible to infection and disorders such as systemic lupus, diabetes, cancer and autoimmune and neurodegenerative disease.[191,216]

Downstream of apoptotic signaling, recognition of cells marked for apoptosis by cells of the germline is mediated by phagocytic receptors including the cell death protein, CED-1.[191] CED-1, analogous to the mammalian scavenger receptor from endothelial cells (SREC), is a single transmembrane protein characterized by several EGF-like repeats on the extracellular amino terminal and an intracellular signaling sequence associated with residues 1 and 20 of the carboxyl terminus.[191,217] The repeating EGF units, comprised of eight cysteine units, are known to facilitate ligand-receptor interaction.[191] CED-1

recognizes exposed phospholipids associated with the plasma membrane indicative of plasma membrane disruption characteristic of dying cells. CED-1 activity is dependant upon a number of other proteins including CED-7, an ABC transporter, the large GTPase Dynamin (DYN-1), phosphatidlyinositol-3-phosphate (PI3P) and the small GTPase, Ras related protein-7 (RAB-7) as well the receptor mediated endocytosis-8 protein (RME-8).[191,217]

Interaction with neighboring cells targeted for apoptosis results in increased CED-1 localization or "clustering" at the plasma membrane in a CED-7 dependent fashion.[217] It is believed that CED-7 participates in CED-1 recognition of exposed phospholipids by promoting rearrangement or transport of plasma membrane lipid components of apoptotic cells. *Ced-1* mutants display increased vulnerability to pathogenic killing especially in response to challenge with live *S. enterica* and *E. coli* indicating its important role in innate immunity.[191]

CED-1 functionality is also dependent upon activity of DYN-1, a Dynamin protein homolog in *C. elegans*. DYN-1 is required for CED-1 mediated uptake by facilitating vesicular fission during phagosome formation. It is now known that DYN-1 regulates the rate of phagolysosome formation by controlling the recruitment and localization of RAB-7 and PI3P to the phagosomal membrane.[218] Therefore, *dyn-1* mutants exhibit significant decreases in uptake of apoptotic cells.[218]

Additionally, CED-1 activation is required for the expression of genes critical during pathogenic infection and ER stress.[191] These include ten *pqn/abu* genes which when upregulated reduce susceptibility to infection by *S. enterica.*[191] The prion-like Q/N-

rich domain (*pqn*) and activated in blocked unfolded protein response (*abu*) family of genes encode proteins that are upregulated in the ER during the stress response. *Pqn/abu* regulated proteins facilitate survival during ER stress and have been shown to increase lifespan in *C. elegans*.[219] CED-1 mutants do not properly express *pqn/abu* genes and are more susceptible to infection by pathogenic bacteria including live *S. enterica*.[191]

Previously, we have observed increased phagocytosis in coelomocytes isolated from *L. terrestris* feeding on dietary herbs and spices. Using the *C. elegans* model, we monitored changes in the expression of proteins critical during the engulfment process including DYN-1 and RME-8, in response to treatment with herbs and spices.

4.2 Methods

4.2.1 *C. elegans* growth conditions

Transgenic worms expressing GFP promoter fusions to genes involved in innate immunity were obtained from the Caenorhabditis Genetics Center (CGC) (Table 34). *C. elegans* were maintained according to standard protocols.[220] Worms were maintained at 18-20°C on Nematode Growth Medium (NGM) agar (0.3% NaCl, 1.7% agar, 0.25% peptone, 5 mg/ml cholesterol, 1M KPO4, 1M MgSO4, 1M CaCl2 which was aseptically poured into 35mm or 60 mm petri plates using a peristaltic pump.[221]

Table 34. Transgenic *C. elegans* strains used.

Strain	Gene	Strain	Gene
BW1940	*dbl-1*	BC13348	*gst-4*
BC11466	*tir-1*	CF1553	*sod-3*
IG692	*nlp-29*	BC13292	*dym-1*
BC10545	*nsy-1*	DH1336	*rme-8*
BC14074	*daf-2*	GS1912	*pymo-3*
BC14279	*isp-1*	GS1826	*plisp*
CL2166	*gst-4*	OE3010	*trx-1*

4.2.2 Treatment of *C. elegans*

The low molecular weight fraction of turmeric, ginger, oregano and rosemary was extracted from freeze dried powders by heating 0.05 g powder in 1 ml DH_2O for 30 minutes at 60°C. Vacuum filtration was then used to obtain the low molecular weight fraction. Finally, extracts were then sterilized by filtration using a 0.2 μm filter. Extract treatments (top 2 herbs and spices) were added at 0.1% (v/v), 0.5% (v/v) and 1% (v/v) to the NGM solution just prior to pouring.[222] Plates were inoculated with 50 μl of *E. coli* OP50 overnight cultures mixed with 0.0% (v/v), 0.1% (v/v), 0.5% (v/v) or 1.0% (v/v) extract, wrapped in parafilm and incubated for 9 hours at 37°C. Plates were then allowed to cool to room temperature. Two mature adult worms were transferred by picking to treatment plates and allowed to lay eggs, hatch and grow to the L4 to mature adult stage. Picks were fashioned with 32 gauge platinum wire fused to the tip of a Pasteur pipet and heat sterilized between transfers.[220]

4.2.3 Imaging and quantification of gene expression

Two adult worms from each plate were randomly selected to be imaged (n=6). Worms were temporarily immobilized by chilling on ice for five minutes prior to imaging. The changes in gene expression were imaged using the Nikon SMZ1500 fluorescence microscope with Ri1 CCD camera and the relative fluorescence with respect to control was quantified using the NIH ImageJ software (Figure 26).

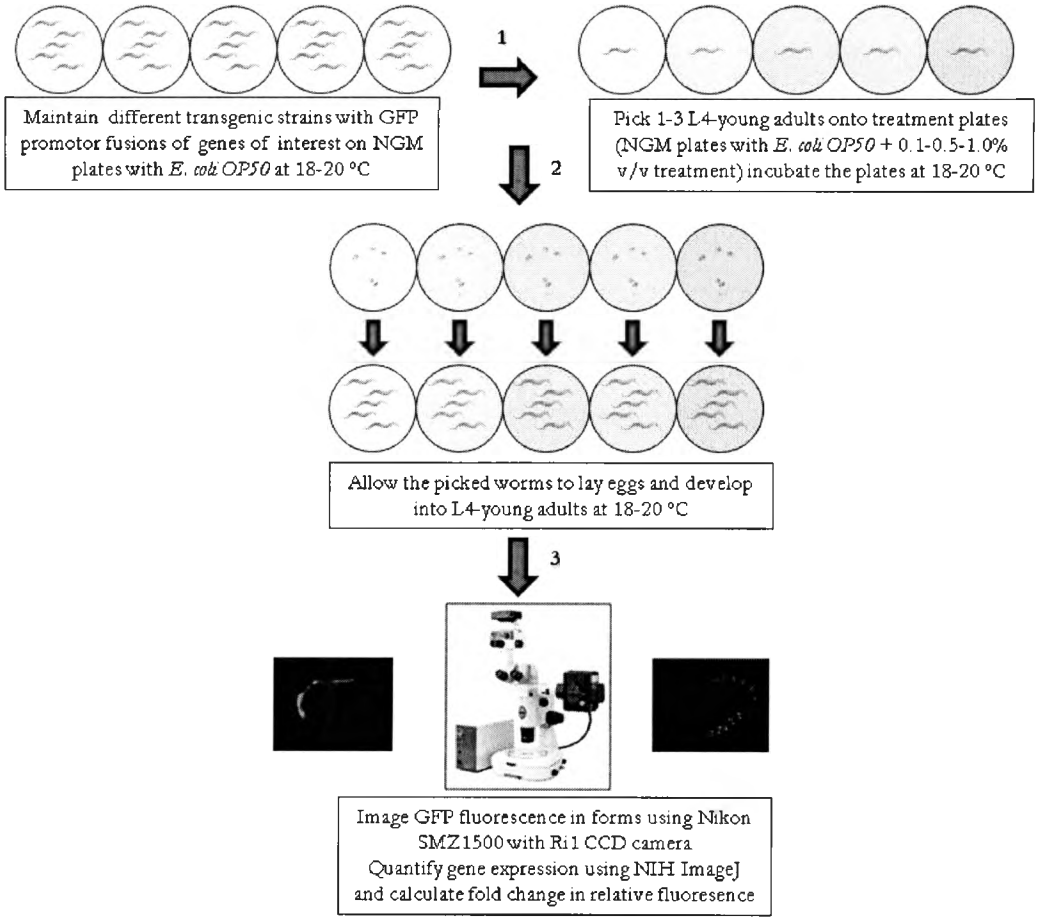

Figure 27. *C. elegans* model for studying innate immunity.

4.2.4 Statistical Analysis

Statistical significance was determined using a Student's two tailed t-test. Treatments were compared to control C. elegans feeding on NGM only. Statistical significance was indicated by p values of <0.05.

4.3 Results *C. elegans*

4.3.1 Effect of dietary herbs and spices on *dbl-1* expression in *C. elegans*

Because TGF-β signaling mediates important innate immune functions during pathogenic attack such as increased production of antimicrobial peptides (AMP) and Caenacin antibacterial factors, we were interested in measuring potential changes in the expression of *dbl-1*, the TGF-β ligand and bone morphogenic-like protein homolog in *C. elegans* in response to treatment with herbs and spices. *C. elegans* feeding on NGM and *E. coli OP50* were established as controls and experimental worms were fed various extracts at 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) and *E. coli OP50* from birth to adulthood. To evaluate for changes in *dbl-1* expression, we used the transgenic strain BW1940. We found that in worms feeding on turmeric at 0.1% (v/v), 0.5% (v/v) or 1.0% (v/v) *dbl-1* was upregulated by 1.23 fold (p=0.966), 1.78 fold (p=0.002) and 1.71 fold (p=0.000), respectively, compared to control (Table 35, Figure 28).

In response to treatment with ginger, there was very little change in *dbl-1* expression although at 1.0% (v/v) a 1.3 fold change was observed (p=0.54) (Table 35). Fold changes for worms feeding on 0.1% (v/v) and 0.5% (v/v) ginger were 1.0 (p=0.020) and 0.9 (p=0.000) of control. *Dbl-1* was also upregulated in worms feeding on rosemary

at all of the concentrations tested (Table 35). In worms treated with 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) rosemary, *dbl-1* expression changed by 1.06 fold (p=0.298) and 1.12 fold (p=0.264), and 1.46 fold (p=0.000) respectively. For worms feeding on 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) oregano, *dbl-1* expression was upregulated by 1.3 fold (p=0.34), 1.1 fold (p=0.55) and 1.1 fold (p=0.67), respectively (Table 35).

Table 35. Effect of herbs and spices on *dbl-1* expression in *C. elegans*. Fold change in fluorescence intensity in *C. elegans* treated with various extracts at 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) compared to control, as measured using the Nikon SMZ1500 fluorescence microscope and Ri1 CCD camera for imaging and NIH ImageJ software for quantification. FC- Fold change. *-Indicates significantly different from control ($p<0.05$). n=6.

DBL-1 n=6			
Treatment	**Conc. (% v/v)**	**FC**	***P value***
Turmeric	**0.1**	**1.23**	**0.966**
	0.5	**1.78**	**0.002***
	1	**1.71**	**0.000***
Ginger	**0.1**	**1.01**	**0.022***
	0.5	**0.91**	**0.000***
	1	**1.28**	**0.54**
Rosemary	**0.1**	**1.06**	**0.298**
	0.5	**1.12**	**0.264**
	1	**1.46**	**0.000***
Oregano	**0.1**	**1.26**	**0.344**
	0.5	**1.1**	**0.552**
	1	**1.07**	**0.672**

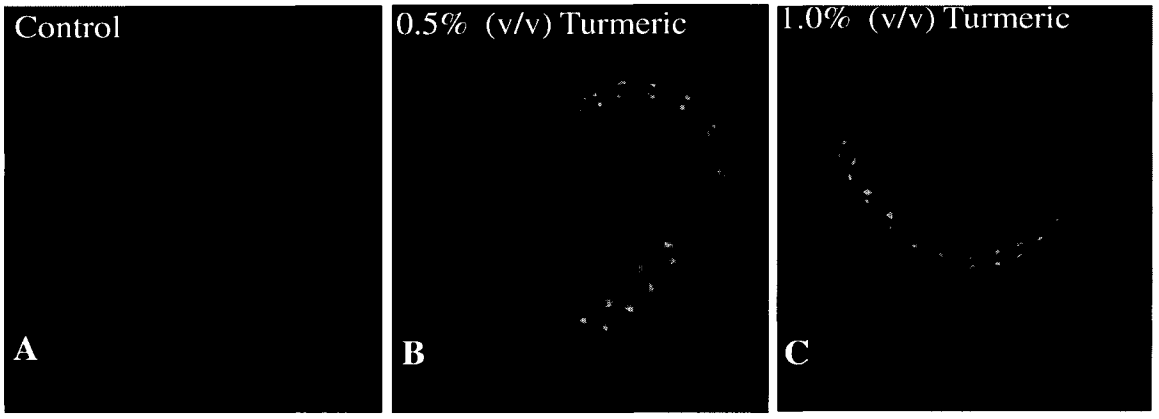

Figure 28. Expression of *dbl-1*::GFP in *C. elegans* feeding on turmeric at varying concentrations. A- 0.0% (v/v) turmeric. B- 0.5% (v/v) turmeric. C- 1.0% (v/v) turmeric.

4.3.2 Effect of dietary herbs and spices on p38 MAPK signaling in *C. elegans*

The highly conserved p38 MAPK pathway is critical during the innate immune response to opportunistic infection to pathogens including *P. aeruginosa*, among others.[207] This protein cassette, which includes NSY-1, SEK-1, PMK-1 signal primarily through TIR-1 (SARM homolog) to regulate expression of a number of humoral factors such as NLP-29 and the lysozymes following pathogen recognition.[207,223] Our objective was to determine the effect of dietary spices and herbs on the expression of *tir-1* and *nsy-1* involved in MAPK signaling as well as one of important downstream products, *nlp*-29. To test this, we used the transgenic strains BC11466, BC10545 and IG692 carrying GFP promoter fusions to *tir-1, nsy-1* and *nlp-29*, respectively. Although there were slight variations between treatments and treatment concentrations, overall we observed increased expression of the antimicrobial peptide *nlp*-29 even though the upstream proteins TIR-1 and NSY-1 were actually down regulated (Table 36, 37).

In *C. elegans* feeding on turmeric, expression of *tir-1* was down regulated 0.6 fold (p=0.010) and 0.5 fold (p=0.133) in worms feeding on 0.1% (v/v) and 1.0% (v/v) turmeric, respectively (Table 36). However, *tir-1* expression increased, although not significantly, in response to treatment with 0.5% (v/v) by 1.2 fold (p=0.084). *Nsy-1*, downstream of TIR-1 in the p38 MAPK cascade was also down regulated in worms feeding on turmeric at all concentrations (Table 36). For worms feeding on 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) turmeric, *nsy-1* expression decreased 0.84 fold (p=0.404), 0.67 fold (p=0.153) and 0.94 (p=0.934) of the control, respectively. However, *nlp-29* expression increased 1.2 fold (p=0.325) in response to treatment with 0.1% (v/v) turmeric and significantly increased following treatment with 0.5% (v/v) and 1.0% (v/v of the spice by 1.72 fold (p=0.002) and 1.65 fold (p=0.000), respectively (Table 37, Figure 29).

A similar effect was observed in worms treated with varying concentrations of ginger (Table 36, 37). Although *tir-1* and *nsy-1* were down regulated by an average of 0.53 fold and 0.71 fold, respectively, the antibacterial factor *nlp-29* was upregulated 1.25 fold on average. Significant decreases in *tir-1* expression occurred in worms feeding on 0.1% by 0.50 fold (p=0.022) and 0.60 fold (p=0.024) in worms feeding on 1.0%. *Nsy-1* expression was also significantly lower than control following treatment with 0.1%, 0.5% and 1.0% by 0.67 fold (p=0.000), 0.73 fold (p=0.000) and 0.72 fold (p=0.000) of the control, respectively (Table 36). *Nlp-29* was upregulated 1.14 fold (p=0.217) and 1.77 fold (p=0.250) in worms feeding on 0.1% (v/v) and 0.5% (v/v) ginger, respectively (Table 37). However, *nlp-29* expression was down regulated in response to treatment with 1.0% (v/v) ginger by 0.84 fold (p=0.000) compared to control.

For worms feeding on rosemary, *tir-1* expression remained relatively unchanged (Table 36). *Tir-1* expression changed by 1.07 fold (p=0.009) 1.05 (p=0.031) and 1.05 fold (p=0.060) of the control in worms feeding on 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) rosemary, respectively. *Nsy-1* on average was also down regulated by 0.93 fold in worms feeding on rosemary, compared to control. At 0.1% (v/v) rosemary, *nsy-1* was unchanged. However, for worms feeding on 0.5% (v/v), and 1.0% (v/v) *nsy-1* decreased by 0.87 fold (p=0.053) and 0.91 fold (p=0.180) compared to control. The antimicrobial peptide *nlp-29* was upregulated compared to the control in worms feeding on rosemary at all concentrations tested (Table 37). Fold changes for worms feeding on 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) rosemary were 1.06 (p=0.299), 1.12 (p=0.264) and 1.46 (p=0.000), respectively.

In *C. elegans* feeding on oregano, *tir-1* expression decreased in a dose dependent manner (Table 36). For worms feeding on 0.1% (v/v) *tir-1* expression was unchanged compared to control but at 0.5% (v/v) and 1.0% (v/v) oregano, *tir-1* expression decreased by 0.9 fold (p=0.235) and 0.4 fold (p=0.030). A similar effect was also observed when we measured *Nsy-1* in worms feeding on oregano (Table 36). At 0.1% (v/v) oregano, *nsy-1* expression was upregulated 1.3 fold (p=0.419) compared to control, but in response to treatment with 0.5% (v/v) and 1.0% (v/v) oregano, *nsy-1* was down regulated 0.97 fold (p=0.763) and 0.81 fold (p=0.272). *Nlp-29* expression, however, was upregulated 1.06 fold (p=0.299), 1.12 fold (p=0.264) and 1.46 fold (p=0.000) in worms feeding on 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) oregano, respectively (Table 37).

Because we observed upregulation of *nlp-29* in response to the treatment independent of *tir-1* and *nsy-1* upregulation we were interested in measuring changes to

the antioxidant enzyme thioredoxin-1 (TRX-1) a known repressor of NSY-1. To do this, we used the transgenic strain OE3010. Interestingly, we observed significant increases in *trx-1* expression as a result of treatment with all of the extracts tested (Table 36). Compared to the control, *trx-1* expression significantly increased in worms feeding on 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) turmeric by 1.59 fold (p=0.000), 1.37 fold (p=0.000) and 1.43 fold (p=0.004), respectively. For worms treated with ginger at 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) over the life cycle, *trx-1* expression was upregulated 1.41 fold (p=0.001), 1.38 fold (p=0.181) and 1.40 fold (p=0.001) of the control, respectively.

Trx-1 expression significantly increased in worms consuming rosemary at 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) by 1.53 fold (p=0.003), 1.40 fold (p=0.001) and 1.43 fold (p=0.000) compared to control, respectively. Similarly, treatment with oregano resulted in upregulation of *trx-1* by 1.25 fold (p=0.025), 1.78 fold (p=0.191) and 1.18 fold (p=0.090) of the control in worms feeding on 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) of the herb, respectively.

Table 36. Effect of herbs and spices on *tir-1, nsy-1* expression in *C. elegans*. Relative change in fluorescence intensity in *C. elegans* treated with various extracts at concentrations of 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) compared to control, as measured using the Nikon SMZ1500 fluorescence microscope, R1 CCD camera for imaging and NIH ImageJ software for quantification. FC- Fold change. *-Indicates significantly different from control ($p<0.05$). n=6.

Treatment	TIR-1 n=6			NSY-1 n=6		
	Conc. (% v/v)	*FC*	*P value*	*Conc. (% v/v)*	*FC*	*P value*
Turmeric	0.1	0.6	0.010*	0.1	0.84	0.404
	0.5	1.2	0.084	0.5	0.67	0.153
	1	0.5	0.133	1	0.94	0.934
Ginger	0.1	0.5	0.022*	0.1	0.67	0.000*
	0.5	0.5	0.312	0.5	0.73	0.000*
	1	0.6	0.024*	1	0.72	0.000*
Rosemary	0.1	1.1	0.009*	0.1	1	0.969
	0.5	1	0.031*	0.5	0.87	0.053
	1	1.1	0.06	1	0.91	0.18
Oregano	0.1	1	0.178	0.1	1.3	0.419
	0.5	0.9	0.235	0.5	0.97	0.763
	1	0.4	0.030*	1	0.81	0.272

Table 37. Effect of herbs and spices on *nlp-29* and *trx-1* expression in *C. elegans*. Relative change in fluorescence intensity in *C. elegans* treated with various extracts at concentrations of 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) compared to control, as measured using the Nikon SMZ1500 fluorescence microscope, Ri1 CCD camera for imaging and NIH ImageJ software for quantification. FC- Fold change. *-Indicates significantly different from control ($p<0.05$). n=6.

	Gene					
	NLP-29 n=6			TRX-1 n=6		
Treatment	*Conc. (% v/v)*	*FC*	*P value*	*Conc. (% v/v)*	*FC*	*P value*
Turmeric	0.1	1.19	0.325	0.1	1.59	0.000*
	0.5	1.72	0.002*	0.5	1.37	0.000*
	1	1.65	0.000*	1	1.43	0.004*
Ginger	0.1	1.14	0.217	0.1	1.41	0.001*
	0.5	1.77	0.25	0.5	1.38	0.181
	1	0.84	0.000*	1	1.4	0.001*
Rosemary	0.1	1.06	0.299	0.1	1.53	0.003*
	0.5	1.12	0.264	0.5	1.4	0.001*
	1	1.46	0.000*	1	1.43	0.000*
Oregano	0.1	1.41	0.053	0.1	1.25	0.025*
	0.5	1.11	0.553	0.5	1.78	0.191
	1	1.07	0.675	1	1.18	0.09

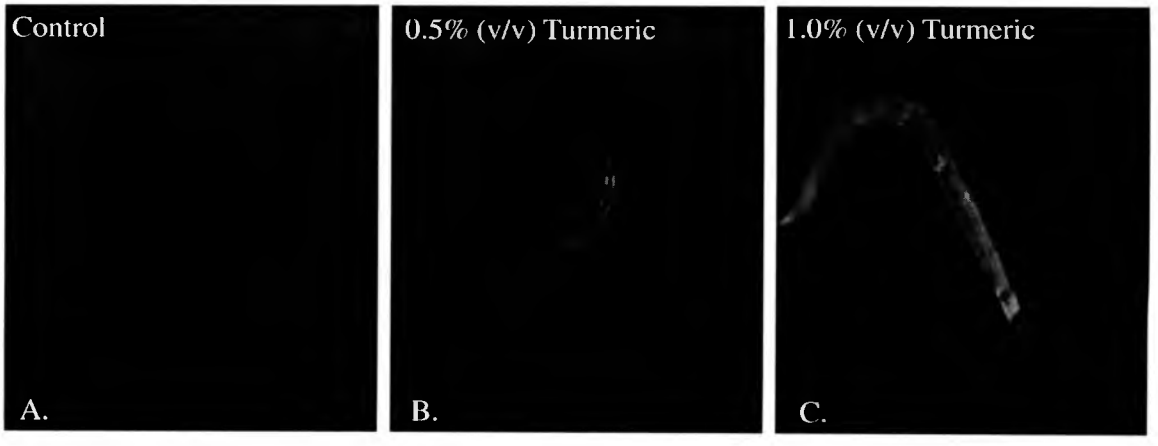

Figure 29. Expression of *nlp-29*::GFP in *C. elegans* feeding on turmeric at varying concentrations. A- 0.0% (v/v) turmeric. B- 0.5% (v/v) turmeric. C- 1.0% (v/v) turmeric.

4.3.3 Effect of dietary herbs and spices on DAF-2/IGFR signaling in *C. elegans*

Insulin signaling through the DAF-2/IGFR in *C. elegans* regulates a number of metabolic processes related to growth and metabolism in addition to its role in pathogen defense.[192,224] Hormetic responses to physiological and environmental stressors are in part mediated by genes under the transcriptional regulation by DAF-16 (FOXO) and SKN-1 (Nrf-2) in *C. elegans*.[192,224] Nuclear accumulation of DAF-16 is associated with increased expression of superoxide dismutase *(sod-3)* and heat shock protein *(hsp-16.2).* However, active DAF-2/IGFR signaling is associated with phosphorylation of DAF-16 thereby preventing nuclear translocation.[212] Conversely, activation of DAF-2/IGFR promotes dissociation of SKN-1 from GSK-3 thereby enabling its nuclear translocation and accumulation leading to expression of glutathione-S-transferase *(gst-4)* and iron sulfur protein *(isp-1).*[206,225]

Here, we measured changes in gene expression of *daf-2*, *sod-3* and *hsp-16.2*, using the *C. elegans* transgenic strains BC14074, CF1553 and GS1829, which carried GFP promoter fusions to the respective genes. Although we observed slight differences between treatments, we observed similar patterns of expression in response to treatment with the four extracts (Table 38). On average, *daf-2* expression was relatively unchanged in response to the treatments and *sod-3* and *hsp-16.2*, which are negatively regulated by active DAF-2 signaling, were down regulated. Specifically, in worms feeding on 0.1% (v/v) 0.5% (v/v) and 1.0% (v/v) turmeric, *daf-2* expression was 0.95 fold (p=0.215), 1.29 fold (p=0.348) and 0.94 fold (p=0.084) of the control, respectively (Table 38).

Interestingly, *sod-3* expression in worms treated with turmeric was down regulated by all of the concentrations tested (Table 38). In worms feeding on 0.1% (v/v),

0.5% (v/v) and 1.0% (v/v) turmeric *sod-3* was down regulated 0.56 fold (p=0.013), 0.64 fold (p=0.569) and 0.49 fold (p=0.010) compared to control, respectively. We also observed *hsp-16.2* expression to be down regulated 0.88 fold (p=0.000), and 0.9 fold (p=0.000) following treatment with 0.1% (v/v) and 0.5% (v/v) turmeric, respectively. However, *hsp-16.2* expression increased by 1.12 fold (p=0.421) in worms feeding on 1.0% (v/v) turmeric.

In response to treatment with 0.1% (v/v) and 1.0% (v/v) ginger, *daf-2* was significantly down regulated 0.88 (p=0.001) fold and 0.81 fold (p=0.000) compared to control, respectively (Table 38). *Daf-2* was unchanged in worms treated with 0.5% (v/v) ginger, however. *Sod-3* expression decreased significantly in response to the treatment with ginger at all concentrations tested (Figure 30). Fold changes were 0.61 (p=0.000), 0.63 (p=0.001) and 0.81 (p=0.000) of the control in worms feeding on 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v), respectively. Similar to treatment with turmeric, expression of *hsp-16.2* remained relatively unchanged in response to treatment with ginger. For worms consuming 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) ginger, *hsp-16.2* was 0.99 fold (p=0.198), 1.01 fold (p=0.153) and 0.99 fold (p=0.706) of the control, respectively.

Treatment with rosemary resulted in a dose dependent decrease in *daf-2* expression (Table 38). For worms consuming 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) rosemary, *daf-2* expression was 1.03 fold (p=0.119), 0.94 fold (p=0.201) and 0.8 fold (p=0.000) of control. *Sod-3* expression was also down regulated in worms feeding on rosemary at 0.1% (v/), 0.5% (v/v), and 1.0% (v/v) by 0.88 fold (p=0.297), 0.94 fold (p=0.027) and 0.94 fold (p=0.431), respectively. However, *hsp-16.2* expression was significantly upregulated in worms feeding on all concentrations of rosemary. *Hsp-16.2*

was 1.21 fold (p=0.001), 1.22 fold (p=0.003) and 1.19 fold (p=0.002) of control in worms feeding on 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) rosemary, respectively.

Treatment with oregano resulted in essentially no change in *daf-2* expression (Table 38). For worms feeding on 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v), *daf-2* expression was 1.02 fold (p=0.109), 1.04 fold (p=0.029) and 1.03 fold (p=0.043) of control, respectively. However, a dose dependent decrease in *sod-3* expression was observed following treatment with oregano. At 0.1% (v/v) oregano *sod-3* increased by 1.25 fold (p=0.051) compared to control but at 0.5% (v/v) and 1.0% (v/v) oregano, *sod-3* was down regulated by 0.88 fold (p=0.315) and 0.85 fold (p=0.162), respectively. *Hsp-16.2* was significantly down regulated as a result of treatment with all concentrations tested. For worms feeding on 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v), *hsp16.2* was 0.65 fold (p=0.000), 0.53 fold (p=0.000) and 0.50 fold (p=0.000) of control, respectively.

Table 38. Effect of herbs and spices on *daf-2, sod-3* and *hsp16.2* expression in *C. elegans*. Relative change in fluorescence intensity in *C. elegans* treated with various extracts at concentrations of 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) compared to control, as measured using the Nikon SMZ1500 fluorescence microscope, Ri1 CCD camera for imaging and NIH ImageJ software for quantification. FC- Fold change. *-Indicates significantly different from control ($p<0.05$). n=6.

	Gene								
	DAF-2 n=6			SOD-3 n=6			HSP16.2 n=6		
Treatment	*Conc. (v/v %)*	*FC*	*P value*	*Conc. (v/v %)*	*FC*	*P value*	*Conc. (v/v %)*	*FC*	*P value*
Turmeric	0.1	0.95	0.215	0.1	0.56	0.013*	0.1	0.88	0.000*
	0.5	1.29	0.348	0.5	0.64	0.569	0.5	0.9	0.000*
	1	0.97	0.084	1	0.49	0.010*	1	1.12	0.421
Ginger	0.1	0.88	0.001*	0.1	0.61	0.000*	0.1	0.99	0.198
	0.5	1	0.982	0.5	0.63	0.001*	0.5	1.01	0.153
	1	0.81	0.000*	1	0.81	0.000*	1	0.99	0.706
Rosemary	0.1	1.03	0.119	0.1	0.88	0.297	0.1	1.21	0.001*
	0.5	0.94	0.201	0.5	0.94	0.027*	0.5	1.22	0.003*
	1	0.8	0.000*	1	0.94	0.431	1	1.19	0.002*
Oregano	0.1	1.02	0.109	0.1	1.25	0.051	0.1	0.65	0.000*
	0.5	1.04	0.029*	0.5	0.88	0.315	0.5	0.53	0.000*
	1	1.03	0.043*	1	0.85	0.162	1	0.5	0.000*

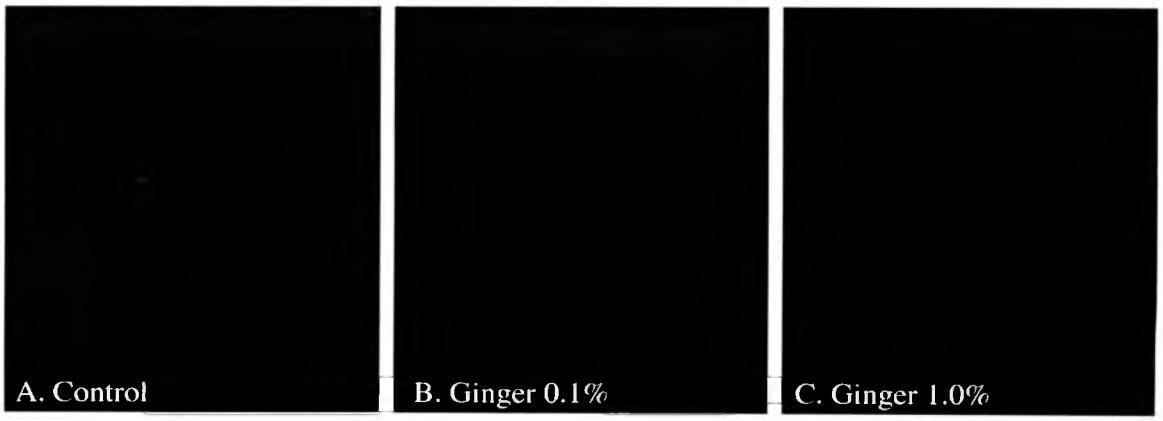

Figure 30. Expression of *sod-3*::GFP in *C. elegans* feeding on ginger at varying concentrations. A- 0.0% (v/v) ginger. B- 0.1% (v/v) ginger. C- 1.0% (v/v) ginger.

Additionally, we measured changes in expression of *gst-4* and *isp-1*, under direct transcriptional regulation by SKN-1 (Nrf2), in response to treatment with turmeric, ginger, rosemary and oregano. *Gst-4* and *isp-1* were measured using the transgenic strains CL2166 and BC14279, respectively. We found *gst-4* expression to be significantly upregulated in response to all the treatments at all concentrations tested and was particularly high in worms feeding on rosemary and oregano of the *Lamiaceae* family of herbs (Table 39). *Isp-1* expression was also upregulated following treatment of all four extracts tested although expression was highest in worms feeding on turmeric and ginger of the *Zingiberaceae* spices (Table 39).

For worms feeding on turmeric at 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v), *gst-4* expression was upregulated 1.72 fold (p=0.004), 1.7 fold (p=0.004) and 1.7 fold (p=0.002) of the control, respectively (Table 39). *Isp-1* expression changed by 1.58 fold, (p=0.061) 1.25 fold (p=0.062) and 1.53 fold (p=0.003) compared to control in worms feeding on 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) turmeric, respectively (Table 39). *Gst-4* expression significantly increased in worms feeding on ginger at 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) by 2.11 fold (p=0.000), 1.81 fold (p=0.001) and 1.76 fold (p=0.000), respectively (Table 39). Significant increases in *isp-1* were also observed as a result of treatment with 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) ginger by 1.32 fold (p=0.000), 1.79 fold (p=0.032) and 1.92 fold (p=0.000), respectively (Table 39).

For worms feeding on rosemary at 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v), *gst-4* was upregulated 1.11 fold (p=0.019), 2.39 fold (p=0.000) and 2.77 fold (p=0.007), respectively (Table 39, Figure 31). *Isp-1* increased in worms treated with 0.1% and 0.5% (v/v) rosemary but decreased in worms feeding on 1.0% (v/v). Fold changes with respect

to control were 1.08 (p=0.679), 1.65 (p=0.037) and 0.90 (p=0.066) (Table 39). Treatment with oregano resulted in significant increases in *gst-4* expression by 2.70 fold (p=0.039), 2.72 fold (p=0.001) and 2.84 fold (p=0.000) in worms consuming 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) of the herb, respectively (Table 39). Increased *isp-1* expression was also observed in worms feeding on oregano at 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) by 1.15 fold (p=0.133), 1.46 fold (p=0.000) and 1.36 fold (p=0.091), compared to control, respectively (Table 39).

Table 39. Effect of herbs and spices on *gst-4* and *isp-1* expression in *C. elegans*. Fold change in fluorescence intensity in *C. elegans* treated with various extracts at concentrations of 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) compared to control, as measured using the Nikon SMZ1500 fluorescence microscope, Ri1 CCD camera for imaging and NIH ImageJ software for quantification. FC- Fold change. *-Indicates significantly different from control ($p<0.05$). n=6.

	Gene					
	GST-4 n=6			ISP-1 n=6		
Treatment	*Conc. (% v/v)*	*FC*	*P value*	*Conc. (% v/v)*	*FC*	*P value*
Turmeric	0.1	1.72	0.004*	0.1	1.58	0.061
	0.5	1.7	0.004*	0.5	1.28	0.062
	1	1.7	0.002*	1	1.53	0.003*
Ginger	0.1	2.11	0.000*	0.1	1.32	0.000*
	0.5	1.81	0.001*	0.5	1.79	0.032*
	1	1.76	0.000*	1	1.92	0.000*
Rosemary	0.1	1.11	0.019*	0.1	1.08	0.679
	0.5	2.39	0.000*	0.5	1.65	0.037*
	1	2.77	0.007*	1	0.9	0.066
Oregano	0.1	2.7	0.039*	0.1	1.15	0.133
	0.5	2.72	0.001*	0.5	1.46	0.000*
	1	2.84	0.000*	1	1.36	0.091

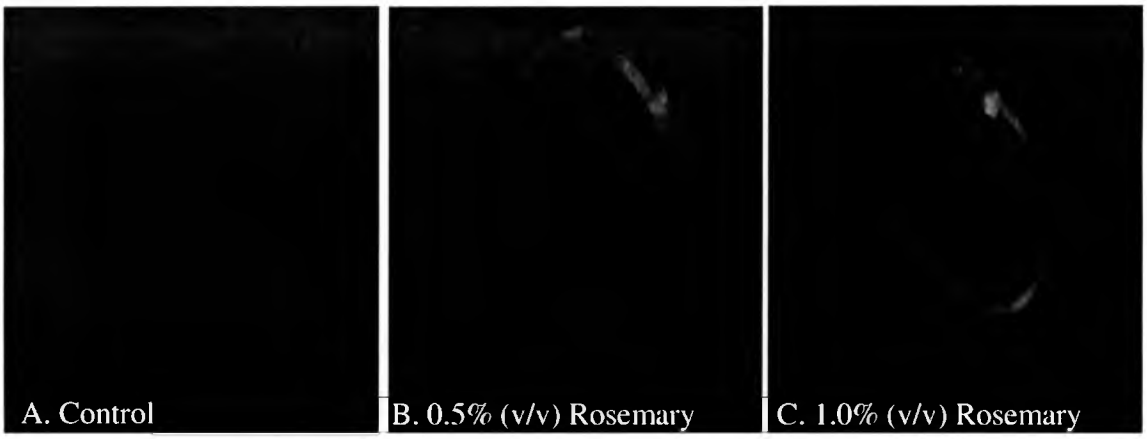

Figure 31. Expression of *gst-4*::GFP in *C. elegans* feeding on rosemary at varying concentrations. A- 0.0% (v/v) rosemary. B- 0.5% (v/v) rosemary. C- 1.0% (v/v) rosemary.

4.3.4 Effect of dietary herbs and spices on apoptosis signaling in *C. elegans*

Recognition and rapid removal of non-self epitopes and cells undergoing apoptosis represents an important component of the innate immune response. The clearance of these materials is critical to prevent the release of pro-inflammatory or toxic compounds by necrotic and pathogenic cells.[226] In *C. elegans,* apoptosis and subsequent endocytosis is orchestrated by a number of cell death (CED) proteins.[226] The scavenger receptor CED-1, homolog to the mammalian SREC, is especially important during the initial events of endocytosis. Its function is dependent, in part, on dynamin, a highly conserved large GTP binding protein that localizes to the plasma membrane in association with CED-1 during pseudopod extension.[226,227] *Dyn-1* mutants therefore, exhibit impaired endocytic uptake of apoptotic cells.[226] In this study, we were interested in measuring potential changes in *dyn-1* expression in *C. elegans* as a result of treatment

with turmeric, ginger, rosemary and oregano. The transgenic strain BC13292 carrying GFP fused to the promoter region of *dyn-1* was used.

We also measured changes in expression of *rme-8*, a protein required during receptor mediated endocytosis, in response to treatment with dietary herbs and spices. RME-8 has recently been identified as a DnaJ domain containing co-chaperone to the heat shock protein 70 (Hsc70) molecular chaperone which functions in the release of clathrin coated vesicles following endocytosis.[228] It is also thought to play an important role during phagosome formation by promoting RAB-5 and RAB-7 localization at the phagosomal membrane. DH1336 was used to monitor changes in *rme-8* expression.

Dyn-1 expression was upregulated in worms feeding on 0.1% (v/v) turmeric by 1.38 fold (p=0.178) compared to control, but was significantly down regulated by 0.81 fold (p=0.008) in worms feeding on 0.5% (v/v) of the spice. At 1.0% (vv) turmeric, *dyn-1* increased by 1.06 fold (p=0.359) compared to the control. *Rme-8* expression in worms consuming 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) turmeric was down regulated by 0.88 fold (p=0.116), 0.91 fold (p=0.184) and 0.83 fold (p=0.831) of the control, respectively (Table 40).

Dyn-1 was also down regulated in worms treated with ginger at 0.1% (v/v) and 1.0% (v/v) by 0.92 fold (p=0.339) and 0.65 fold (p=0.005) of the control, respectively (Table 40). However, *dyn-1* expression increased by 3.75 fold (p=0.414) in worms feeding on ginger at 0.5% (v/v) compared to the control. Additionally, *rme-8* expression significantly increased in worms feeding on 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) ginger

by 1.71 fold (p=0.000), 1.77 fold (p=0.000) and 1.66 fold (p=0.000) of the control, respectively (Table 40).

On average, treatment with rosemary resulted in increased expression of both *dyn-1* and *rme-8* (Table 40). Compared to control, *dyn-1* expression in worms consuming 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) rosemary changed by 1.44 fold (p=0.047), 2.32 fold (p=0.053) and 1.07 fold (p=0.152), respectively (Figure 33). *Rme-8* expression in response to treatment with 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) rosemary was 0.99 fold (p=0.863), 1.05 fold (p=0.339) and 1.11 fold (p=0.002) of the control, respectively (Table 40).

Dyn-1 expression increased significantly in worms consuming oregano at 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) (Table 40). Compared to control, *dyn-1* expression changed by 1.49 fold (p=0.009), 1.39 fold (p=0.000), and 1.28 fold (p=0.002). Additionally, *rme-8* expression was significantly upregulated in worms treated with oregano at all three concentrations tested. Fold changes with respect to control were 1.49 (p=0.000), 1.54 (p=0.000) and 1.33 (p=0.000) in worms feeding on 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v), respectively (Table 40).

Table 40. Effect of herbs and spices on *dyn-1* and *rme-8* in *C. elegans*. Relative change in fluorescence intensity in *C. elegans* treated with various extracts at concentrations of 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) compared to control, as measured using the Nikon SMZ1500 fluorescence microscope, Ri1 CCD camera for imaging and NIH ImageJ software for quantification. FC- Fold change. *-Indicates significantly different from control ($p<0.05$). n=6.

Treatment	Gene					
	DYN-1 n=6			RME-8 n=6		
	Conc. (% v/v)	*FC*	*P value*	*Conc. (% v/v)*	*FC*	*P value*
Turmeric	0.1	1.38	0.178	0.1	0.88	0.116
	0.5	0.81	0.008*	0.5	0.91	0.184
	1	1.06	0.359	1	0.83	0.831
Ginger	0.1	0.92	0.339	0.1	1.71	0.000*
	0.5	3.75	0.414	0.5	1.77	0.000*
	1	0.65	0.005*	1	1.66	0.000*
Rosemary	0.1	1.44	0.047*	0.1	0.99	0.863
	0.5	2.32	0.053	0.5	1.05	0.339
	1	1.07	0.152	1	1.11	0.002*
Oregano	0.1	1.49	0.009*	0.1	1.49	0.000*
	0.5	1.39	0.000*	0.5	1.54	0.000*
	1	1.28	0.002*	1	1.33	0.000*

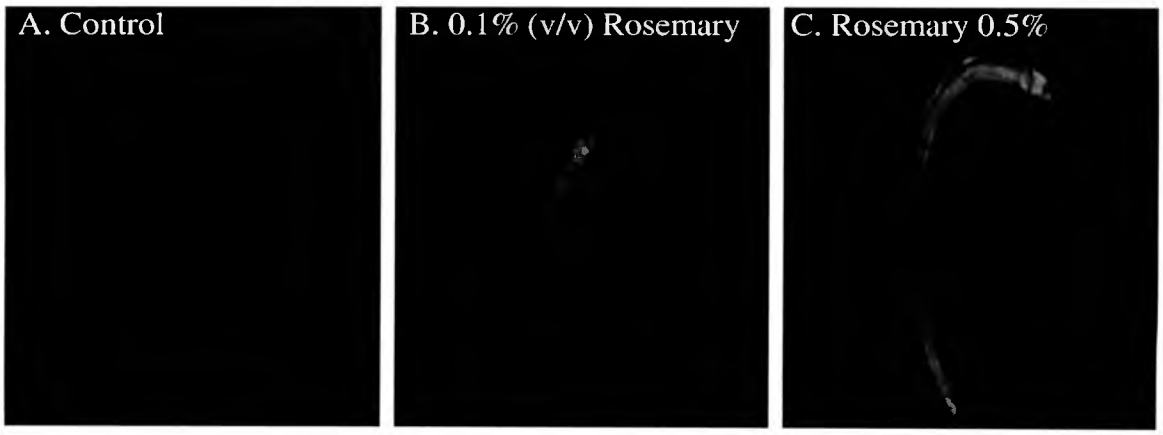

Figure 32. Expression of *dyn-1*::GFP in *C. elegans* feeding on rosemary at varying concentrations. A- 0.0% (v/v) rosemary. B- 0.1% (v/v) rosemary. C- 0.5% (v/v) rosemary.

4.4 Discussion *C. elegans*

4.4.1 Overall effect of dietary herbs and spices on *dbl-1* expression in *C. elegans*

In mammals, cellular proliferation, differentiation and apoptosis are regulated in part by TGF-β signaling.[196] Signal transduction following BMP interaction with the TGF-β receptor initiates a phosphorylation cascade generating the heterotrimeric complex Smad-2/ Smad-3/Smad-4, capable of nuclear translocation and induction of a number of genes involved in these processes.[229] TGF-β signaling is a known inducer of differentiation of multipotential stem cells and myeloid progenitor cells by regulating the expression of required transcription factors, growth factors and their respective receptors.[196,230]

In *C. elegans*, TGF-β signaling mediated by the BMP homolog DBL-1, plays an important role in the innate immune response in addition to its traditional role in development.[231] It is now well established that *dbl-1* mutants exhibit increased vulnerability to infection especially by fungal pathogens including *D. coniospora*. This is primarily because TGF-β signaling, activated by the transforming growth factor, DBL-1, promotes expression of genes encoding antimicrobial peptides such as lectins and lysozymes in the intestinal lumen which is in direct and constant contact with soil borne pathogens.[232] Additionally, DBL-1 promotes expression of Caenacin antimicrobial peptides in neuronal tissues during fungal infection.[232] Previously in our lab we have observed significant increases in the total number of coelomocytes as well as the neutrophil population in *L. terrestris* feeding on *Zingiberaceae* spices and *Lamiaceae* herbs. Because TGF-β signaling is a well known regulator of cellular proliferation and myeloid differentiation, we monitored changes in the TGF-β ligand, DBL-1, to

investigate if phytochemicals in herbs and spices are capable of modulating proteins involved in this pathway.

Using the transgenic strain BW1940, we observed an overall increase in *dbl-1* expression in worms feeding on turmeric, ginger, rosemary and oregano over the life cycle (Figure 33). Because TGF-β signaling is highly conserved among species, upregulation of *dbl-1* expression in *L. terrestris* as a result of treatment with dietary and herbs and spices is likely. Moreover, activation of TGF- β signaling in *L. terrestris* could, at least in part, explain the increased differentiation of myeloid cells into neutrophil-like coelomocytes that we previously observed. However, further analysis to understand the mechanism of action by which phytochemicals present in dietary herbs and spices activate TGF-β signaling using genetic approaches such as RNA interference would be required.

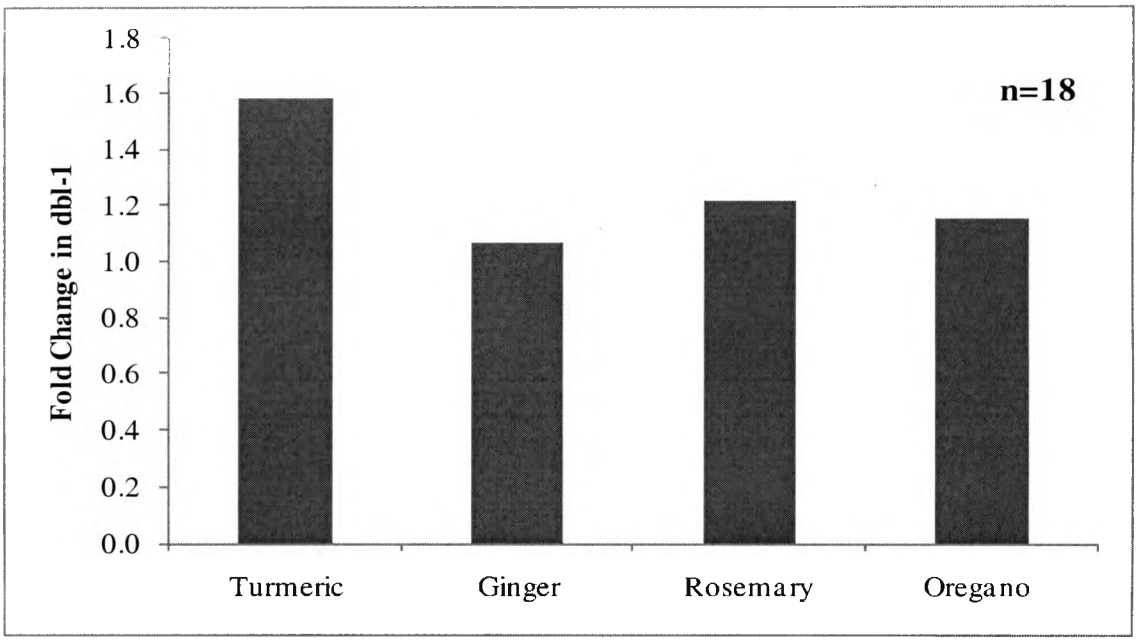

Figure 33. Overall fold change in *dbl-1* expression in *C. elegans*. Fold changes calculated as the average fold change of treatment with 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) of herb or spice, compared to control worms feeding on NGM only.

4.4.2 Overall effect of dietary herbs and spices on p38 MAPK signaling in *C. elegans*

In *C. elegans*, p38 MAPK signaling regulates expression of a number of antimicrobial factors involved in the innate immune response through the TIR-1/NSY-1/SEK-1/PMK-1 cassette.[223] Activation of this pathway as a result of infection results in PMK-1 mediated activation of the transcription factor ATF-7, which regulates expression of bactericidal proteins such as C-type lectins, and NLP-29.[205] Additionally, p38 MAPK signaling is critical during the oxidative stress response by promoting expression of antioxidant enzymes and phase II detoxification proteins including GST-4 and GCS-1 under the transcriptional regulation by SKN-1 (Nrf2).[206]

In this study, we monitored changes in the expression of *tir-1*, *nsy-1* as well as *nlp-29, gst-4* and *isp-1*. Overall, we observed decreased expression of *tir-1* and *nsy-1* in

response to treatment with turmeric, ginger, rosemary and oregano (Figure 34). However, our results show *nlp-29, gst-4* and *isp-1* to be upregulated in response to the treatments overall (Figure 34). It is now known that a number of signaling pathways converge with the traditional p38 MAPK/PMK-1 pathway to control gene expression of proteins involved in the oxidative stress and innate immune response.[233-235]

Ziegler et al. recently observed induction of *nlp*-29 expression following activation of a G protein coupled receptor (GPCR) and TPA-1, a phospholipase C (PLC) homolog in *C. elegans*.[234] Activated PLC stimulates diacylglycerol (DAG) synthesis which in turn triggers activation of protein kinase C (PKC).[235] Ziegler et al. also demonstrated TIR-1 to be a direct target of PKC and therefore upstream of the p38 MAPK cascade.[234] In mammals, PKC is a well known activator of the transcription factor NF-κB via interaction with TLR adapter proteins TIRAP and TRAM.[234] Alternative targets of PKC include DKF-2, a protein kinase D (PKD) homolog in *C. elegans*. DKF-2 has also been shown to be an effector molecule in activation of p38 MAPK signaling via interaction and activation of PMK-1.[233]

MAP-kinase kinase-4 has also been shown to activate p38 MAPK signaling through activation of PMK-1.[236] In 2008 Kell et al. demonstrated that *mkk-4* RNA interference resulted in inhibition of SKN-1 nuclear accumulation suggesting yet another kinase involved in SKN-1 activation.[225] Therefore, it is possible that phytochemicals in dietary herbs and spices may contribute to activation of PMK-1 through non-traditional p38 MAPK signaling thereby resulting in increased expression of *nlp-29*.

Additionally, the observed decreased expression of *nsy*-1 was associated with increased expression of *trx*-1 overall. TRX-1 is now known to be a repressor of NSY-1 activation. This is because under normal conditions, TRX-1 heterodimerizes with NSY-1 in the cytosol thereby inhibiting NSY-1 phosphorylation and activation. However, under oxidative stress conditions, this complex is dissociated as a result of interaction with reactive oxygen species.[208] It is therefore possible that the increased TRX-1 expression as a result of treatment with dietary herbs and spices may explain the decrease in NSY-1 expression.

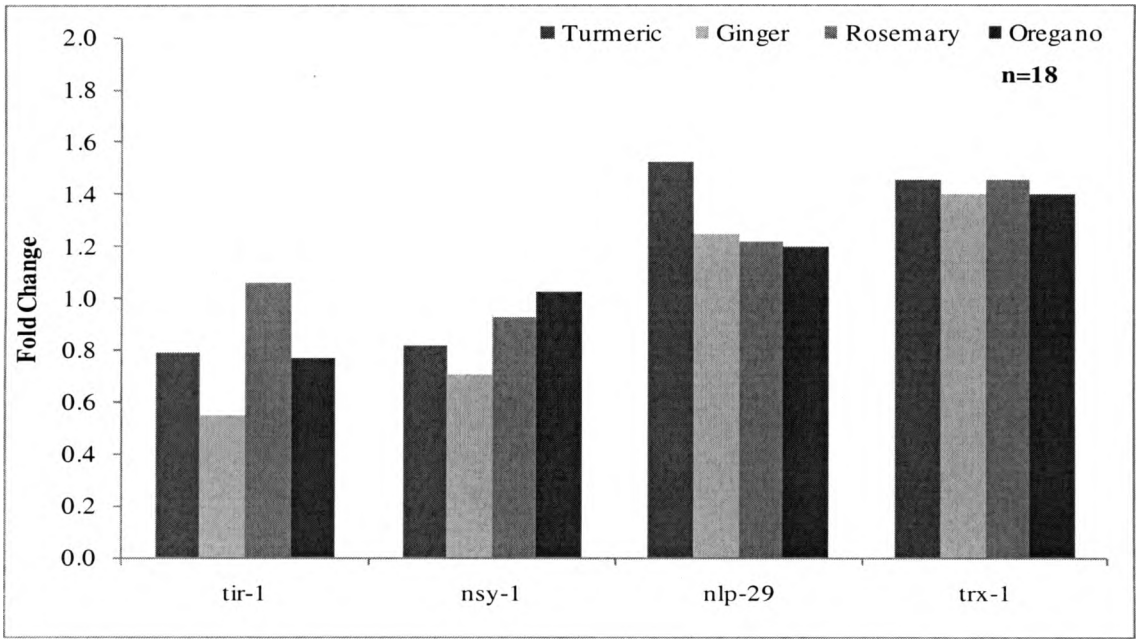

Figure 34. Overall fold change in *tir-1, nsy-1, nlp-29* and *trx-1* expression in *C. elegans*. Fold changes calculated as the average fold change of treatment with 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) of herb or spice, compared to control worms feeding on NGM only.

4.4.3 Overall effect of dietary herbs and spices on DAF-2/IGFR signaling in *C. elegans*

Insulin signaling through the evolutionarily conserved IGF-1 signaling pathway is now known to regulate a number of important physiological processes including those associated with aging and the stress response.[211] Decreased IGF-1 signaling is associated with increased longevity and increased resistance to environmental and oxidative stress.[212] This is primarily true because IGF-1 signaling inhibits activation of the FOXO transcription factor required for expression of antioxidant enzymes and stress response proteins such as superoxide dismutase-3 *(sod-3)* and the heat shock proteins *(hsp-16.2)*.

Insulin signaling is conserved in *C. elegans* and is mediated through the DAF-2/IGFR-1 receptor which when activated inhibits DAF-16 (FOXO) activation.[212] Microarray analysis has shown upregulation of *hsp-16.2*, and *sod-3* in *daf-2* mutants.[210] DAF-2 signaling leading to phosphorylation and activation of AGE-1 (PI3K) and protein kinase B (AKT) are associated with DAF-16 phosphorylation thereby inhibiting its nuclear translocation.[192,211] In the present study, we evaluated changes in expression of *daf-2, sod-3* and *hsp-16.2* in *C. elegans* feeding on dietary herbs and spices over the life cycle. Overall, we did not observe a significant change in *daf-2* expression in response to any of the treatments (Figure 35). However, we observed down regulation of *sod-3* and *hsp-16.2* by 0.79 fold and 0.93 fold, respectively. These results suggest active *daf-2* signaling resulting in phosphorylation of DAF-16 thereby inhibiting expression of DAF-16 target genes.

We also observed an overall increase in *gst-4* and *isp-1* expression in response to the treatments with *Zingiberaceae* spices and *Lamiaceae* herbs. These genes are under

transcriptional regulation by the SKN-1 transcription factor, which is retained in the cytosol bound to GSK-3. However, it is now known that AKT (protein kinase B) has been shown to directly interact with and inhibit GSK-3. Therefore, GSK-3 phosphorylation by AKT during an active DAF-2/IGFR pathway may interfere with GSK repression of SKN-1 thereby enabling nuclear accumulation and expression of the target genes, *gst*-4 and *isp*-1.[225] Therefore, it is possible that active DAF-2 signaling as a result of the treatments may contribute to activation of SKN-1 and the observed upregulation of *gst*-4 and *isp*-1. However, future studies using genetic knock outs and/or RNA interference would be required to better understand the impact of dietary phytochemicals on DAF-2/IGFR signaling.

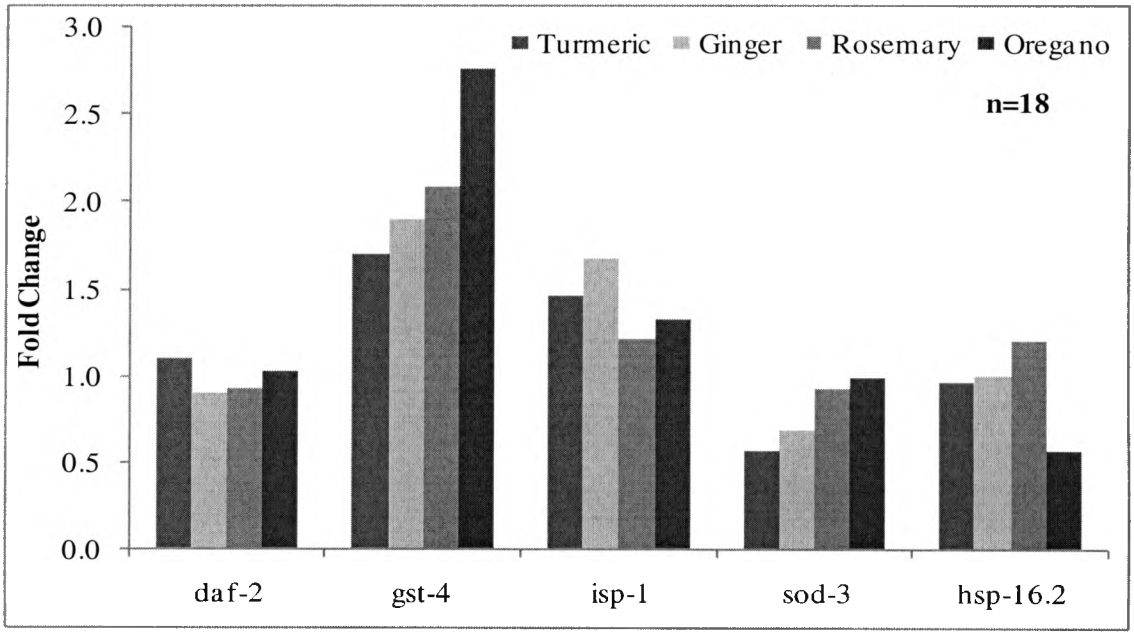

Figure 35. Overall fold change in *daf-2, gst-4, isp-1, sod-3 and hsp-16.2* expression in *C. elegans*. Fold changes calculated as the average fold change of treatment with 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) of herb or spice, compared to control worms feeding on NGM only.

4.4.4 Overall effect of dietary herbs and spices on apoptosis signaling in *C. elegans*

Rapid and efficient recognition and removal of pathogenic and apoptotic cells is an important function of innate immunity.[216,217] Professional phagocytes recognize specific markers such as pathogenic associated molecular patterns (PAMPs) or exposed phospholipids such as phosphatidylserine on apoptotic cells and initiate the endocytic process.[217] Inadequate uptake of apoptotic cells is associated with development of chronic diseases such as cancer, diabetes, and Alzheimer's disease.[191,216]

In *C. elegans*, clearance of these cells is mediated in part by the highly conserved CED-1/CED-6/CED-7 pathway and the CED-2/CED-5/CED-12 pathway.[191] Initiation of endocytosis occurs as a result of recognition of apoptotic cells by CED-1, homologous to the mammalian CD91. CED-1 activity is dependent on a number of supporting proteins including CED-7, DYN-1 and RME-8, among others. CED-7, an ABC transporter homolog, is required for ligand presentation to CED-1 as well as CED-1 clustering at the plasma membrane.[191,218] DYN-1 is a GTPase dynamin homolog in *C. elegans* that is required for CED-1 uptake of apoptotic cells and functions during vesicular fission and phagolysosome formation.[218] RME-8, a DnaJ containing molecular co-chaperone is required for receptor mediated endocytosis during phagosomes formation by promoting RAB-5 and RAB-7 recruitment at the phagosomal membrane.[228]

In the present study we monitored changes in gene expression of *dyn-1* and *rme-8* in *C. elegans* treated with turmeric, ginger, rosemary or oregano over the life cycle. Overall, we observed upregulation of *dyn-1* expression by 1.14 fold (Figure 36). We also observed a 1.27 fold increase overall in *rme-8* expression as a result of the treatments

(Figure 36). These findings suggest that phytochemicals present in these herbs and spices may potentially contribute to enhanced recognition and uptake of apoptotic cells in *C. elegans* by upregulating expression of *dyn-1* and *rme-8*.

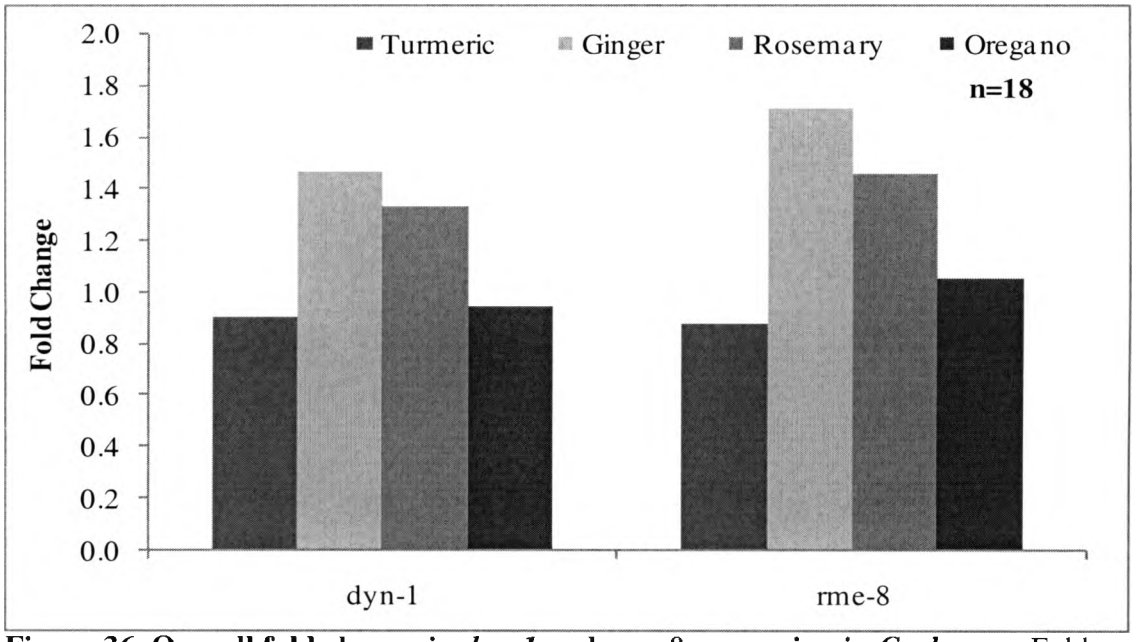

Figure 36. Overall fold change in *dyn-1* and *rme-8* expression in *C. elegans*. Fold changes calculated as the average fold change of treatment with 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) of herb or spice, compared to control worms feeding on NGM only.

4. 5 Future directions

4.5.1 Future studies using *L. terrestris*

In the current study, we have observed significant physiological changes in fundamental aspects of innate immunity *in vivo* as a result of consumption of dietary herbs and spices over the life cycle. Using *L. terrestris*, we show that phytochemicals in *Zingiberaceae* spices and *Lamiaceae* herbs may stimulate hematopoiesis and differentiation of myeloid precursors which may contribute to increased phagocytosis and a more controlled oxidative degradation during respiratory burst. Moreover, because these changes primarily occurred concomitantly with increases in extracellular nitric oxide, we believe that nitric oxide may be acting as a signaling molecule to activate pathways which trigger these processes.

Future studies using quantitative RT-PCR would be necessary to evaluate the effect of these phytochemicals on the expression of genes critical to the innate immune response. We are especially interested in measuring changes in expression of genes involved in signaling pathways regulated by nitric oxide (NO) and the antioxidant response element (ARE). Currently, expressed sequence tags (EST's) for many of these genes have been identified and primers have been designed and obtained for these studies (Table 41).

Table 41. EST's of genes involved in innate immunity in *L. terrestris*. The following sequences were accessed from LumbriBASE and primers were designed using the NIH primer blast program (PUBMED).

Protein	Protein Function
Toll	Toll-like receptor proteins involved in pathogen recognition (LPS)
Nitric Oxide Synthase (NOS)	Catalyzes synthesis of NO
Coelomic Cytolytic Factor	Pattern Recognition Protein Binds to PAMPs such as O-antigen of LPS Functionally analogous to TNF
Lumbricin, Lysozyme	Antimicrobial peptide against gram +/- bacteria
NFκB	Nuclear transcription factor involved in transcription of ~400 genes associated with immune response
Myeloid Protein	Adaptor protein in signaling pathways mediated by TLR's and IL-1 receptors leading to activation of NFκB and transcription of genes involved in immune response
Microsomal glutathione S-transferase	Enzyme that catalysis the conjunction of glutathione to electrophiles
Catalase	Antioxidant enzyme that catalyzes the removal of hydrogen peroxide
Glutathione Peroxidase	Catalyzes reduction of hydrogen peroxide
Superoxide Dismutase	Dismutates superoxide anions
Thioredoxin Peroxidase	Antioxidant enzyme that reduces hydrogen peroxide to thioredoxin

Additionally, because one of the primary functions of innate immunity involves pathogen recognition and clearance, it would be important to evaluate if these phytochemicals in herbs and spices are capable of modulating the innate immune response during a pathogenic challenge. Here we observed enhanced uptake and degradation of *S. cerevisiae* in coelomocytes isolated from *L. terrestris* feeding on commonly consumed herbs and spices. Future studies using common pathogens such as *Pseudomonas aeruginosa* or *Staphylococcus aureus* as a challenge would be important to evaluate the effect of plant secondary metabolites on important aspects of innate immunity. Moreover, because we observed increased total coelomocyte and neutrophil

count as well as increased coelomocyte viability as a result of these phytochemicals, it would interesting to evaluate if pre-treatment with dietary herbs and spices could have a protective effect during a bacterial challenge.

4.5.2. Future studies using *C. elegans*

In the present study, we have partially characterized the genetic effects of dietary herbs and spices using a number of transgenic *C. elegans* carrying GFP constructs to genes involved in four primary innate immune signaling pathways. Overall, we observed significant changes in gene expression of proteins involved in multiple signaling pathways including TGF-β signaling, p38 MAPK signaling, DAF-2/IGFR signaling and the UPR response. However, future studies using different molecular approaches are necessary to better understand the exact mechanism of action by which these plant secondary metabolites exert their effects. Such techniques include RNA interference (RNAi) to sequentially silence genes involved these four pathways in order to ascertain potential molecular targets of phytochemicals in herbs and spices. Additionally, activation of the transcription factors SKN-1, DAF-16 and ATF-7 regulated by p38 MAPK and DAF-2/IGFR signaling can be determined by western blot for the phosphorylated forms of these proteins.

4.5.3 Characterization of phytochemicals in herbs and spices

In the current study we evaluated the effect of whole dietary herbs and spices on the innate immune system. Therefore, because herbs and spices are rich in a diverse range of phytochemicals and plant secondary metabolites,[13] it would be interesting to isolate the different chemical constituents and identify the bioactive compounds that may be

contributing the observed effects. Phytochemical analysis could be carried out by fractionation into the major phytochemical classes and characterized using high performance liquid chromatography (HPLC) and mass spectrometry (MS).[237]

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