THE PHYTOCHEMICAL INVESTIGATION, BREEDING AND ARTHROPOD REPELLENT EFFICACY OF *NEPETA CATARIA*

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ABSTRACT OF THE DISSERTATION

The Phytochemical Investigation, Breeding and Arthropod Repellent Efficacy of *Nepeta Cataria*

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Dr. James E. Simon

While notoriously known for inducing the euphoric effect on felines, *Nepeta cataria* (catnip) is emerging as a natural product and source of a variety of compounds useful for human health. *Nepeta cataria* produces essential oil in glandular trichomes on leaf and floral surfaces as well as a variety of non-volatile compounds that have been shown to be medicinally active. This plant species is becoming increasingly popular for its use as a feline attractant, medicinal therapeutic and as a naturally sourced insect repellent. This research shows that the bioactives in catnip can be as effective as a repellant against mosquitoes and ticks as DEET. With the new developments and current use of catnip as a natural product, this dissertation focuses on standardizing plants and extracts for applications of its bioactives for emerging areas of public health and interest. An investigation into the genetic inheritance of the key bioactive compound nepetalactone, was conducted as a foundational base for plant breeding to develop improved *N. cataria* populations. Breeding was conducted to select for increased biomass, total essential oil and increased nepetalactone production in the aromatic volatile oil. Two new plant varieties

were developed, cv. CR9 and cv. CR3 each with significantly improved total essential oil and as rich sources of nepetalactone. Associated studies examining where such bioactive compounds accumulate, as well as the antioxidant and anti-inflammatory activities were conducted. This dissertation then focused on examining the repellency efficacy that *N. cataria* population's essential oils have against disease vectoring insects such as *A. aegypti* mosquitoes and *D. variabilis* as well as *I. scapularis* ticks by developing dose response curves to determine the minimum concentration required of the essential to achieve complete repellency of the disease vectors. Finally, *N. cataria* essential oil was incorporated into a series of formulations to extend the duration of acceptable repellency of the essential oil while also examining the skin toxicity associated with one of the effective formulations.

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Dedication

This dissertation is dedicated to the men and women of the armed forces who have lost their lives defending the liberties of the United States of America.

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Chapter 1 Introduction

Throughout human history, natural products have been manufactured by processing naturally occurring organisms into more purified extracts for many purposes. These natural products have been developed from animals, plants and fungi for many years either directly or indirectly and are desired to increase one's quality of life[1]. While the domestication of animals and nutrient providing crops such as corn and wheat are common knowledge, often the selection of natural products providing novel uses to humans is overlooked or misunderstood. As early as 3,000 BC, Egyptians made papyrus scrolls from fibers of the *Cyperus papyrus* plant to record their history and events of their daily lives[2]. Cloth and dyes were also made with the natural resources they found around them while biblical references say the gifts normally reserved for kings, identified as frankincense and myrrh, were gifts to Jesus Christ upon his birth[3,4]. Natural products have also served as part of ritualistic ceremonies where naturally derived entheogens have been used to express devotion, thanks and to seek spiritual involvement from deities[5]. There is no shortage of examples of humans using natural products to influence and improve their lifestyles as early adopters of natural products maintained a competitive advantage over individuals that did not utilize these rich resources benefiting from either their medicinal or ethnobotanical uses[6].

Commonly, natural products have long been used in the past as they are today as therapeutic agents to treat medical disorders and many drugs have been developed through the investigation into naturally occurring compounds[7]. Healing practices by indigenous populations have been recorded for over 5,000 years in which natural products derived from plants were central to the healing process[8,9]. More accepted pain management

techniques have been at the epicenter of major pharmaceutical development from natural products ever since the use of opium by German scientist Johann Wolfgang von Goethe and the subsequent isolation of the morphine alkaloid by Friedrich Wilhelm Sertürner in the early 19th century[10]. In 1827, Emanuel Merck started isolating morphine and other medications forming the nosiness model of the modern day pharmaceutical industry[11]. Since then, the demand for new and more effective naturally derived pharmaceutical solutions to medical disorders has driven modern pharmaceutical companies to continue their pursuit of drug development by investigating naturally occurring compounds[12].

While using natural products to treat medical disorders is well documented, proactive approaches to not contracting infections from disease vectoring insects utilizing natural products have also been recognized and documented in writings for thousands of years[13]. Images depicting plants being literally hung around houses or burnt nearby have been found illustrating the ethnobotanical use of these natural products to drive the insects away showing that just like the development of the pharmaceutical industry, insect repellent manufacturing had its start with natural derivatives that are still used to this day[14]. While DEET has been the standard repellent since its development as a pesticide for the United States Department of Agriculture and further adoption by the Department of Defense as a repellent, natural products have shown efficacy comparable to DEET at repelling the same insects vectoring deadly diseases[15,16]. Recently, natural products have become of greater interest in replacing synthetics as alternatives to repelling disease vectoring insects due to efficacy and public sentiment toward naturally occurring compounds[17]. With an estimated 1.5 million deaths per year due to arthropod-borne diseases, this clear and present danger

to the global population's public health must be addressed to limit the spread of these disease and protect individuals from them[18].

This dissertation investigates the plant species *Nepeta cataria* (Fam. Lamiaceae), commonly known as catnip to take advantage of its ethnobotanical use as a natural product. Identifying the volatile compounds and their patterns of inheritance throughout the parents and progeny of different *N. cataria* plants will aid in the breeding and isolation of desired naturally produced compounds. Further work focused on the selection and development of two unique new catnip varieties, cv. CR9 and cv. CR3 with differential chemistry but both developed as part of a larger plant breeding program to increase the total essential oil content in comparison to commercially available catnips and to ensure a significantly higher accumulation of the bioactive compound, nepetalactone in the aromatic volatile or essential oil. Investigating the non-volatile compounds will allow us identify potential medicinally bioactive compounds further supporting its current holistic use. Finally, due to the previous literature that suggests *N. cataria* essential oil as an arthropod repellent, testing and formulations will be made to validate the essential oil as a repellent and to create skin safe formulations developed from this natural product.

1.1 Natural Products Overview

Natural products are a chemical or substance created by a living organism that is exploited for use by individuals. These products can be in the form of cloths, dyes, building materials and most notably medicinal products. As mentioned, these products have been used for thousands of years and continue to be a major avenue of exploration today[2,7]. Different cultures and societies around the world have used natural products either directly or

indirectly. Animals, fungi and plants have long been used as natural products while sometimes maintaining a strong spiritual connection to them. Animal products have long been recorded throughout human history for the use of clothing, satchels, weapons made from bones and numerous other products to help individuals inhabit certain regions of the globe that otherwise would have been uninhabitable[19]. Musical instruments such as the bagpipe were made from animal parts with the bladder or hide serving as the windchest[20]. Natural products made from fungi are similarly just as important and in 1944 Selman Waksman developed streptomycin from *Actinomyces griseus*, a natural product used to treat tuberculosis, an infectious disease that has estimated to have killed 1.3 million people in 2017[21,22]. Without this discovery, millions more people a year would be infected and die from the infectious pathogen *Mycobacterium tuberculosis*. While natural products have been developed from organisms from multiple kingdoms, more commonly, natural products are immediately associated with plants in the form a flavoring or spice, supplement, or pharmaceutically pure extract designed to treat specific ailments.

1.1.1 Natural Products Derived from Plants

There is no shortage of examples of using plants as natural products and almost every tissue of plants has been used in one way or another to form a natural product. Natural products can be sourced from the primary and secondary metabolites from a plant where primary metabolites are metabolites required for the normal growth (amino acids and sugars) and physiological development of a plant and secondary metabolites are metabolites associated with the plant interacting with its environment or defense from a pathogen attack (volatile essential oils and non-volatile polyphenols)[23]. While the use of plants for food is common knowledge, fibers from roots and stems have been used to make cloth, paper, weapons and

other forms of products in which tension is required[2,24]. In 2009, the commercial timber industry in the Amazon is estimated to have $15.4 billion dollars in value remaining[25]. Alkaloids obtained from the leaves and fruits of plants have been used historically both medicinally and immorally to treat patients pain or to cause harm to individuals respectively[10,26]. Chemical compounds isolated from plants have also been developed into recreational substances that cause powerful temporary physiological changes in an individual that are so powerful, many countries have deemed them illegal giving rise to powerful, violent crime syndicates seeking to control the billion-dollar illicit markets associated with them[27,28]. Elicitation of plant secondary metabolites with hormones in cell cultures for the pharmaceutical industry has shown great progress in increasing their yields in comparison to traditional breeding methods and processing technologies[29].

1.1.1.1 Volatile Secondary Metabolites

Volatile secondary metabolites are a diverse sect of chemical compounds with a high vapor pressure under ambient conditions and these chemicals serve many purposes within a plant including, but not limited to, pollinator attraction, stress relief and pathogen defense[30]. These chemicals can be synthesized from various biosynthetic precursors including fatty acids, amino acids and throughout the terpenoid synthetic pathway[31]. Terpenoids are the largest group of natural products and constitute over 20,000 different compounds[32]. In non-biological systems, terpenoids are developed through the joining of individual isoprene units in a "head to tail" method to create the carbon skeletons to be further modified creating the diverse array of compounds **(Figure 1-1, page 6)**[33]. Each isoprene units consists of five carbons and the nomenclature for compounds containing one, two, three

and four isoprene units is a hemiterpene, monoterpene, sesquiterpene and diterpene respectively[33].

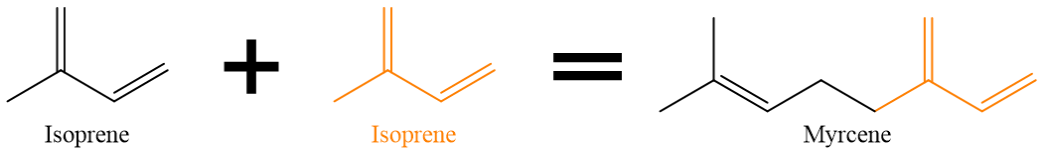

Figure 1-1: Diagram illustrating the creation of terpenoids from individual isoprene units following the "head to tail" rule in non-biological systems.

In biological systems, isopentenyl diphosphate (IPP) is the energized molecule used to create terpenoids, not individual isoprene units and this pathway with the addition of additional IPP units provides the backbone for further organism specific modifications similar to the isoprene additions **(Figure 1-2, page 6)**[33]. These terpenoids can be further modified into cyclic forms or remain acyclic void of any further modifications.

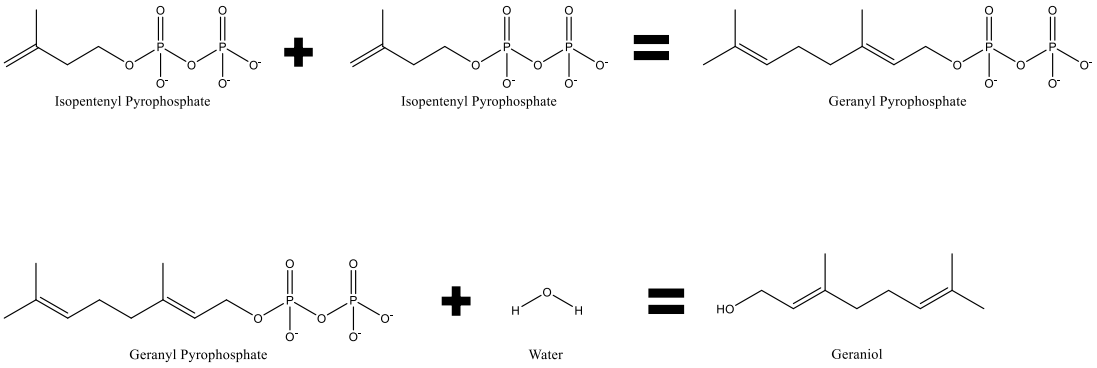

Figure 1-2: Diagram illustrating the creation of terpenoids from individual isopentenyl pyrophosphate units following the "head to tail" rule in biological systems with subsequent hydrolysis to form the monoterpenoid geraniol.

Terpenoid synthesis in plants was thought to only occur through the mevalonate pathway in the cytoplasm and mitochondria, however recent has shown that terpenoid synthesis can be carried out in plastids through the non-mevalonate pathway to form the backbones of many terpenoids **(Figure 1-3, page 7)**[34]. On a cellular level, terpenoid biosynthesis is compartmentalized in resin ducts and glandular trichomes throughout the plants with the most well known site being the glandular trichomes of a plant that reside on the leaf epidermis[32,35].

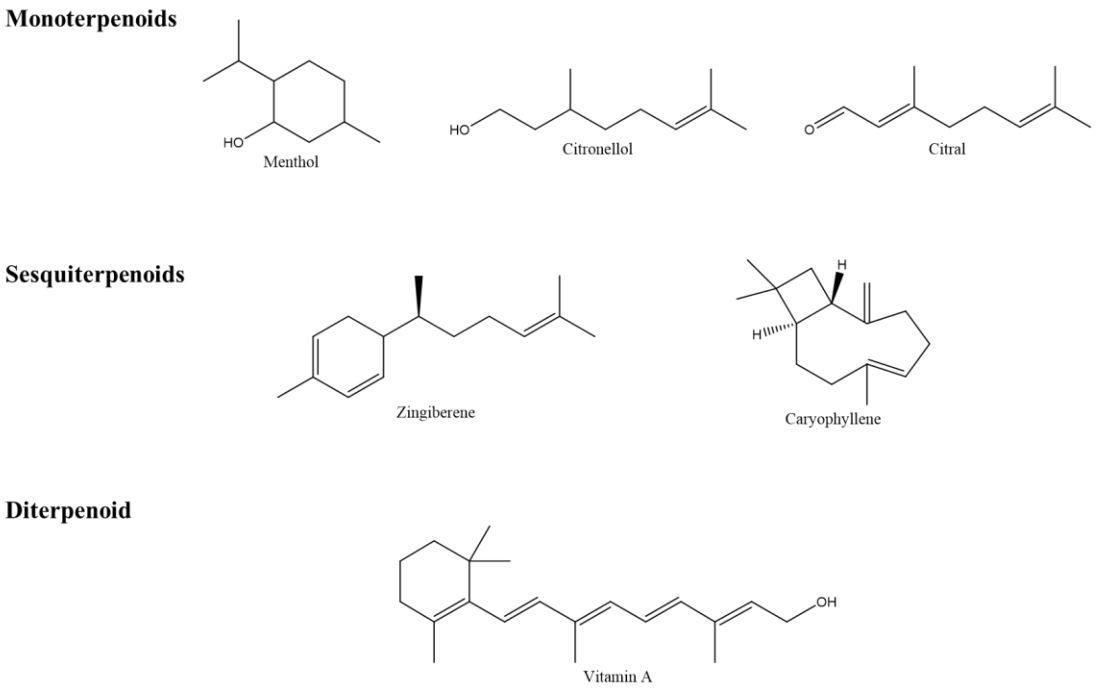

Figure 1-3: Bioactive terpenoids found in biological systems. The monoterpenoids menthol, citronellol and citral are formed the by the addition of two isopentenyl pyrophosphate units. The sesquiterpenoids zingiberene and caryophyllene are formed the addition of three isopentenyl pyrophosphate units. The diterpenoid is formed by two monoterpenes.

1.1.1.2 Non-volatile Secondary Metabolites

Non-volatile secondary metabolites consist of a wide diversity of compounds in contrast to volatile compounds differ in that they have a low vapor pressure and are synthesized throughout many pathways. One of the most commonly studied natural products are alkaloids, in which the interpretation of the definition changes depending on your field of study, but for the biologist it is a biologically active and heterocyclic compound that contains at least one nitrogen and has pharmacological or ecological use **(Figure 1-4, page 9)**[36]. One of the oldest known uses of alkaloids as a medical treatment is the use of opium in the recipe for Theriak, an anti-poison recipe using snake meat and other substances used by the Greco-Romans that was eventually purified by Emanuel Merck and gave rise to the modern day pharmaceutical industry[11,33]. Just like the use of essential oils as a medicinal therapeutic, there is no shortage of examples of using alkaloids to treat medical disorders in which cocaine, morphine, caffeine and vinblastine have been purified to treat specific ailments[33].

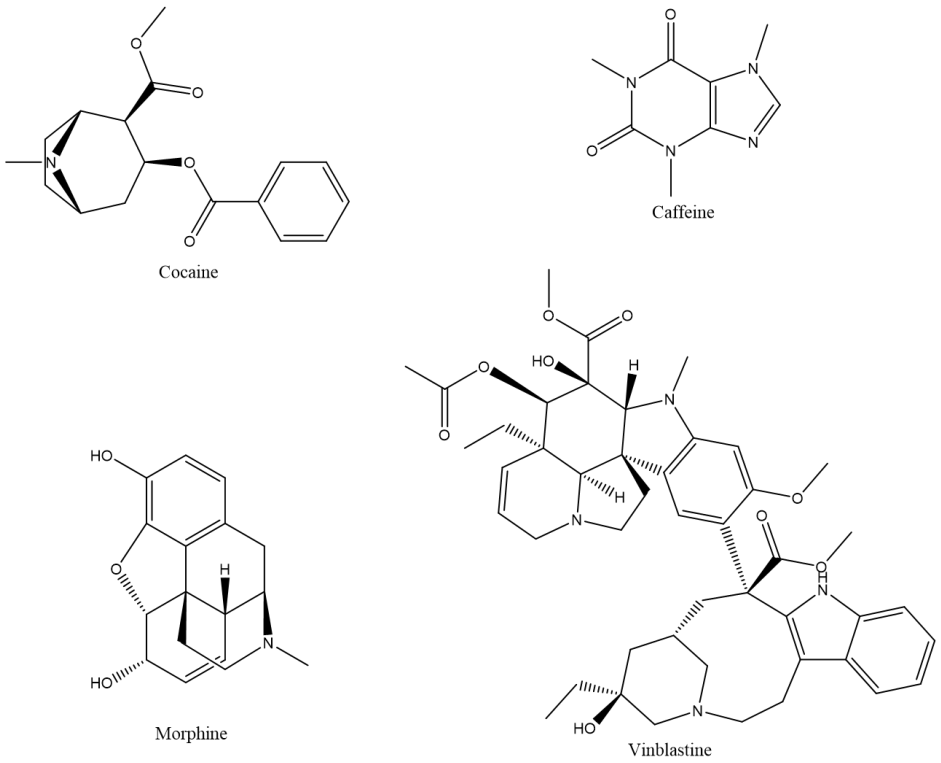

Figure 1-4: Selected isolated alkaloids purified from plant material that are utilized today by the pharmaceutical industry.

Another major class of non-volatile compounds produced by plants are polyphenols **(Figure 1-5, page 10)**. This class of compounds is associated with environmental stress management for plants by reducing free radicals within their cells known as reactive oxygen species that cause membrane and enzyme instability[37,38]. Polyphenols act by reducing free electrons that arise in an organism capitulating the radicals ability to cause damage through oxidation[39]. Polyphenols are characterized by the presence of more than one aromatic hydroxyl group and are commonly consumed from natural sources such as fruits, vegetables and herbs[40]. Polyphenols have been historically used as a natural source of compounds to tan hides and make inks prior to the understanding of them as a medicinal therapeutic[41]. More recently, the medicinal value of the non-volatile compounds produced

by plants have gained notoriety and individuals that frequently consume polyphenol rich foods have shown to have a reduced efficacy towards getting cancer, cardiovascular diseases, neurodegenerative diseases and inflammation due to the chemical structure [42,43]. The claims that these non-volatile extracts are capable of treating a wide range of medical issues, along with the volatile compounds, have helped create a market for herbal supplements and in 2017, this industry recorded $30.2 billion in revenue and $2.6 billion in profit[44]. This industry is expected to continue to grow as new consumers enter it and new products are developed for them.

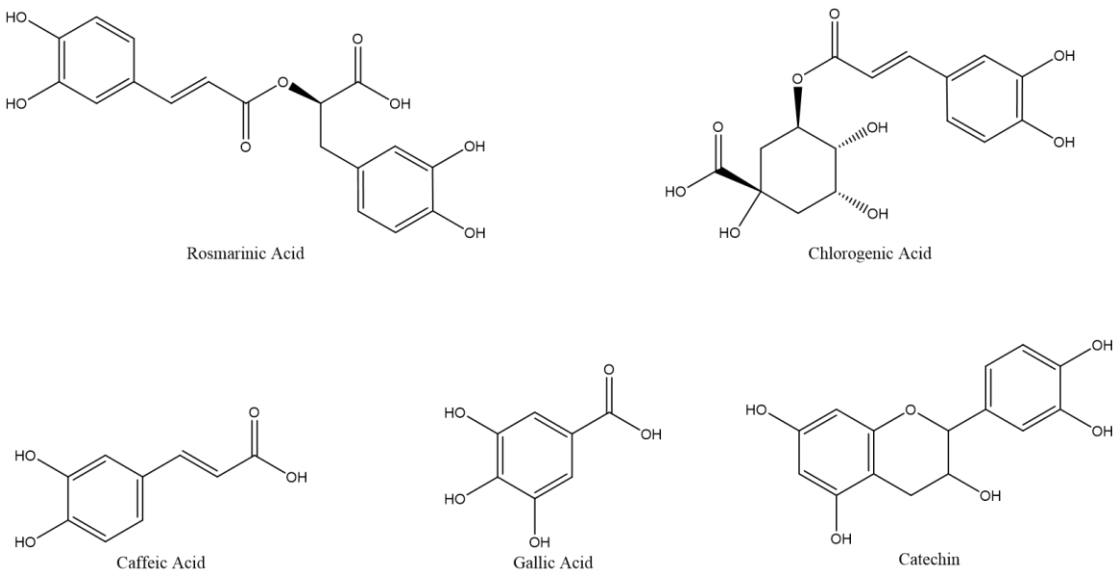

Figure 1-5: Selected isolated polyphenols produced by plants illustrating the multiple aromatic rings and hydroxyl groups.

One subclass of polyphenols is known as flavonoids and they contain fifteen carbon atoms, two six-membered rings linked with a three-carbon unit and may contain a third ring

(Figure 1-6, page 11)[45]. The flavonoids can exist by themselves or combine the monomers to form dimers or oligomers[33]. These flavonoids are stored in vacuoles and serve many physiological purposes such as environmental stress relief, ecological relations such as pollinator attraction and defense[33]. These flavonoids can occur in various forms where the aglycone has no derivatives, a glycosylated flavonoid has a sugar attached to it and many more derivatives that contribute to their large diversity. The radical scavenging capabilities of flavonoids contributes to the medicinal potential as an antimicrobial, antibacterial, antifungal and anti-inflammatory agent[45].

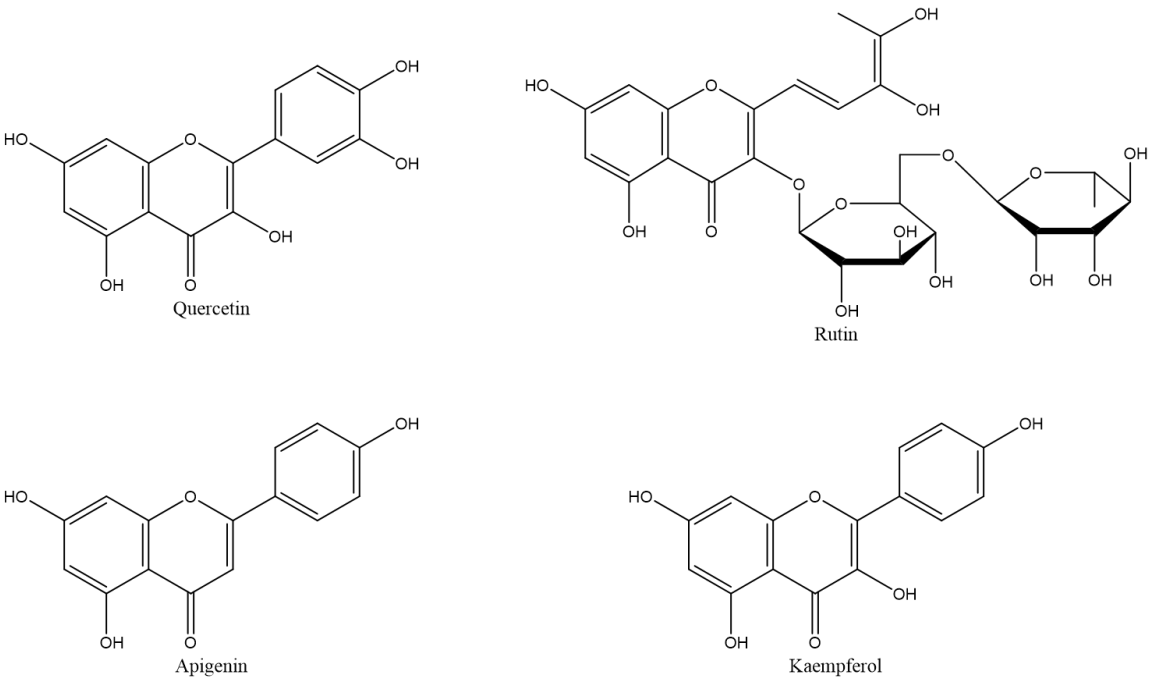

Figure 1-6: Selected isolated flavonoids that exhibit bioactive properties.

1.2 *Nepeta cataria* as a Natural Product

1.2.1 Lamiaceae Phylogeny

The plant kingdom consists of eukaryotic organisms that possess photosynthetic capabilities, cell walls, spores and maintain sedentary behavior[46]. *Nepeta cataria* is vascular, seed reproducing, flowering, dicot that was originally classified by Carl Linnaeus in 1800 in his second volume of *Species Plantarum* and has since been reclassified multiple times[46,47]. Catnip *Nepeta cataria*, an aromatic herb from southwestern Asia, is best known for causing a euphoric effect on domestic cats and other members of the feline family due to the volatile compound nepetalactone contained in the essential oil of the plant and long been recognized for its phytochemical and medicinal properties[62,47,48,49,50,51]. *Nepeta cataria*, is a member of the Lamiaceae plant family or more commonly referred to as the mint family[52]. This family contains over 200 genera and over 6,000 species[46]. The Lamiaceae family is characterized by having hermaphroditic flowers on plants with four sided (square) stems that are herbs or shrubs with short stalked glandular trichomes producing aromatic essential oils[46]. The *Nepeta* genus is a member of the Lamiaceae family and contains at least 150 to 250 species depending on the characters used for analysis[48,53,54].

1.2.2 Natural Products Derived from the Lamiaceae Family

Natural products sourced from the Lamiaceae family provide a wealth of variety in the compounds they produce and contribute to the annual $20.7 billion seasoning, spice and sauce market in the United States[55]. The plants incorporated into this family are found throughout the globe and have contributed to the development and economic gain of rural communities[56,57,58,59,60]. Some plants such as various mint species have been used historically to treat many different types of medical ailments[61]. However, the attribute most

associated with members of this plant family is that they are highly aromatic with many species renown for culinary uses such as basil, mints, oregano, rosemary, sage and thyme while several such as peppermint, spearmint, clary sage and others are grown to distill their volatile essential oils for use in foods, flavors, personal hygiene, cosmetics, industrial products and the pharmaceutical industry[62,63]. Plants such as mint, salvia, sage and oregano also produce non-volatile compounds of interest that possess medicinal value as well, though the plant family is recognized more for its volatile essential oils[64,65]. The plants in this family also serve non medicinal and non culinary purposes such as *Hyptis suaveolens* that has been used as a natural product by individuals in West Africa as an effective insect repellent for many years as well[66]. These natural products are sourced and used as raw materials for further purification into commodities purchased by individuals for many applications.

1.2.2.1 Volatiles Produced by the Lamiaceae Family

In 2017, American companies profited $79.5 million in sales from essential oils and the industry is expected to grow in the coming years illustrating the importance that the Lamiaceae plant family has toward natural products derived from them[67]. Volatile essential oils are incorporated into man products used in the food, flavoring, cosmetic and pharmaceutical industries. The essential oil profiles change when dealing with different genera, species and environmental conditions as well[68,69,70]. In various sweet basil (*Ocimum basilicum*) varieties, linalool, camphor, citral, α-humulene, eucalyptol, eugenol, methyl chavicol, methyl cinnamate and methyl eugenol have been produced within this species[71]. In various *Mentha* species, mint plants not only produced the characteristic volatile menthol, used in many consume products, but they also produce menthol,

menthone, isomenthone, carvone, piperitone oxide and eucalyptol **(Figure 1-7, page 14)**[72]. In oregano (*Origanum vulgare*), varieties are known to be rich in, carvacrol and thymol, with many other aromatic volatiles such as sabinene and linalool in their essential oil. These compounds are desired by many industries for multiple purposes and are globally sourced.

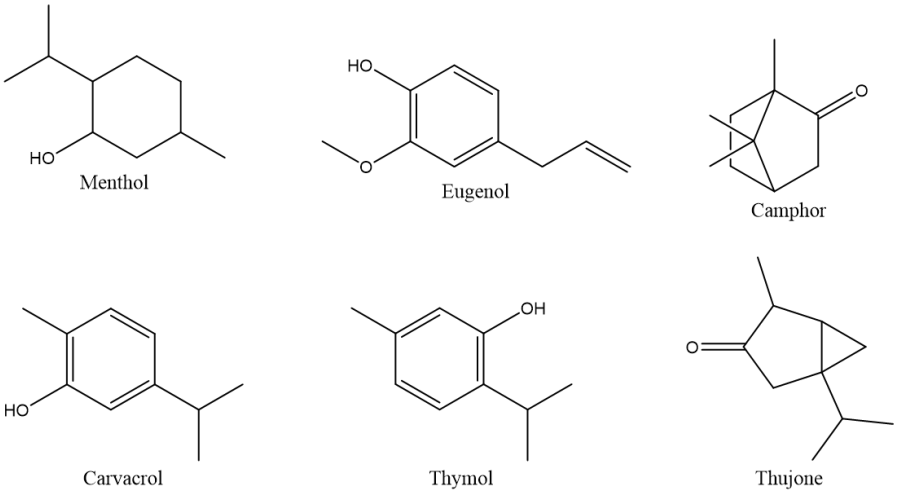

Figure 1-7: Selected volatile compounds sourced from the *Lamiaceae* family.

1.2.2.2 Medicinally Bioactive Polyphenols from the Lamiaceae Family

Polyphenols biosynthesized in members of the Lamiaceae family have acetocholinesterase inhibitory activity indicating that they could be useful in enhancing the chlorogenic transmission of messages for people suffering from Alzheimer's Disease[73]. Polyphenols from oregano and other Lamiaceae species inhibited DPP-IV, which is responsible for insulin secretion, warranting further investigation into Lamiaceae derived polyphenols for diabetes management[74]. Apigenin, luteolin, and pebrellin are commonly found in the plant tissues of Lamiaceae plants[75,76]. Anti-inflammatory activity has also been confirmed in many members of this family by measuring the production of NO after the addition of an

extract to lipopolysaccharide (LPS)-induced RAW 264.7 cells to examine whether the extract inhibits the activity of inducible nitric oxide synthase (iNOS), a main indicator for an inflammatory response within an organism[77]. Mint and oregano polyphenols such as rosmarinic, oleanolic and ursolic acid inhibit iNOS and sequential NO production[83,84]. Apigenin is an inhibitor of the transcriptional activator of COX-2 and iNOS resulting in reduced inflammation in LPS-induced RAW 264.7 murine macrophages[78]. Apigenin reduced TNF-α-induced monocyte adhesion to endothelial cells suggesting anti-inflammatory activity in the human vascular system[79]. Luteolins extracted from dandelion flowers have also been shown to inhibit the transcriptional activation of COX-2 and iNOS in LPS-induced RAW 264.7 murine macrophages resulting in a reduction of inflammation suggesting medicinal value from members of the Lamiaceae family that also produce luteolin[80]. Another *in vivo* murine study showed that luteolin reduced vascular inflammation in mice and that it also suppressed the IKBα/NF-κB signaling pathway resulting in protected endothelial cells to TNF-α- induced monocyte adhesion[81]. Aqueous extracts from *Schizonepeta tenuifolia* (Japanese catnip) reduced inflammation *in vivo* in carrageenan induced paw edema mice further inciting a need for the investigation of Lamiaceae species with respect to anti-inflammatory activity[82]. Anti-oxidant activity is well reported within the Lamiaceae plant family with lavender, mint, oregano and lemon balm being just some of the species reported to contain high concentrations of bioactive polyphenols and other compounds exhibiting radical scavenging capabilities **(Figure 1-8, page 16)**[83,84,85].

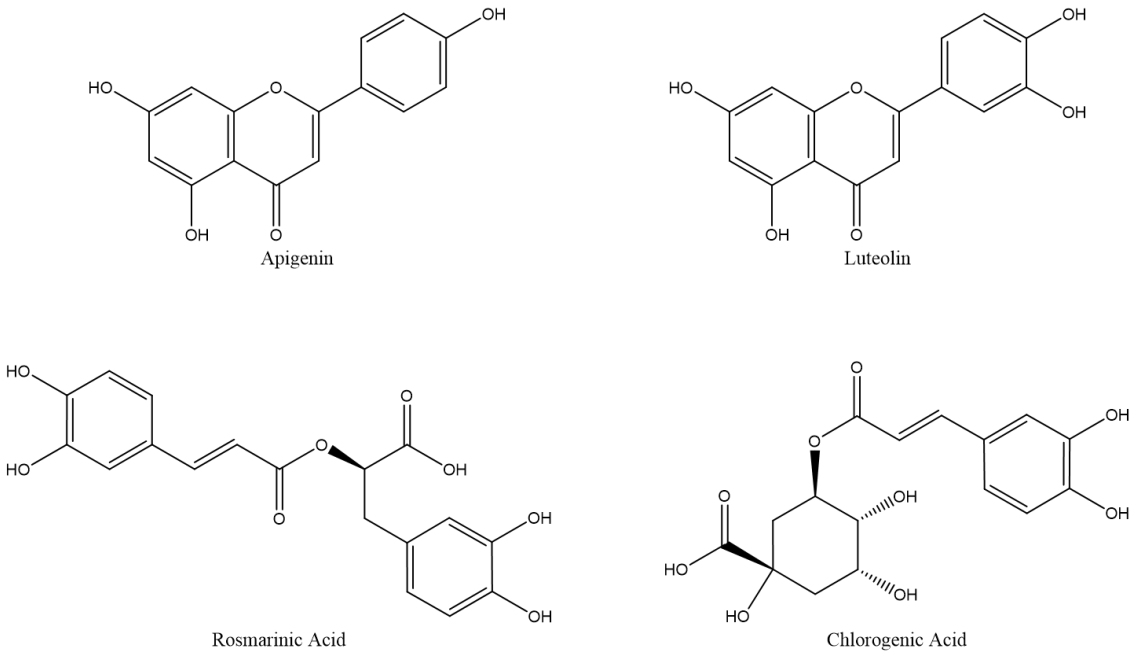

Figure 1-8: Selected non-volatile compounds produced by plants in the Lamiaceae family.

1.2.3 Breeding of Lamiaceae Plants for Natural Products

Efforts to increase the yields of desired natural products have been a focus of plant breeders since civilizations were indirectly selecting wheat for less pronounced awns on the seed head and teosinte selection that eventually developed into corn for yields and sugar content[86,87]. Historically, traditional breeding techniques were void of modern molecular tools in which transgenic plants are created to enhance the production or quality of the desired natural products being made by the plants. In the past, plant breeding techniques were plant and character dependent that broadly could be categorized into two paths where plants are either inbred to increase homozygosity or crossed to other desirable parents to

increase heterozygosity for future inbreeding. Outside of the Lamiaceae family, plants have been bred to increase cellulose production within plants for biofuel purposes such as corn and sorghum while cotton plants (*Gossypium sp.*) have been bred to increase the amount and quality of their fibers[88,89,90]. Breeding for the resistance to pathogens has been accomplished as well, in which tomato plants (*Lycopersicon hirsutum*) have been developed to be resistant to the Yellow Lead Curl Virus[91,92].

Within the Lamiaceae family, these minor crops have had little breeding efforts gone into them in comparison to our staple food. Efforts have been to increase to overall biomass, essential oil and the compounds of interest yields as well as insect and disease resistant. Disease resistance should also be prioritized in members of this family since infected plants can have decreased biomass, essential oil and compound of interest yields. New peppermint plants (*Mentha × piperita L.*) have been developed to increase the biomass and essential oil content for farmers that supply fresh, dried and distilled essential oils to their customers[93]. Three different cultivars of basil (*Ocimum sp.*) showed different chemical profiles in their essential oils that dramatically changed the aroma profile, an increase in linalool, from the traditional sweet basils[94]. Breeding programs to increase the carvacrol concentration, the compound of interest, in Greek Oregano (*Origanum vulgare*) has also been performed[95]. Another breeding project focused on creating a mint plant that shows resistance to mint wilt, a disease caused by the soil borne fungus *Verticillium dahlia* that is detrimental to the production of mints and causes crop losses[96].

1.2.4 *Nepeta cataria* Morphology

Nepeta cataria plants have the common characters associated with the Lamiaceae plant family and *Nepeta* genus. They are shrubs with square stems, simple opposite leaves where

the inflorescence is terminal in verticillaster units[46]. Due to the morphological nature of the bilabiate bisexual flowers, this plant can self-pollinate and can outcross[97]. Like many of the other members of this family, the aromatic volatiles of catnip are produced in the glandular trichomes in the leaf epidermis and floral tissue[98]. There are many types of trichomes on the surface of the leaves with peltate glands and type 1 capitate hairs being present, both where essential oils are accumulated[99].

1.2.5 *Nepeta cataria* as a Natural Product

There are many uses for *Nepeta cataria*. The essential oils biosynthesized in the trichomes of the *Nepeta* genus produce many volatile compounds. Stereoisomers of pinene, ocimene, terpineol, limonene and caryophyllenes are present in the essential oil of *Nepeta cataria* as well as aromatic citrus compounds such as geraniol, linalool and citronellol[100,101]. While catnip produces many different types of compounds within its essential oil, it is most famously known for producing nepetalactones; volatile monoterpenoids that are responsible for its characteristic aroma[50,102]. While many variations of the iridoid nepetalactone are possible, the two main stereoisomers of nepetalactone produced are the Z, E and E, Z nepetalactone isomers (**Figure 1-9, 18**).

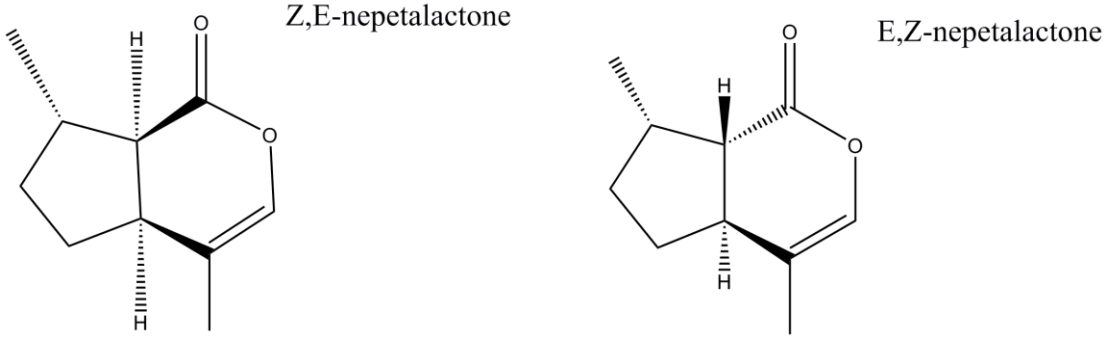

Figure 1-9: Chemical structures of the two main nepetalactones in *Nepeta cataria*. The structures of Z, E-nepetalactone and E, Z-nepetalactone; the two main nepetalactone isomers produced by *N. cataria*.

These nepetalactones produced in the essential oil of nepetalactone are the desired bioactive products from *N. cataria* and are extracted from the glandular trichomes in myriad ways. Solvent, hydrodistillation, steam distillation, ultrasonic and supercritical CO_2 extractions are among a variety of extraction methods to procure essential oils from aromatic plants[103].

1.2.6 *Nepeta cataria* as a Feline Attractant

The use of catnip as a feline attractant is the most common use of this plant. Pet stores sell dried catnip leaves or essential oil extracts to elicit the characteristic responses of sniffling, licking, chewing and rubbing in felines[104,105,106]. While not all cats are affected by catnip extracts and it is hypothesized to be inherited by autosomal dominance, natural products developed for this reason are commonly purchased and used on all members of the feline family[107]. From a toxicological point of view, when force fed to felines in concentrations of up to 80 milligrams, no physiological or histological issues were observed indicating that at these concentrations, indicating that nepetalactones are safe for cats to consume[51].

1.2.6.1 Holistic Use of *Nepeta cataria*

Catnip is also consumed as an herbal tea purported in traditional cultures to be useful for treating medical disorders such as inflammation, digestive ailments, infantile colic, toothaches, used as a cold remedy, anxiety and as a blood depurative[108,109]. Native American tribes would make catnip tea and drink it multiple times a day to help treat their ailments even after the introduction of western medicine[110]. African American slaves as well as their owners used catnip for the same purposes in the southern Appalachian Mountains and today it is still used by African Americans to treat infantile colic and

digestive issues[111]. Europeans and the Chinese have previously used catnip leaves in their cooking and herbal infusions[112,113].

1.2.7 *Nepeta cataria* as a Medicinal Therapeutic

While catnip is well known for producing volatile monoterpene iridoid nepetalactones, the compounds responsible for eliciting euphoria in felines, members of this genus also produce a wide array of non-volatile medicinally bioactive polyphenols that are often overlooked[48,50,51,]. Both the volatile and non-volatile compounds contain in *N. cataria* have been investigated to have medicinal bioactivity such as anti-fungal, anti-microbial, anti-oxidant, anti-inflammatory and relieve symptoms associated with the flu and the common cold[114,115,116,117]. The examination *N. cataria's* medicinal properties appears to justify its historical and present use of the plant given the presence of bioactive natural products.

1.2.7.1 Antioxidant Activity in the *Nepeta* Genus

Plants produce antioxidants to relieve themselves from the stresses associated with excess sunlight, anaerobic conditions and other environmental stresses such as salinity[118]. While many types of radicals can form through escaped electrons, reactive oxygen species are generated in plants under aerobic conditions near the sites of electron transport chains and are formed when free electrons that escape the electron transport chain, react with oxygen creating a reactive chemical with an unpaired electron[119]. The superoxide radical (O_2^-) and the hydroxyl radical (OH^-) are two examples of reactive oxygen species made when electrons react with O_2 and OH respectively. When high concentrations of reactive oxygen species build up in a plant, they can react with proteins, membranes, organelles and DNA causing denaturation, instability, degradation and mutations respectively. Plants produce polyphenols that act as antioxidants to quench the radicals formed during stressful

conditions to maintain homeostasis and not alter their normal physiological functions. The same mechanism from which plants reduce reactive oxygen species through their polyphenols is the same mechanism that acts when individuals consume polyphenol rich foods to combat illness that are associated with long term exposure to radicals. Antioxidant capacity is well reported within the *Nepeta* genus and has been shown to be effective at capturing free radicals by multiple assays illustrating the importance that the volatiles and non-volatile compounds have towards this activity[120,121]. In *N. cataria* methanol extracts and essential oils were tested independently of one another and they both have significant antioxidant properties by the DPPH and β-carotene/linoleic antioxidant assays. Harvesting times also influence the radical scavenging capabilities in *N. cataria* and it is expected that the maturity of the plant effects this[122]. Efforts to investigate the different plant parts within and intraspecies population needs to be investigated since *N. cataria* plants have a long history of established holistic use to form production guidelines for companies selling catnip products as a supplement.

1.2.7.2 Inflammatory Pathway and Anti-Inflammatory Activity in the *Nepeta* Genus

The inflammatory response is an innate response conserved throughout many members of the animal kingdom and is used to protect organisms from physical, mechanical, stresses and from other organismal attack[123]. The inflammatory pathway is activated through a metabolic cascade of different proteins being activated through specific interactions with the environment such as the previously mentioned interactions followed by the subsequent transcription of pro-inflammatory proteins to further respond to the stimuli[123]. Inflammation can result in many different responses within an organism however, if the pathway is left unchecked, constantly being expressed due to environmental interactions

or an overactive autoimmune response, the organism can suffer due to a hyperimmune response for specific pathogens[123]. In humans, many chronic autoimmune inflammatory diseases throughout different organs have been contributed to an overactive inflammatory response such as asthma, rheumatoid arthritis, inflammatory bowel disease, and psoriasis[124]. In contrast, if the inflammatory pathway is not active and is metabolically dysfunctional, it will not be able to correctly respond to the stimulus and infections as well as a non-response can be the end result since the inflammatory response is involved in almost all types of injury and infection. The inflammatory response can be broken down into two pathways, the innate and adaptive response where the innate response occurs right after a stimulus with the recruitment of neutrophils[123]. The adaptive response is more familiarly known as the immune system and is when a variety of cells generate specific antibodies, so leukocytes can recognize them[123].

The innate inflammatory response is also known as the acute inflammatory response in which neutrophils (white blood cells involved in the innate inflammatory response) travel to the site of physical injury or pathogen invasion guided by the release of chemokines and cytokines by affected cells[125]. The symptoms of pain, redness and heat in affected cells are characteristic of an acute inflammatory response[125]. There are numerous physiological changes that occur when the innate inflammatory pathway is initiated with the first change being within endothelial cells, cells that coat lymphatic and blood vessels[126]. Once endothelial cells are activated the express adhesion factors on the cell surface which allows neutrophils to attach themselves to it after being guided by pro-inflammatory cytokines and chemokines[126]. After activation, endothelial cells undergo physiological changes releasing proteins and other metabolites into the effected area encouraging neutrophil

transport to that area[125]. When the inflammatory pathway is activated by pathogens, neutrophils destroy them through phagocytosis after identification where adaptive immunity is utilized where the metabolites are used to create pathogen specific antibodies and in specialized cells (known as antigen presenting cells), present fragments of the lysed cell to label other detrimental microorganism so specialized leukocytes (T-cells) can be more selective and efficient at removing them[126].

The adaptive inflammatory response differs from the innate response by the type of leukocyte that that ingests a microorganism and the subsequent action taken in the inflammatory pathway[125]. Antigen presenting cells can be a differentiated form of leukocytes and engulf microorganisms like neutrophils however, these cells take fragments of the lysed pathogen and present them to T-cells in specialized receptors on the cell surface[123,125]. Regulatory T-cells then release cytokines to recruit, attract and stimulate the production of other T-cells[123,125]. Specific cytotoxic T-cells then recognize specific antigens from infected cells, bind to them and release proteins that destabilize the membrane of that cell[123,125]. Once the threat is gone, regulatory T-cells deactivate the production of cytotoxic T-cells and activate the production of memory T-cells, cells that stay in circulation patrolling for the recurrence of the pathogen[123,125].

While the inflammatory response is an elegant evolutionarily accomplishment to protect an organisms well being from injury, pathogens and other environmental disturbances, the pathway can also become dysfunctional responding to stimuli[123]. Asthma is the result of the inflammatory pathway recognizing an allergen in the respiratory tract that results in increased cytokine expression and subsequent transcriptional responses that increase mucus production in the lungs and constriction of the proximal airway[127]. Pneumonia is

similar to asthma where the inflammatory response to a bacterial or viral infection is producing excessive fluids in the lung causing shortness of breath and chest pain[123]. Psoriasis is an autoimmune inflammatory skin disease where an abnormal amount of keratinocytes (epidermal cells), T-cells and dendritic cells remain in the infected area after the allergen is gone causing rashes, heat and itching sensations[128]. Efforts to reduce inflammation by using anti-inflammatory drugs are widely used derived from synthetic and naturally occurring compounds[129,130].

1.2.8 Mosquito Epidemic and Insect Repellents

Mosquitoes vector the deadliest diseases on the planet killing an estimated half a million people annually by Malaria, Yellow fever and the Dengue virus alone[131,132,133]. Recently, a newer threat known as the Zika virus reemerged and is being rapidly spread by *Aedes aegypti* mosquitoes, as well as from a viremic mother to her newborn and by sexual intercourse throughout the western hemisphere, potentially causing neurological disorders and microcephaly[134]. Female mosquitoes seek out hosts in search of a blood meal for reproduction providing an opportunity for the infectious agent to enter the host while the mosquito is feeding[135]. The common symptoms of all these diseases are rashes, a high fever and chills that all complicate emergency room diagnosis due to the similarities, resulting in a missed or delayed diagnosis that could result in mortality[136]. Some diseases such as the West Nile virus show very little symptoms and can go undiagnosed until movement loss or neurological illness[137]. Disease vectoring mosquitoes cover the world and efforts to control the spread of diseases, identify new repellents and to educate people lowering the risk of infection are currently being implemented on a multinational level[138,139,140]. DDT (1,1'-(2,2,2-Trichloroethane-1,1-diyl)bis(4-chlorobenzene)) is an pinsecticide that was

used globally in the past to kill mosquitoes but its usage has been severely curtailed due to its negative environmental impacts[141]. Educating individuals about mosquito control in regions of the globe where there is a high rate of disease incidence on how to manage mosquito populations, has been effective at reducing infections[142,143].

Insect repellents developed by government and private industries are very effective at deterring mosquitoes and protecting the users from contracting these diseases. DEET (N, N-Diethyl-3-methylbenzamide) has been the benchmark of insect repellents since its development for the United States Army to use in tropical regions where there is a high incidence of insect transmitted diseases[144]. Picaradin, developed by Bayer, is very effective at repelling mosquitoes as well[145]. While DEET is extremely effective, it is not as volatile as other insect repellents and leads to a limited range of efficacy in repelling mosquitoes and other insects[146]. Whereas another critical concern associated with the consistent use of DEET is its potential toxicity. Studies have shown DEET inhibits human acetyl cholinesterase, modulates GPCRs and inhibits ion channels[147,148,149,150,151]. Numerous publications exist urging caution in its use and claim that DEET is unsuitable for young children and pregnant females, though the Centers for Disease Control and Protection (CDC; Atlanta, GA) still recommends the use of it for vector protection[152,153,154]. DEET is also absorbed through the skin at a high rate and special formulations are required to reduce transdermal absorption[155]. However, other reviews suggest that it does not cause adverse health effects[156]. The costs associated with the production of DEET have made it unaffordable to many of those living in regions affected by disease vectoring mosquitoes. Between the costs, access, and public concern about the dangers of DEET individuals and families are preferring natural insect repellants over DEET.

While many regions of the world have access to DEET and other effective insect repellents, natural products still serve as a primary source of repellents in areas where people cannot afford or do not have access to them. Specialty crops cultivated to produce a wide array of insect repellents in all areas of the globe and efforts to repel disease-causing insects from natural sources have been found dating back to before the Common Era[157]. Ethnobotanical resources have led to the identification of plants that can be used to repel insects[14,158]. Volatizing citronella and geraniol from lemongrass oil as well as neem oil are the most common natural sources of mosquito repellents[159,160]. In 2005, the CDC endorsed para-Menthane-3, 8-diol (PMD), a steam distillate product from the leaves of the Australian lemon-scented gum tree as a mosquito repellent[161]. Insect repellents derived from natural sources such as volatile essential oils have been shown to be as efficient at repelling mosquitoes as DEET, at least for up to an hour, and their current use to supplement or replace the use of DEET is substantial and is expected to increase**Error! Bookmark not defined.**,[162,163].

1.2.8.1 *Nepeta cataria* as Mosquito Repellent

Natural insect repellent formulations using essential oils from aromatic plants in the Lamiaceae family are sourced from the glandular trichomes on the epidermis of leaves and flowers. Plants biosynthesize multiple compounds in their essential oil, and breeding programs have increased the bioactive compounds concentration within the essential oil of multiple species of plants across the kingdom[164,165,166,167,168]. Other members from the Lamiaceae family such as Bush mint (*Hyptis suaveolens), Endostemon tereticaulis*, and three basil species have demonstrated that their essential oils can act as a mosquito repellent comparable to DEET[169,170]. While normally cultivated for the pet toy industry as a safe

attractant to cats and for ornamental applications, recent research has shown that essential oils from catnip are an efficient insect repellent and are at least comparable to repelling insects to the industry standard repellent DEET with far less toxicity [51,171,172,173,174]. Other tests showed that *Nepeta cataria* (catnip), has had its essential oils containing various nepetalactone stereoisomers tested and the results showed that it is comparable to DEET at repelling mosquitoes, while offering better spatial repellency [174,175,176]. Catnips volatile oil effectively repels mosquitoes, including the females that carry the plasmodium that causes malaria and those that transmit yellow fever, filariasis, the West Nile virus and encephalitis for a total of six different mosquito species repelled[163,174,177,178]. In one study, 41 different plant species were tested for repellency toward three species of mosquitos that carry pathogens and *N.* cataria was one of the top five plants (Litsea (*Litsea cubeba*), Cajeput (*Melaleuca leucadendron*), Niaouli (*Melaleuca quinquenervia*), Violet (*Viola odorata*), and catnip (*Nepeta cataria*) whose oil exhibited repellency[163]. Like many genera of the Lamiaceae plant family, the *Nepeta* genus contains species that produce a wide array of non-volatile and volatile compounds in their essential oil with nepetalactones, β-caryophyllene, nerol, citronellol and geraniol present[179,180,181].

Insects can often discriminate different isomers, perceiving them differently from one another and the stereochemistry of an insect repellent compound can alter its efficacy as a repellent[182,183,184]. In *A. aegypti* mosquitoes, efficacy of the repellant A220 compound varied three to four times when different isomers were tested and this was contributed to the mechanics of the repellent receptor system within the mosquito[182]. Other studies have also suggested that the molecular structure of a repellent compound influences the repellent activity in *A. aegypti* mosquitoes including picaridin[185]. Due to the different perception of

repellents by arthropods, efforts to find the most effective isomer need to be taken when design a naturally sourced repellent as these compounds are not always produced in the same concentrations due to genetic and environmental influences[186].

Members of the *Nepeta* genus produce many different stereoisomers of nepetalactone within their essential oils **(Figure 1-9**, **page 18)**[100,187,188]. Efforts to investigate the differences in the two main crude essential oil chemotypes (Z, E-nepetalactone dominated or E, Z-nepetalactone dominated) and the isomers of nepetalactone with respect to repellency in mosquitoes have been investigated[177,189]. A World Health Organization (WHO) approved topical application bioassay showed the different essential oil chemotypes were comparable to DEET at repelling *Anopheles gambiae* in a forearm assay from a steam distilled product[177]. The Z, E- and E, Z-nepetalactones showed similar efficacy at repelling *A. gambiae* in another WHO approved topical application bioassay where the crude oils and purified compounds were not statistically different from one another at repelling the mosquitoes[177]. A biting deterrent assay in *A. aegypti* showed no difference in the crude oils and purified nepetalactones in the ability to stop feeding[189]. The Z, E isomer can also be hydrogenated to form dihydronepetalactone 2 that is as effective at repelling two species of mosquitoes as well as DEET and offers complete protection for up to five hours in experiments involving human subjects[171].

1.2.8.2 *Nepeta cataria* as an Arthropod Repellent

Catnip essential oils and the bioactive compound nepetalactone has been shown to repel disease vectoring insects other than mosquitoes. The Z, E isomer has shown significant repellency towards house and stable flies as well as being shown that catnip derived nepetalactones are an oviposition repellent[173,190,191,192]. The peach-potato aphid is also

repelled by nepetalactones suggesting that *N. cataria* could be evaluated as an organic pesticide for peach orchards and potato fields[193]. In addition, both the American and German cockroach, that harbor disease causing organisms, were repelled by the nepetalactones present in *Nepeta cataria* and showed better repellency than DEET[172,173,175]. Common brown ticks and the deer tick that harbor the bacterium responsible for Lyme disease are repelled by the nepetalactones in *N. cataria* as well as the dihydronepetalactones[171,177]. Three species subterranean termites that eat away at houses and other various wood-based structures causing significant financial loss were also repelled by the nepetalactones found in catnip oil[194,195,196]. The Z, E nepetalactone isomer was also efficient in repelling many common house dust mite species and poultry mites[177,197]. In a body contact assay involving harvester ants, mortality was achieved faster with the Z, E isomer than the other nepetalactones in catnip[198]. A commercial repellent has been patented that utilizes the nepetalactones derived from *N. cataria*[199]. Pilot programs have been implemented to assess the ability to commercially produce the nepetalactones from *N. cataria* yet commercial viability of using catnip oil has been limited by the high cost of the essential oil due to the physiological characteristics and agrotechnical production constraints with the currently available catnip plants[200].

1.3 Essential Oil Based Formulations as Arthropod Repellents

While insect repellent developed from natural sources such as essential oils have been effective at repelling disease vectoring arthropods, the main problem with using crude essential oils is their physical property of being volatile and that they are not effective for long periods of time compared to formulations made with them to increase their duration[201]. With the exception of efficacy of essential oils as insect repellents, this is the

most investigated area of essential oil based insect repellent research. Using liquid paraffins and polyethylene glycol's varying in molecular weight, waxes and encapsulation techniques, insect repellent formulations made of essential oils have shown an increased period of effective repellency against these arthropods[14,200]. Most of these formulations incorporate hydrophobic stabilizers to regulate the delivery of the essential oils in a more controlled manner and are generally recognized as safe. Liquid paraffins consist entirely of carbon and hydrogen denoting them as hydrocarbons, their physical properties of interest (solubility, stabilization of bioactive compound) depend on their chemical structure and have been introduced into formulations up to 20% v/v[201,202]. Polyethylene glycols are polymers made of ethylene glycol monomers and like liquid paraffins **(Figure 1-10, page 30)**, their physical properties (solid or liquid, stabilization of bioactive compound) depend on the number of oligomers that make up the polymer. Essential oils have also been incorporated into various types of paraffin based waxes for controlled releases and the animal natural product beeswax however, the chemical composition of beeswax can change due to the species of bee that is making it, making the standardization of beeswax products more difficult than synthetics waxes[203].

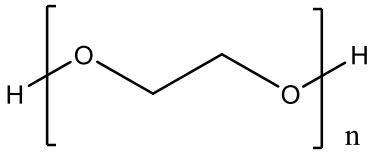

Figure 1-10: Polyethylene glycol general formula. Brackets indicate monomer.

1.3.1 Encapsulation of Essential Oils

Encapsulating essential oils refers to the process of loading essential oils into the core a membrane bound vesicle or wall based sphere to form an inclusion complex to increase the stability of the essential oils[204]. There are many types of methods to produce an encapsulated essential oil. One of the most common methods to create an encapsulated essential oil is to use cyclodextrins, a starch byproduct and cyclic oligosaccharide **(Figure 1-11, page 32)**[205]. There are three main types of cyclodextrins that differ in size, α-cyclodextrin, β-cyclodextrin and γ-cyclodextrin and increase in core size respectively[206]. The stability of the cyclodextrins depends on the stress on the bonds that holds them together and size as well as the polarity of the molecules within the essential oil[205]. Since chemical attributes change with different essential oils, the proper cyclodextrin to encapsulate specific essential oils should be one that is proper size and chemically stable[205,207,208].

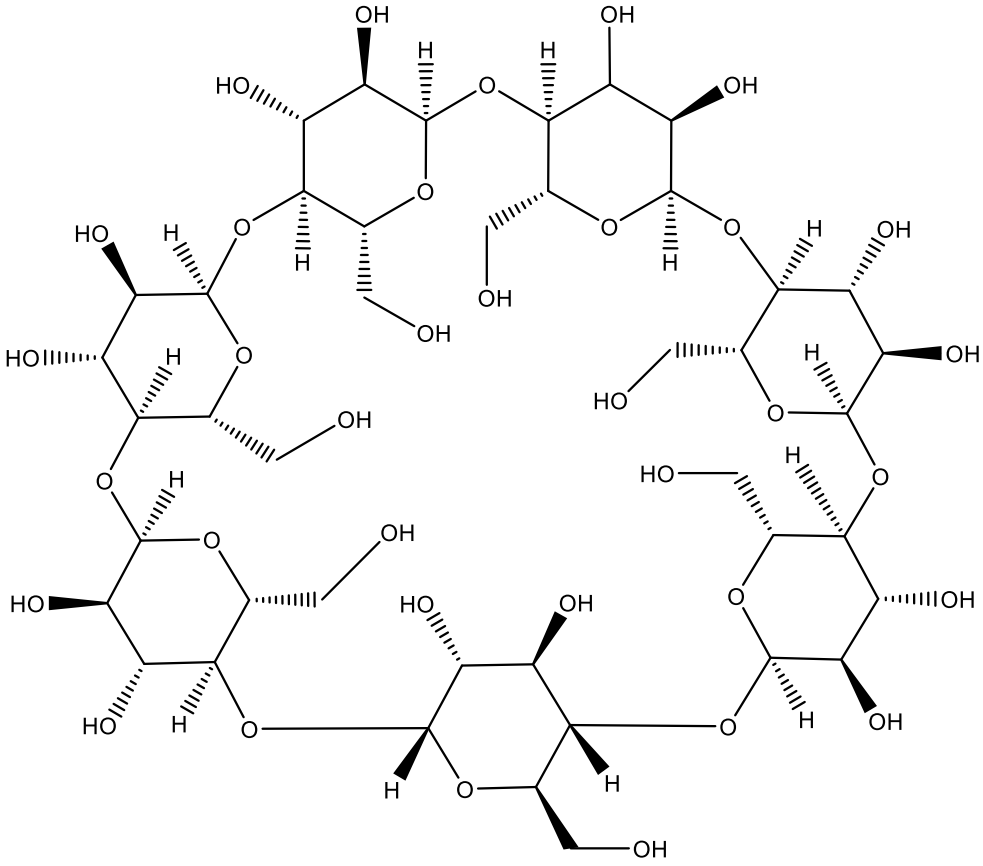

Figure 1-11: β-cyclodextrin chemical structure illustrating the cavity/core capable of holding volatile chemicals.

1.4 Insect Repellent Toxicity

While insect repellents provide a user with an effective means of deterring disease vectoring insects, there are safety concerns over using them as synthetic and natural product based repellents can have negative toxicological effects[209]. As previously mentioned, serious concerns over the safety and use of DEET have arisen due to its ability to inhibit acetylcholinesterase activity and ion channels as well as being absorbed through the skin rapidly[147,148,149,150,151,155]. However, conflicting research shows that mammals have low sensitivity to DEET and that the use of it is still suggested[153,210].

Essential oils have the public perception of being safer than synthetic repellents, but they can still induce various types of toxicity in humans[211]. The toxicity of an essential oil, like all substances, is dependent on many factors such as the concentration or dose, the mode of administration and the reactivity of the compound[211]. Acute and chronic toxicological effects of essential oils include but are not limited to poisonings, skin irritation and respiratory ailments[211]. While essential oils contain many compounds, often one or more of the components of the essential oil contribute to its toxicity and since they are not regulated by the Food and Drug Administration they can be easily adulterated[212]. Poisonings due to essential oils are mostly contributed to the user ingesting it in its pure form or in high concetrations[211]. Essential oils applied to the skin can lead to epidermal cell cytotoxicity due to absorption and elicit an immune response treating the essential oil as an allergen causing skin sensitization[213]. Inhaling essential oils can cause localized and systemic irritation due to the large surface area of the lungs and direct access to vascular networks[211].

1.4.1 Skin Sensitization Pathway

The most common method of using insect repellents is applying a liquid spray or stick formulation directly on exposed skin. However, when considering essential oil based insect repellents, one must address skin sensitization capabilities of the formulation on the skin due to the inherent reactivity of the essential oils. Essential oils applied in their crude form have shown epidermal skin cell necrosis and changes in the physiology of the cells after application and after dilution and subsequent formulation, the negative effects of the essential oil were minimized[214]. Allergic Contact Dermatitis (ACD) is medical condition from the skin sensitization pathway and is a delayed response to an allergen that elicits an

immune response by recruiting T-lymphocytes that induce hypersensitivity in epidermal cells following exposure to the allergen[215].

Allergic Contact Dermatitis (ACD) is a condition in which repeated exposure to an allergen or hapten results in the hypersensitization of epidermal skin cells[216]. Skin sensitization occurs in two phases, the sensitization or induction phase and the elicitation phase[215,217]. The sensitization phase starts with haptens gaining access to the skin cells and the binding of them to skin proteins[215,216]. Langerhans cells (dendritic cells of the skin) bind to this complex and differentiate into mature dendritic cells while migrating to the lymph node where they present the complex to T-lymphocytes[215,216]. Effector and memory T-cells then clonally expand before release into the blood stream[215,216]. The elicitation phase begins with T-lymphocytes being released into the blood stream causing sensitization and upon re-exposure of the haptens to the individual, epidermal cells release proinflammatory cytokines (molecules that have effects on other cells) and chemokines (molecules that recruit T-cells to an area) drawing the T-cells into the epidermis[215,216]. Newly infiltrated T-cells into the epidermis release cytokines triggering keratinocytes to release chemokines resulting in increased T-cell recruitment to that area[215,216]. Allergic contact dermatitis then develops as the skin is inflamed due to repeat exposure[215,216].

1.5 Dissertation Hypothesis and Objectives

This dissertation focused on standardizing, improving and developing *N. cataria* as a natural product for individuals that use *N. cataria* as a feline attractant, medicinal therapeutic and insect repellent. The first objective was to understand the inheritance of nepetalactone within *N. cataria* plants as we hypothesized that nepetalactone expression

will be dominant to other terpenoid production and that isolating these genes within a population will increase the production of these desired characteristics. Our second objective was to develop a high biomass, essential oil and nepetalactone yielding *N. cataria* line and we hypothesized that selecting and inbreeding the top performing plants will isolate the genes for high production of the desired characteristics and that we will be able to develop a population that outperforms the current commercial alternatives. Our third objective was to identify, quantitate and validate the medicinally active polyphenols in catnip as we hypothesized that the plant would contain bioactive compounds that could be responsible for the anti-inflammatory activity as a basis for which the plant has been and still is used. Through these objectives, the knowledge gained would allow the beginnings of harvesting guidelines for this crop. This latter part while not a central core focus of the dissertation would contribute and support the applied translational aspects of the more basic work given *N. cataria* is being introduced as a nutraceutical and insect repellant source. Our hypothesis is that these polyphenols and insect repellants will be produced in different concentrations throughout the different plant tissues in an intraspecies population. Our final objectives will be to validate the new *N. cataria* populations for repellency efficacy against disease vectoring insects by establishing dose response curves and to increase the duration of repellency by developing skin safe formulations to delay the volatilization of the essential oil. We hypothesize that incorporating the crude *N. cataria* essential oil into wax, polyether and other high weight molecular polymers will increase the duration that *N. cataria* essential oils will be active at repelling these insects and reduce the skin sensitization capabilities of crude *N. cataria* essential oil.

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Chapter 2 Inheritance of Nepetalactone Expression in *Nepeta cataria*

Commercially available varieties and populations of *N. cataria* do not produce sufficient biomass, nor accumulate sufficient essential oil (as in mg/plant; or kgs EO/ha) and most produce low to moderate amounts of nepetalactone (as a function of the rel. % constituent in the total EO). To develop an open pollinated high nepetalactone yielding populations of *N. cataria*, the inheritance of nepetalactone expression needs to be understood to determine which plants should be subjected to selection and inbreeding to improve a desired population. Determining the number of effective factors controlling high nepetalactone production would allow breeders to target their breeding programs accordingly to generate the correct populations for isolating the desired phenotype. Likewise, the genetics effects of these factors could be determined and aid in the breeding of the desired phenotype as well. Two essential oil chemotypes of *N. cataria*, one that produces many monoterpenoids such as citronellol, citral and geraniol (CN3) and one that is dominated by the production of nepetalactone (CR5). These parents were used and crossed with one another to generate a full-sibling family to determine the genetic control of nepetalactone production over three trial periods to aid in breeding projects focused on essential oils in *N. cataria*. The inheritance of nepetalactone production was determined by evaluating the six related members of the CR5 x CN3 family over three time periods in a research greenhouse to assesses and minimize the environmental variation respectively. The segregating populations had their essential oils analyzed by gas chromatography through headspace analysis to rapidly identify the volatiles associated with the essential oils. This analysis allowed the quantification of the aromatic volatiles oils that were captured in the static headspace system, including the essential oil constituents of interest to determine the

variance components within each population over three trial periods, as well as the variance associated with the different trial periods, to determine if there are significant differences within the six populations, the number of effective factors and the genetic effects controlling high nepetalactone producing.

2.1 Introduction

Catnip, Nepeta cataria (Fam. Lamiaceae), are short lived perennials known for producing volatile essential oils in glandular trichomes on the leaf epidermis responsible for eliciting euphoria in felines[1,2]. Recently, *N. cataria* essential oil has been shown to be a potent insect repellent, being able to repel disease vectoring insects such as *A. aegypti* and *A. gambiae* mosquitoes that vector the Yellow Fever and Malaria virus respectively as well as *I. scapularis* ticks that vector the Lyme disease[3,4,5]. The bioactive secondary metabolites responsible for these repellent properties within the essential oil of *N. cataria* plants is the volatile monoterpenoid nepetalactone[5]. While this compound has many different isomers, the most commonly found nepetalactone isomers present in the essential oil of various *Nepeta* species is the Z, E and E, Z isomer[6,7,8].

The wide diversity of plants within the *Nepeta* species as contributed to the production of many different types of monoterpenoids in addition to nepetalactones with menthol, caryophyllene, β-caryophyllene, various pinenes and humulenes being detected in the essential oil of various *Nepeta* species[8]. In reference to these two distinct chemotypes, *N. cataria* essential oil is either dominated by nepetalactone production or by having numerous ‘lemon’ scented compounds present as well such as neral, geraniol, geranial and

citronellol present in it[9]. These compounds have different ecological impacts and this species could have evolved to produce both chemotypes in many ways.

These volatile secondary metabolites are all produced from the terpenoid synthetic pathway in which individual terpenes are joined together in a head to tail synthetic pathway[10]. The addition of these five carbon units allows the assembly of numerous different compounds containing individual isoprene units creating the largest class of plant secondary metabolites[11]. While the genetic control of the pathway forming the carbon backbones of terpenoids is well understood the control of the biosynthesis of species specific metabolites has been shown to be regulated by different genes[12]. Breeding programs focused on manipulating the volatile secondary metabolites in the terpenoid pathway must be concerned with the genetic control of the metabolites within the pathway of the desired compounds to produce a desired population.

To create a population of plants with desired terpenoids, the genetic factors controlling the expression of those terpenoids need to be isolated into a single homogenous population within the targered species. In *N. cataria*, the desired terpenoids are the various nepetalactones isomers and understanding the inheritance of these nepetalactones against other terpenoids will aid in the breeding of new populations of *N. cataria* to combine high nepetalactone production with the other phenotype of the breeding program. In this study, we examined the inheritance of high nepetalactone production within the essential oil of *N. cataria* to aid breeders in their *N. cataria* breeding programs. Here, a six-member full-sibling population was generated using two inbred parents expressing high and low nepetalactone production to determine the genetic control of high nepetalactone production. This study was conducted over the 2017 summer in a research greenhouse in

which the six-member population was tested in three independent trials to minimize the environmental influence on the phenotype. Trials that have similar F2 variances will be pooled in all subsequent statistical analysis. The number of effective factors will be estimated by five different methods and the gene effects will be estimated by the Hayman's analysis[13,14,15].

2.2 Materials and Methods

2.2.1 Six Generation, Full-Sibling Family Formation

The parental genotypes used in this study were selected after two generations of inbreeding within the Rutgers Research Greenhouses and a field trial to evaluate the uniformity in the production of the desired essential oil chemotypes. The first parent, CR5 demonstrated consistent Z, E-nepetalactone production and did not produce any of the 'lemon' scented compounds while the second parent CN3 produced the lemon scented compounds in addition to Z, E-nepetalactone thereby lowering the overall concentration of it within the essential oil.

To determine the inheritance of nepetalactone within *N. cataria* plants a full-sibling six-member generation mating design consisting of a parent producing large amounts of Z, E-nepetalactone (CR5) was crossed to another parent producing much less nepetalactone (CN3), an F1 (maternal parent CR5), an F1L (maternal parent CN3), F2's, an F1 backcross to CR5 (BCcr5) and an F1 backcross to CN3 (BCn3) to measure gene effects[13].

To generate the six-member full-sibling populations, vegetative clones of the two genetically distinct populations CR5 and CN3 were made by making cuttings at the terminal nodes and dipping them in Hormodin 2, 0.3% indole-3-butyric acid (IBA) to

induce root formation and placed in Fafard Grow Mix 2 (Sun Gro Horticulture, Agawam, MA) in a mist house until the roots developed. Once the plants flowered, these populations served as the source for pollen and eggs to generate the selfed parental, F1 and backcross generations. The F1L populations was used to create the F2 population since initial testing showed that this generation's population expressed high quantities of nepetalactone and none of the lemon scented compounds, confirming the purposeful cross was made. The selfed parental and F2 populations were generated by covering the clonal parental and germinated F1 flower whorls in glassine bags respectively until seed formation. Both F1 and both backcross populations were made by emasculating immature, unopened flowers in whorls in the morning and hand pollinating them 24 hours later. The pollinated whorls were then covered in glassine bags until seed formation.

2.2.2 Greenhouse Trials and Experimental Design

All generations from the full-sibling population were evaluated over the 2017 summer in three independent trials at the Rutgers Research Greenhouses in New Brunswick, NJ. This was done to remove as much environmental influence as possible that contributes to the variability within the generations of the essential oils. In each trial, the six-member population's generations were germinated at the same time in 128 cell planting flats in Fafard Grow Mix 2 (Sun Gro Horticulture, Agawam, MA). Each trial was germinated about 45 days apart and when they were large enough they were transferred to 15cm pots and randomized in the research greenhouse. Trial one was started on March 15th, 2017 and consisted of 14 CR5, 15 CN3, 10 F1, 10 F1L, 112 F2, 45 BCcr5 and 105 BCcn3 plants. Trial two was conducted from May 1st, 2017 and consisted of 13 CR5, 13 CN3, 12 F1, 11 F1L, 108 F2, 47 BCcr5 and 61 BCcn3 plants. Trial three was conducted from June 15th,

2017 and consisted of 13 CR5, 12 CN3, 17 F1, 17 F1L, 100 F2, 48 BCcr5 and 65 BCcn3 plants. In total, 40 CR5, 40 CN3, 39 F1, 38 F1L, 320 F2, 140 BCcr5 and 231 BCcn3 were used in this study.

2.2.3 Population Sampling, GC/MS Conditions and Aromatic Volatile Compound Identification

Within each trial, each plant from each generation was sampled when the main inflorescence was at full flower. The leaf that was sampled was second to the inflorescence on the main stem. All sampling was done early morning until each trial was completed where ca. 200mg of leaf was placed in a headspace vial before sampling.

Headspace sampling was performed on a Shimadzu gas chromatograph 2010 Plus with an AOC-6000 autosampler. The samples were pre-equilibrated to 90°C prior to each run and each sample incubated for 5min at 250rpm to ensure reproducibility. The samples were separated on a SH-Rxi-5Sil MS column heated from 35°C with a hold of 4min to 250°C with a hold of 1.25min at 20°C/min and the injection volume was 10ul's. The inlet temperature was 250°C with a 10:1 split injection. A Shimadzu TQ8040 MS was used for compound identification. The ion source temperature was set to 200°C, the interface temperature was set to 250°C, the solvent cut time was 3.5min, and the detector voltage was set to 0.2kV with a threshold of 1000.

Peak integration percentages were generated using the GCMSsolution v4.3© software from Shimadzu Corporation. Individual identities were determined by comparing the mass spectral results to current literature and screening them in the NIST05.LIB, NIST05s.LIB, W10N14.lib and the W10N14R.lib mass spectral libraries. Citronellol, citral (neral and

geranial) and geraniol were identified by comparison to co-injected authenticated standards obtained from Sigma Aldrich (St. Louis, MO).

2.2.4 Statistical Design

Generation means, variances and frequencies were calculated independently for each of the three trials over the experimental period using PROC MEANS (SAS version 9.4; SAS Institute, Cary, NC). A Bartlett's test was used on the F2's generations nepetalactone concentrations to determine if all three trial's data could be pooled in further analysis[16,17]. The F1 and F1L populations had their generation means subjected to a two-sample t-test to investigate maternal effects on nepetalactone production and to see if the data could be pooled for subsequent statistical analysis[17].

PROC MIXED was used to perform an analysis of variance (ANOVA) between the trials that exhibit homogenous F2 variances, testing the different generations with respect to nepetalactone concentration where the generation was fixed because the controlled, purposeful crosses were performed with prior knowledge of the nepetalactone concentrations. Variance estimates were calculated for the various trials, generations and the trial·generation interaction using restricted maximum likelihood. Mean separations were performed to determine significant differences among the generation by analyzing their least significant difference ($\alpha = 0.05$).

A Chi-square test was used to determine the goodness of fit to the single dominant gene model based on the number of observed and expected high nepetalactone producing plants using the F2 and the BCcn3 populations expected frequencies that were 3:1 and 1:1 respectively. Estimations on the number of genes or polygenes involved in nepetalactone

production was performed using the SASQuant 1.3 program in which five separate methods determined the minimum number of effective factors involved[18]. The estimation methods used in SASQuant where an overall mean was calculated were the Wright's method, Mather's method, and Lande's methods I–III[13,15,19].

Backcrossing the F1 populations back into the CN3 parent allows for the estimation of main and epistatic gene effects affecting nepetalactone concentration. Using SASQuant that calculated the Hayman's gene effects, standard errors and Students significance levels, a least square regression was used to analyze the six related genetic effects known as the overall mean, additive, dominant, additive·additive, additive·dominant and dominant·dominant effects[14,18].

2.3 Results

During the 2017 summer at the Rutgers Research Greenhouses, the CR5 parent, both F1's and the BCcr5 populations maintained consistently low variation throughout the growth trials **(Table 2-1, page 53)**. The CN3 parent, the BCcn3 plants as well as the F2 populations did not exhibit consistent variation throughout the growth trials as their means both increased and decreased throughout the experimental trials suggesting environmental influences. The results of the Bartlett's test for homogeneity shows that the variances within the F2 populations across the different trials were not homogenous ($P > 0.05$). The CN3 population in the first trial produced much less nepetalactone and more lemon-scented compounds than the other two trials with much less variation around its mean. Trial 2 and trial 3 data were then pooled in the subsequent statistical analysis as their F2 variances were homogenous. The F1 and F1 average trial generation's means, 94.1% and 94.7

respectively were not statistically different (P > 0.05) from one another suggesting that nepetalactone production is not maternally inherited. The F1 and F1L generations data was then pooled in subsequent statistical analyses.

	Trial	Generations						
		CR5	**CN3**	**F1**	**F1L**	**BCcr5**	**BCcn3**	**F2**
Means	**Trial 1**	0.963	0.367	0.942	0.955	0.953	0.819	0.807
	Trial 2	0.964	0.588	0.942	0.948	0.955	0.790	0.869
	Trial 3	0.955	0.530	0.940	0.939	0.952	0.785	0.879
Var	**Trial 1**	0.000	0.005	0.001	0.000	0.000	0.047	0.046
	Trial 2	0.000	0.020	0.000	0.000	0.000	0.034	0.013
	Trial 3	0.001	0.008	0.000	0.001	0.000	0.013	0.009
N	**Trial 1**	14	15	10	10	45	105	112
	Trial 2	13	13	12	11	47	61	108
	Trial 3	13	12	17	17	48	65	100

Table 2-1: Generation Means and Variances. Means and variances of the nepetalactone concentration in the six-generation full-sibling populations created from the CR5xCN3 cross during the three trial periods in the summer of 2017. The CR5 was the high nepetalactone producing *N. cataria* plant, CN3 was the low nepetalactone producing plant, F1 was the cross generated from using CR5 as the maternal parent, F1L was the cross generated from using CN3 as the maternal parent, BCcr5 was the backcross to the high nepetalactone producing plant, BCcn3 was the backcross to the low nepetalactone producing plant and the F2 population is the result of selfing the F1 generation. F1 and F1L populations were not significantly different (P > 0.05) according to Fisher's two-sample t test.

There was a significant difference ($P < 0.05$) in the production of nepetalactone between the full-sibling six-member generations **(Table 2-2, page 55; Table 2-3, page 55)** but the generations mean nepetalactone concentration did not differ between trials 2 and 3 ($P > 0.05$). The two parental generations differed significantly ($P < 0.05$) in the production of their nepetalactone concentrations with CR5 producing 95.9% and CN3 producing 56.1% within their essential oils. The pooled F1 generation showed a significant difference ($P < 0.05$) in the production of nepetalactone, 94.2%, when compared to the CN3 generation suggesting that high nepetalactone production is dominate over lower nepetalactone production. The BCcr5 generation produced nepetalactone at the same concentration, 95.4%, that was significantly similar ($P < 0.05$) to both the CR5 parent and F1 generation while the BCcn3 generation's nepetalactone concentration, 78.7%, was significantly different ($P < 0.05$) from the CR5 and F1 generation. Finally, the F2 generation's mean nepetalactone production was 87.4% and was statistically different to all the other generations ($P < 0.05$).

Source	**DF**	**Mean Square**	**Variance**
Gen*	5	1.646017	-

Trial	1	0.072855	0.00003
Trial$_x$Gen	5	0.064786	0.00098

*** Significant at P = 0.05.**

Table 2-2: Mean square and variance component estimates for nepetalactone concentration in trials 2 and 3 in CR5xCN3 full-sibling populations pooled over the 2017 summer.

Generation	N	Mean	Group	SE
CR5	26	0.959	A	0.0201
CN3	25	0.561	D	0.0205
F1	57	0.942	A	0.0136
F2	208	0.874	B	0.0071
BCcr5	95	0.954	A	0.0105
BCcn3	126	0.787	C	0.0091

Table 2-3: Least square means for high nepetalactone concentrations in the six generations pooled from trials 2 and 3 of the full-sib population generated from the CR5xCN3 in the 2017 summer. Values followed by the same letter are not significantly different at P = 0.05 level according to least significant difference (LSD).

2.3.1 Determining Trial Variation

The ANOVA calculations used to estimate the amount of variance contributed from the two trial periods determined that the trial and trial by generation effects were not significant ($P > 0.05$) and much lower than the variance from the generations. This data shows that the variation in the different trial periods was much less relative to the F2 and BCcn3 generations indicating that the phenotypic variation within the population contributed to

the genetic differences within the six-member populations. Since the total phenotypic variation can be attributed to the summation of genetic and environmental variation, when the phenotypic variation is largely attributed to genetic variation and environmental variation is minimized, genetic estimates can be determined to identify how the phenotype is controlled or inherited.

2.3.2 Gene Action

The pooled F2 and BCcn3 populations failed the chi-square test for single dominant gene action indicating that there is more than one locus controlling high nepetalactone concentration. The different methods for estimating the number of effective factors involved in the production of high nepetalactone concentration generated different results (**Table 2-4, page 57**). In terms of the number of effective factors, estimates that generated negative numbers were considered zero, though the negative results are presented to contribute to the accumulation of knowledge in which they may be properly analyzed in future meta-analysis[20,21]. The Wright, Mather and Jinks, Landes I, Landes II and Landes III methods of estimating effective factors estimated 3.8, -221, 2.7, -55.3 and 1.3 effective factors respectively. When calculating the average amount of the genes involved the Mather and Jinks as well as the Landes II methods results were changed to zero and the overall mean for the estimated number of effective factors is 1.44[18]. This result was then interpreted as 2 as the number of effective factors are considered whole numbers.

Effective Factor Estimation Results		
Method	Estimate	Estimate (0)
Wright	3.8	3.8

Mather and Jinks	-221	0
Landes I	2.7	2.1
Landes II	-55.3	0
Landes III	1.3	1.3
Mean	-53.7	1.44

Table 2-4: Effective Factor Estimation Results. The results of the five different methods for estimating the number of effective factors contributing to a phenotype. Estimates that yielded a negative result were considered zero, though the original results are presented for further meta-analysis.

2.3.3 Gene Effects

The results of the Hayman's analysis showed that there was a significant amount ($P < 0.05$) of additive and dominant variation contributing to the overall variance of the six-member full-sibling population while the interaction gene effects additive x additive, additive x dominant and dominant x dominant were not significant ($P > 0.05$) (**Table 2-5, page 58**). The significant additive gene effect contributing to the variation associated with the population shows that this variation is controlled by a heritable allele effecting the nepetalactone expression. The significant dominant gene effect contributing the variation shows that the nepetalactone concentration that deviates from the mean is associated with a dominant gene. This analysis shows that the epistatic influences were not significant in the production of high nepetalactone concentration.

Hayman's Results	**Students T**

Gene Effect	Estimate	SE	P value
Mean	0.87	0.01	< 0.0001
Additive	0.17	0.02	< 0.0001
Dominant	0.17	0.08	0.0293
Additive x additive	-0.01	0.06	0.8070
Additive x dominant	-0.03	0.03	0.2603
Dominant x dominant	-0.06	0.12	0.6081

Table 2-5: Gene Effect Estimates. Gene effect estimates for the six-member population generated by the CR5xCN3 cross from the pooled Trial 2 and 3 data. Probability values determined by t test calculation using standard errors (SE) and df equal to the average number of individuals within segregating generations used to calculate each gene effect.

2.4 Discussion

As expected, the CR5 and CN3 plants produced significantly different amounts of nepetalactone throughout the growing season and the results are similar to other unpublished data in other internal growth trials evaluating the production of nepetalactone concentration in the plants. The F1 and its reciprocal cross were not statistically different ($P > 0.05$) from one another indicating that high nepetalactone concentration is not inherited maternally. The BCcr5 generation only generated high nepetalactone producing populations while the BCcn3 generation generated both high and low nepetalactone producing populations indicating that the genetic factors controlling this expression are transmitted by the CR5 parent since the production of nepetalactone was not always restored when backcrossed to the CN3 parent. While the trials produced nepetalactone in

high concentrations, the variance associated with each generation within the three trials periods came from the presence of the lemon-scented compounds citronellol, citral and geraniol which in turn lowered the relative concentration of nepetalactone within the essential oils. The variance in the F2 populations of these compounds were significantly different between trial 1 and trials 2 and 3, where were statistically similar according to the Bartlett's test for the homogeneity of variances and this is thought to be due to environmental variation within the trial periods. While a greenhouse is supposed to remove environmental influence when conducting inheritance tests, it is possible the growing conditions can change within the greenhouse and effect the phenotype of trait being investigated. Also, the amount of sunlight entering the greenhouse changes throughout the growing season and could contribute to the differential expression in the lemon-scented compounds. Whether the genetic or environmental factors were under more or less pressure to produce nepetalactone, this data illustrates the importance to conduct such evaluations over time even in a greenhouse-controlled environment as environmental factors can manipulate heritable analysis

Within trials 2 and 3, the trial variation from the experiment was very low relative to the variance associated with the six-member full sibling population allowing estimation of the number of loci effecting the genetic control of this trait. The pooled trial 2 and 3 data for the F2 and BCcn3 populations failed the chi-square goodness of fit models for the single dominant gene model indicating that the control of high nepetalactone concentration is controlled by more than one locus. The five methods of estimating the number of effective factors estimate's absolute values were all more than one, and after changing the negative results to zero, they averaged 1.44 signifying that more than one gene (or effective factor)

is responsible for the high nepetalactone production phenotype. Also, according to the Hayman's analysis, the inheritance of dominant alleles is responsible for the phenotype further verifying that the expression of high nepetalactone is due to dominant genetic alleles.

Individuals wanting to incorporate high nepetalactone production within the essential oils of *N. cataria* plants can rogue out the plants that contain recessive alleles and low nepetalactone concentration and select plants that contain the dominant alleles for high nepetalactone expression. Similarly, if the objectives for a *N. cataria* breeding program are high yielding nepetalactone essential oil content and are disease or stress resistance, the CR5 plant can be used as a recurrent backcrossing parent and recessive allele containing plants removed from the subsequent generations to generate a pure line. Molecular markers could be developed from these populations and used in subsequent breeding programs to restore high nepetalactone production. Genomic DNA and/or transcriptional RNA can be compared between the parents, the F1 and F2 populations to determine markers associated with the desired phenotype of high nepetalactone expression to be incorporated into the breeding program. While molecular markers for high nepetalactone concentration can be developed form these populations, the high-throughput ability of headspace sampling would make it easier to evaluate *N. cataria* populations for essential oil content than extracting DNA or RNA for genetic analysis.

The data presented here shows for this first time that the genetic control for high nepetalactone production is dominant to citronellol, citral (neral and geranial) and geraniol production. This dominant phenotype is inherited and is estimated to be controlled by more than one genetic effect according to the five methods utilized to estimate the number of

effective factors. Breeding programs that want to incorporate high nepetalactone producing plants can remove plants producing the lemon-scented compounds containing the recessive alleles contributing to low nepetalactone production.

2.5 References

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Chapter 3 Development of New *Nepeta cataria* Cultivars

As part of a larger plant breeding initiative to improve *N. cataria* as a source of insect repellents, two new populations of catnip were developed in order to generate new cultivars. This was accomplished after multiple rounds of single plant selection (SPS), cloning and selfing to produce two uniform yet distinct populations of *N. cataria* plants. Both new populations were selected for and demonstrated increased total biomass yield, and significantly higher essential oil production as well as higher nepetalactone production when compared to currently available commercial varieties. The two plants that serve as the clonal population that were selfed to produce the two populations were named 'CR9' and 'CR3' and are the results of this multi-year field study originally started in 2001 and completed in 2013. The 'CR9' cultivar and the 'CR3' cultivar differ in the production of different nepetalactone isomers within their essential oil. The 'CR9' cultivar's essential oil profile is dominated by the Z, E nepetalactone isomer while the essential oil produced by the 'CR3' cultivar produces the E, Z nepetalactone isomer is much higher quantities than the Z, E nepetalactone isomer. While the concentration of nepetalactone within the essential oil of both cultivars are comparable to the commercial varieties, the overall nepetalactone yield within both cultivars is much higher due to the significant increase in essential oil production over the currently offered commercial varieties. These two new cultivars were designed to provide the marketplace with an improved catnip that was bred for mechanical cultivation and produced sufficient essential oil to warrant commercial production given the industry need for higher dry biomass (pet toy industry), higher essential oil production (enriched extracts) and nepetalactone production (purified extracts and pharmaceutical products).

3.1 Development of 'CR9': A New Highly Aromatic Catnip *Nepeta cataria* L. Cultivar Rich in Z, E Nepetalactone

3.1.1 Introduction

Catnip, *(Nepeta cataria*, Fam. Lamiaceae), an aromatic herb from southwestern Asia, is best known for causing a euphoric effect on domestic cats and other members of the feline family due to the volatile compound nepetalactone contained in the essential oil of the plant[1,2,3].The aromatic volatiles of catnip are produced in the glandular trichomes in the leaf epidermis[4]. Due to the morphological nature of the bilabiate bisexual flowers, this plant can self-pollinate and also has the ability to outcross[5]. Current production methods utilize seeds and transplants from undomesticated populations. While normally cultivated for the pet toy industry as a safe attractant to cats and for ornamental applications, recent research has shown that essential oils from catnip are an efficient insect repellent and are at least comparable to repelling insects than the industry standard repellent DEET with far less toxicity[3,6,7,8,9].

Catnips volatile oil effectively repels mosquitoes, including the females that carry the plasmodium that causes malaria and those that transmit yellow fever, filariasis, the West Nile virus and encephalitis for a total of six different mosquito species repelled[9,10,11,12]. In one study, 41 different plant species were tested for repellency toward three species of mosquitos that carry pathogens and *N. cataria* was one of the top five plants whose oil exhibited repellency[10]. The Z, E isomer can also be hydrogenated to form dihydronepetalactone 2 that is as effective at repelling two species of mosquitoes as well as DEET and offers complete protection for up to five hours in experiments involving human subjects[6]. The Z, E isomer has shown significant repellency towards house and stable flies as well as being shown that catnip derived nepetalactones are an oviposition

repellent[8,13,14,15]. The peach-potato aphid is also repelled by nepetalactones suggesting that *N. cataria* could be evaluated as an organic pesticide for peach orchards and potato fields[16]. In addition, both the American and German cockroach, that harbor disease causing organisms, were repelled by the nepetalactones present in *Nepeta cataria* and showed better repellency than DEET[8,7,17]. Common brown ticks and the deer tick that harbor the bacterium responsible for Lyme disease are repelled by the nepetalactones in *N. cataria* as well as the dihydronepetalactones[6,11]. Three species subterranean termites that eat away at houses and other various wood based structures causing significant financial loss were also repelled by the nepetalactones found in catnip oil[18,19,20]. The Z, E nepetalactone isomer was also efficient in repelling many common house dust mite species and poultry mites[11,21]. In a body contact assay involving harvester ants, mortality was achieved faster with the Z, E isomer than the other nepetalactones in catnip[22]. A commercial repellent has been patented that utilizes the nepetalactones derived from *N. cataria*[23]. Pilot programs have been implemented to assess the ability to commercially produce the nepetalactones from *N. cataria* yet commercial viability of using catnip oil has been limited by the high cost of the essential oil due to the physiological characteristics of the currently offered catnip plants[24].

Catnip remains still largely undomesticated. Little breeding has been undertaken to improve the catnip's horticultural traits. Relative to most other members of the Lamiaceae family, catnips are susceptible to diseases and environmental stress including poor winter survival in northern temperate zones. Tolerant plants can be perennials but in commercially grown fields they are treated as annuals. Commercial fields from transplants are more expensive as the labor cost is greater and the process is more difficult as the plants die off produce less biomass and exhibit phenotypical architecture that does not lend itself to

efficient mechanization. They also produce lower essential oil yields in comparison with peppermint and spearmint, plants with copious amounts of aromatic oils that can be commercially harvested mechanically. In addition to lower essential oil yields, the plants have not been bred to increase Z, E nepetalactone, the key bioactive constituent found in the volatile aromatic oil. These factors have made the commercialization of catnip as a source for above ground biomass, essential oils and the isolated compound for new insect repellent products most challenging[24]. These factors of catnip make it difficult to effectively mechanize and commercialize to obtain the desired bioactive compound in the volatile oil[24].

'CR9' is the first cultivar of *Nepeta cataria* in North America developed specifically for commercial agricultural production with a more upright growth habit and higher biomass, essential oil and Z, E nepetalactone yield (as a function of the relative percentage of the total essential oil yield). Essential oil from current catnip contains many aromatic volatile compounds including nepetalactone[25]. This cultivar was developed and is distinct from other commercially available sources because it produces a uniform seeded offspring in the desired characteristics. The selfed progeny of CR9 produces higher amounts of biomass, essential oil yields and the essential oil is richer in the production of the bioactive isomer Z, E nepetalactone in these populations. 'CR9' provides a superior type of catnip plant for commercial field production, for dried catnip or for the distilled aromatic essential oils that has multiple applications including the pet toy and insect repellent industries.

3.1.2 Materials and Methods

'CR9' was developed after six different randomized complete block growth trials by selecting the best field performing plants that grew the most upright, survived the winters

in New Jersey, and produced the highest above ground biomass, essential oil and Z, E nepetalactone yields **(Table 3-1, page 189)**. In 2001, the USDA *N. cataria* germplasm was comparatively grown at the Rutgers Clifford E. & Melda Snyder Research Farm, in Pittstown, NJ with a wide range of commercial catnip varieties in a seeded field trial. For two growing seasons, this population of plants had many individual plants that were off types, exhibited poor performance and/or winter injury removed from the study. In 2002, the remaining plants from the best performing USDA line PI# W6 17691 were allowed to outcross by wind and bees, the seed was collected from the remaining individual plants and the new advanced breeding line was formed. In 2005, these seeds were sown in a field trial at the Rutgers Fruit and Ornamental Research Extension Center in Cream Ridge NJ, to identify lines with the desired phenotypic characteristics and to evaluate their uniformity. Only the most promising plants were left in the field, all others were removed. In 2006 after the plants were subjected to the winter season and assessed for winter survival, selections were made on this field with respect to biomass and winter survival by taking cuttings of the individual plants and allowing them to self-pollinate in a research greenhouse. In 2007, those selfed seeds were planted in another two year evaluation at the Rutgers Clifford E. & Melda Snyder Research Farm, in Pittstown, NJ. Selections took place on the second year after the plants were subjected to the winter season However, plant selections were also largely based upon total essential oil production (e.g. yield/plant) and Z, E nepetalactone concentration. The selections from 2008 were then clonally evaluated for two additional years in 2010 and 2011 at the same research farm to ensure minimal environmental influence on the variation of essential oil yields and nepetalactone

concentration. Those clones were then selfed and the seed used in the next growth trial in 2013.

In 2013, the clones demonstrating uniform production of essential oil yields and nepetalactone concentration had their selfed progeny planted in a final seeded field evaluation that year at the New Jersey Agricultural Experiment Station Clifford E. & Melda Snyder Research Farm, in Pittstown, NJ. The progeny of 'CR9' was field grown and compared to commercial seed companies offering catnip (Johnny's Selected Seeds, Albion, ME; Ferry Morse, Norton, MA; Stokes, Buffalo, NY; Territorial Seed Company, Cottage Grove, OR; Richters Herbs, Goodwood, Ontario, Canada). The land was cultivated by disc plowing, raised beds were then mechanically prepared followed by the placement of drip irrigation and plastic mulch. The land was fertilized at 900lbs/acre of 15-15-15 and was irrigated through drip irrigation as needed and as described[24]. The experimental design for 2013 was a randomized complete block design with 10 plants in each of the six lines having their morphological characteristics recorded for each of the three replications. The plants were spaced 61cm apart within the rows and the rows were spaced 274 cm apart. Once the plants were in full flower, morphological characteristics were recorded, the plants were cut back to the ground level after 10 weeks and the entire plot was bulk harvested and dried on site at 37°C using a walk-in forced air commercial Powell Tobacco dryer converted to the drying of herbs and botanicals. Plant height, plant width, leaf length, leaf width and dry weights were recorded. Plant height was measured from the soil level to the flowers down the center of the plant. Plant width was determined by measuring the diameter of the plant. Leaf length was the measurement from the tip of the leaf to the beginning of the petiole on the side that connects to the leaf. The width of the leaf was measured at the basal portion

of the leaf at the largest diameter. Dry weights were determined by recording the weight after plants had lost all the water at the set unified temperature at 37°C. The plants in the field were allowed to grow again to maturity, when they were again bulk harvested as described above and dried on site at 37°C. Essential oil yields were determined by the hydrodistillation of all of the above ground biomass of the plant using a Clevenger-type distillation unit with 100g of dry plant matter. The yields were calculated as percent of dry mass (mg Essential Oil / 100g above ground biomass). Essential oil analysis was performed by quantitatively comparing the samples using a flame ionization detector and qualitatively by identifying the chemical constituents of the oil with mass spectrometry[26].

- 2001
  - Original seeded field establishment for catnip evaluation of the USDA germplasm and commercial lines including USDA germplasm PI # W6 17691.
  - Evaluation for desired morphological characteristics and the rouging out of poor performing plants was performed.
- 2002
  - Plants remaining in 2002 that successfully overwintered from 2001 and exhibited desired morphological characteristics formed the breeding lines (C244, C245, C246, C47, C248, C249, and G1) and were allowed to outcross.
- 2005
  - The outcrossed seeds from lines (C244, C245, C246, C47, C248, C249, and G1) were sown in a field trial and evaluated for desired phenotypic characteristics as well as the rouging out of poor performing plants.
- 2006
  - Plants remaining from the 2005 field trials and which exhibited desired phenotypic characteristics were selected forming the breeding line (CR).
  - (CR) breeding lines were allowed to self-pollinate in a research greenhouse.
- 2007
  - The (CR) line was sown in a field growth trial in which individual plants were identified for desired phenotypic characteristics with emphasis on essential oils.
- 2008
  - Selections of plants (CR1, CR2, CR3, CR4, CR5, CR6, CR7, CR8, and CR9) from the CR breeding line were made after the 2007 winter with emphasis on essential oils.
- 2010
  - Clonal evaluation of advanced breeding lines (CR1, CR2, CR3, CR4, CR5, CR6, CR9) for desired morphological characteristics and essential oil analysis was conducted.
- 2011
  - Clonal evaluation of advanced breeding lines (CR1, CR2, CR3, CR4, CR5, CR6, CR9) for desired morphological characteristics and essential oil analysis was conducted.
  - Breeding lines were allowed to self-pollinate in a research greenhouse.
- 2013
  - Final seeded evaluation of the selfed advanced breeding line 'CR9' the five and commercial lines for comparison.
  - Selection of (CR9) for the new catnip cultivar *Nepeta cataria L. 'CR9'*

Figure 3-1: Genealogy of the new catnip cultivar CR9 (*Nepeta cataria*)

3.1.3 Description

The progeny of the ‘CR9’ cultivar of *N. cataria* have opposite triangular ovate leaves that have crenate edges **(Figure 3-1, page 72)**. All of the leaves are dark to light green. These plants are bushy and flower within 90 days. The white bilabiate flowers are grown on terminal inflorescences in whorls. ‘CR9’ plants were the tallest and had the largest leaves. Once cut back and allowed to regrow, ‘CR9’ plants remained the tallest but the leaf dimensions now resembled the commercial lines in the same study. In central New Jersey’s growing zone six (40.559340, -74.961282), this plant can be harvested twice in a growing season for biomass, essential oils and for Z, E nepetalactone. ‘CR9’ can also be kept from flowering by continually pruning it. The plants serve as an excellent source for pollinators. Bees, butterflies and many other insects frequently visited all the catnips including the progeny of ‘CR9’ plants during the entire flowering period (Figure 1). Chemical characterization of the essential oil of these plants using both GC and MS with alkane standards confirmed the presence of Z, E nepetalactone (**Figure 3-2, page 73; Figure 3-3, page 73)**[27].

Figure 3-2: Single plant photo of catnip cv. CR9 with insect pollinators labeled with yellow arrows.

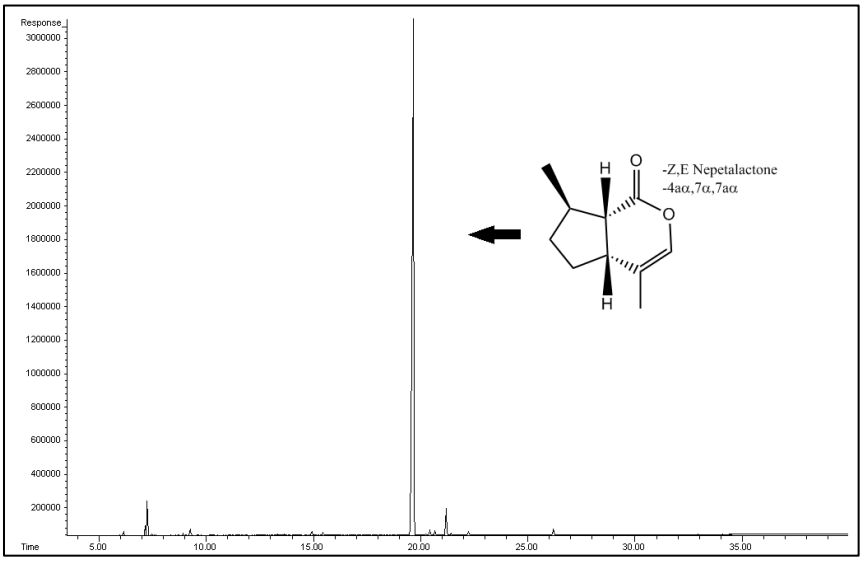

Figure 3-3: Gas chromatogram of the essential oil from catnip cultivar CR9 (*Nepeta cataria)* illustrating the peak of Z, E nepetalactone.

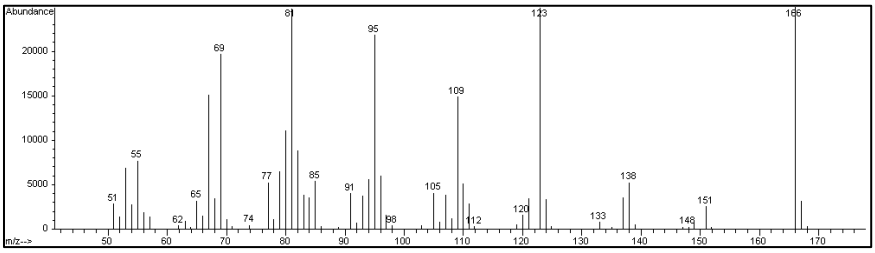

Figure 3-4: Mass spectra of Z, E nepetalactone, the major compound found in the essential oil of catnip 'CR9', (*Nepeta cataria*).

3.1.4 Performance

‘CR9’ performed better than each of the commercial seed companies to which it was compared **(Table 3-1, page 75)**. ‘CR9’ has a mean dry weight per plant of 158.0g per plant on the first harvest and 177.0g on the second harvest for a total of 335g per year with a 33% improvement over the closest commercial line. The essential oil yield per plant is 1.54g per plant on the first harvest and 1.38 g per plant on the second harvest for a total of 2.92g per year with a 54% improvement over the closest commercial line. Z, E nepetalactone yield was 1.34g per plant on the first harvest and 0.35g on the second harvest for a total of 1.7g per year with a 77% improvement over the closest commercial line. On the first harvest the concentration of Z, E nepetalactone was 87% and 25% on the second harvest. All catnip lines evaluated had their concentration of Z, E nepetalactone decrease to lower levels. This new cultivar survived winter conditions and exhibited the least winter injury and die-back compared to the commercial catnips that were evaluated.

2013 Harvest One	Morphological Characteristics					Essential Oil Analysis	
	Plant Height (cm)	Plant Spread (cm)	Leaf Length (cm)	Leaf Width (cm)	Dry Weight Per Plant (g)	Oil Yield Per Plant (g)	Z,E Nepetalactone Yield Per Plant (g)
CR9	65.9 A	92.1 BC	6.0 A	4.8 A	158.0 A	1.54 A	1.34 A
JON	56.5 B	98.0 AB	4.7 B	3.5 B	115.0 BC	1.05 B	0.74 B
RICH	55.4 B	100.9 A	4.8 B	3.6 B	113.3 BC	0.90 B	0.73 B
STOKES	53.3 BC	94.0 ABC	4.9 B	3.7 B	127.7 B	1.21 AB	0.22 C
TERR	55.9 B	101.3 A	4.4 BC	3.3 B	112.3 BC	1.16 AB	0.21 C
CFM	50.4 C	87.5 C	4.0 C	3.4 B	88.0 C	0.46 B	0.06 C

2013 Harvest Two	Morphological Characteristics					Essential Oil Analysis	
	Plant Height (cm)	Plant Spread (cm)	Leaf Length (cm)	Leaf Width (cm)	Dry Weight Per Plant (g)	Oil Yield Per Plant (g)	Z,E Nepetalactone Yield Per Plant (g)
CR9	51.1 A	82.5 AB	5.8 A	4.1 A	177.0 A	1.38 A	0.35 A
CFM	46.3 B	89.7 A	5.7 A	3.7 A	136.0 AB	0.84 B	0.21 B
STOKES	43.3 BC	80.7 AB	5.7 A	3.7 A	115.7 AB	0.73 BC	0.19 BC
RICH	44.3 B	83.1 AB	5.5 A	3.7 A	157.3 AB	0.63 BC	0.16 BC
TERR	43.3 BC	77.5 B	6.0 A	3.9 A	101.7 B	0.50 BC	0.13 BC
JON	39.4 C	79.7 AB	5.8 A	3.9 A	125.0 AB	0.42 C	0.11 C

Table 3-1: Morphological and essential oil characteristics of the new catnip cultivar 'CR9' compared to commercial catnip varieties over two harvests, 2013[z].

[z]CR9=Rutgers new cultivar release; CFM=Ferry Morse Seeds, Norton, MA; JON=Johnny's Selected Seeds, Albion, ME; RICH=Richters Herbs, Goodwood, Ontario, Canada; STOKES= Stokes Seeds, Buffalo, NY; TERR= Territorial Seed Company, Cottage Grove, OR;)

[y]Values within columns followed by the different letters are significantly different according to Duncan's test at $P \leq 0.05$.

3.1.5 Discussion

As a garden herb, ‘CR9’s progeny can live for many additional years on the landscape and could be considered aesthetically attractive with light green soft leaves and a highly pleasant spice-like aroma, however this new cultivar lends itself more to mechanical harvesting as is required for larger-scale essential oil production and was developed for this purpose. Because of the increased essential oil, it makes the commercialization of this catnip cultivar as an essential oil crop more realistic than prior and current catnip lines. Producers of catnip essential oils and extracts designed for the insect repellent industry could increase their income significantly by planting this new cultivar given it was developed as an improved source of essential oil of catnip for larger growers with steam distillation facilities.

3.2 Development of ‘CR3’: A New Highly Aromatic Catnip *Nepeta cataria* L. Cultivar Rich in E, Z and Z, E Nepetalactone

3.2.1 Introduction

As mentioned in section 3.1, *Nepeta cataria* plants are of interest for their biomass, essential oil and nepetalactone and the current commercially available *N. cataria* populations are not adequate to supply the pet toy, essential oil manufacturing and enriched extract industries respectively. The currently offered varieties lack production in each of these desired characteristics as the plants are small and grow laterally making mechanical harvesting difficult as well as producing little essential oil. While *N. cataria* plants can produce many types of volatile terpenoids, the desired compound from the essential oil produced by *Nepeta cataria* is nepetalactone. Two main chemotypes are commonly found in *N. cataria* populations with one chemotype being dominated by the Z, E nepetalactone

isomer while the other chemotype produces two isomers in which the E, Z nepetalactone isomers is produced in higher concentrations than the Z, E nepetalactone isomer[11]. The 'CR9' cultivar's chemical profile only produces the Z, E nepetalactone isomer. A population of *N. cataria* plants that produce the other chemotype would be beneficial because different isomers of the same compound can have different bioactivity including insect repellency[28].

'CR3' was successfully developed alongside 'CR9' and the seeded offspring of this cultivar maintains the growth characteristics needed for commercial agricultural production such as vertical growth higher biomass, essential oil and Z, E nepetalactone yields when compared to current commercially available populations. The selfed progeny of 'CR3' produces higher amounts of biomass, essential oil yields and the essential oil is the different chemotype than 'CR9' by having a high production of E, Z nepetalactone and Z, E nepetalactone. 'CR3' provides a superior type of catnip plant for commercial field production, for dried catnip or for the distilled aromatic essential oils that has multiple applications including the pet toy and insect repellent industries. Individuals producing *N. cataria* natural products on a commercial scale and need the E, Z nepetalactone isomer can produce it by utilizing this population.

3.2.2 Materials and Methods

'CR9' was developed after six different randomized complete block growth trials by selecting the best field performing plants that grew the most upright, survived the winters in New Jersey, and produced the highest above ground biomass, essential oil, E, Z nepetalactone and Z, E nepetalactone yields **(Figure 3-5, page 81)**In 2001, the USDA *N. cataria* germplasm was comparatively grown at the Rutgers Clifford E. & Melda Snyder

Research Farm, in Pittstown, NJ with a wide range of commercial catnip varieties in a seeded field trial. For two growing seasons, this population of plants had many individual plants that were off types, exhibited poor performance and/or winter injury removed from the study. In 2002, the remaining plants from the best performing USDA line PI# W6 17691 were allowed to outcross by wind and bees, the seed was collected from the remaining individual plants and the new advanced breeding line was formed. In 2005, these seeds were sown in a field trial at the Rutgers Fruit and Ornamental Research Extension Center in Cream Ridge NJ, to identify lines with the desired phenotypic characteristics and to evaluate their uniformity. Only the most promising plants were left in the field, all others were removed. In 2006, after the plants were subjected to the winter season and assessed for winter survival, selections were made on this field with respect to biomass and winter survival by taking cuttings of the individual plants and allowing them to self-pollinate in a research greenhouse. In 2007, those selfed seeds were planted in another two year evaluation at the Rutgers Clifford E. & Melda Snyder Research Farm, in Pittstown, NJ. Selections took place on the second year after the plants were subjected to the winter season However, plant selections were also largely based upon total essential oil production (e.g. yield/plant), E, Z nepetalactone and Z, E nepetalactone concentration. The selections from 2008 were then clonally evaluated for two additional years in 2010 and 2011 at the same research farm to ensure minimal environmental influence on the variation of essential oil yields and nepetalactone concentration. Those clones were then selfed and the seed used in the next growth trial in 2013.

In 2013, the clones demonstrating uniform production of essential oil yields and nepetalactone concentration had their selfed progeny planted in a final seeded field

evaluation that year at the New Jersey Agricultural Experiment Station Clifford E. & Melda Snyder Research Farm, in Pittstown, NJ. The progeny of 'CR3' was field grown and compared to commercial seed companies offering catnip (Johnny's Selected Seeds, Albion, ME; Ferry Morse, Norton, MA; Stokes, Buffalo, NY; Territorial Seed Company, Cottage Grove, OR; Richters Herbs, Goodwood, Ontario, Canada). The land was cultivated by disc plowing, raised beds were then mechanically prepared followed by the placement of drip irrigation and plastic mulch. The land was fertilized at 900lbs/acre of 15-15-15 and was irrigated through drip irrigation as needed and as described[24]. The experimental design for 2013 was a randomized complete block design with 10 plants in each of the six lines having their morphological characteristics recorded for each of the three replications. The plants were spaced 61cm apart within the rows and the rows were spaced 274 cm apart. Once the plants were in full flower, morphological characteristics were recorded, the plants were cut back to the ground level after 10 weeks and the entire plot was bulk harvested and dried on site at 37°C using a walk-in forced air commercial Powell Tobacco dryer converted to the drying of herbs and botanicals. Plant height, plant width, leaf length, leaf width and dry weights were recorded. Plant height was measured from the soil level to the flowers down the center of the plant. Plant width was determined by measuring the diameter of the plant. Leaf length was the measurement from the tip of the leaf to the beginning of the petiole on the side that connects to the leaf. The width of the leaf was measured at the basal portion of the leaf at the largest diameter. Dry weights were determined by recording the weight after plants had lost all the water at the set unified temperature at 37°C. The plants in the field were allowed to grow again to maturity, when they were again bulk harvested as described above and dried on site at 37°C. Essential oil yields were determined by the

hydrodistillation of all of the above ground biomass of the plant using a Clevenger-type distillation unit with 100g of dry plant matter. The yields were calculated as percent of dry mass (mg Essential Oil / 100g above ground biomass). Essential oil analysis was performed by quantitatively comparing the samples using a flame ionization detector and qualitatively by identifying the chemical constituents of the oil with mass spectrometry[27].

- 2001
  - Original seeded field establishment for catnip evaluation of the USDA germplasm and commercial lines including USDA germplasm PI # W6 17691.
  - Evaluation for desired morphological characteristics and the rouging out of poor performing plants was performed.
- 2002
  - Plants remaining in 2002 that successfully overwintered from 2001 and exhibited desired morphological characteristics formed the breeding lines (C244, C245, C246, C47, C248, C249, and G1) and were allowed to outcross.
- 2005
  - The outcrossed seeds from lines (C244, C245, C246, C47, C248, C249, and G1) were sown in a field trial and evaluated for desired phenotypic characteristics as well as the rouging out of poor performing plants.
- 2006
  - Plants remaining from the 2005 field trials and which exhibited desired phenotypic characteristics were selected forming the breeding line (CR).
  - (CR) breeding lines were allowed to self-pollinate in a research greenhouse.
- 2007
  - The (CR) line was sown in a field growth trial in which individual plants were identified for desired phenotypic characteristics with emphasis on essential oils.
- 2008
  - Selections of plants (CR1, CR2, CR3, CR4, CR5, CR6, CR7, CR8, and CR9) from the CR breeding line were made after the 2007 winter with emphasis on essential oils.
- 2010
  - Clonal evaluation of advanced breeding lines (CR1, CR2, CR3, CR4, CR5, CR6, CR9) for desired morphological characteristics and essential oil analysis was conducted.
- 2011
  - Clonal evaluation of advanced breeding lines (CR1, CR2, CR3, CR4, CR5, CR6, CR9) for desired morphological characteristics and essential oil analysis was conducted.
  - Breeding lines were allowed to self-pollinate in a research greenhouse.
- 2013
  - Final seeded evaluation of the selfed advanced breeding line 'CR3' the five and commercial lines for comparison.
  - Selection of (CR3) for the new catnip cultivar *Nepeta cataria L. 'CR3'*

Figure 3-5: Genealogy of the new catnip cultivar CR3 (*Nepeta cataria*)

3.2.3 Description

The progeny of the ‘CR3’ cultivar of *N. cataria* have opposite triangular ovate leaves that have crenate edges **(Figure 3-6, page 83)**. All of the leaves are dark to light green. These plants are bushy and flower within 90 days. The white bilabiate flowers are grown on terminal inflorescences in whorls. ‘CR3’ plants were the tallest and had the largest leaves. Once cut back and allowed to regrow, ‘CR3’ plants remained the tallest but the leaf dimensions now resembled the commercial lines in the same study. In central New Jersey’s growing zone six (40.559340, -74.961282), this plant can be harvested twice in a growing season for biomass, essential oils and for both nepetalactone isomer yields. ‘CR3’ can also be kept from flowering by continually pruning it. The plants serve as an excellent source for pollinators. Bees, butterflies and many other insects frequently visited all the catnips including the progeny of ‘CR3’ plants during the entire flowering period. Chemical characterization of the essential oil of these plants using both GC and MS with alkane standards confirmed the presence of both nepetalactones **(Figure 3-7, page 84; Figure 3-8, page 84)**[27].

Figure 3-6: Single plant photo of catnip cv. CR3

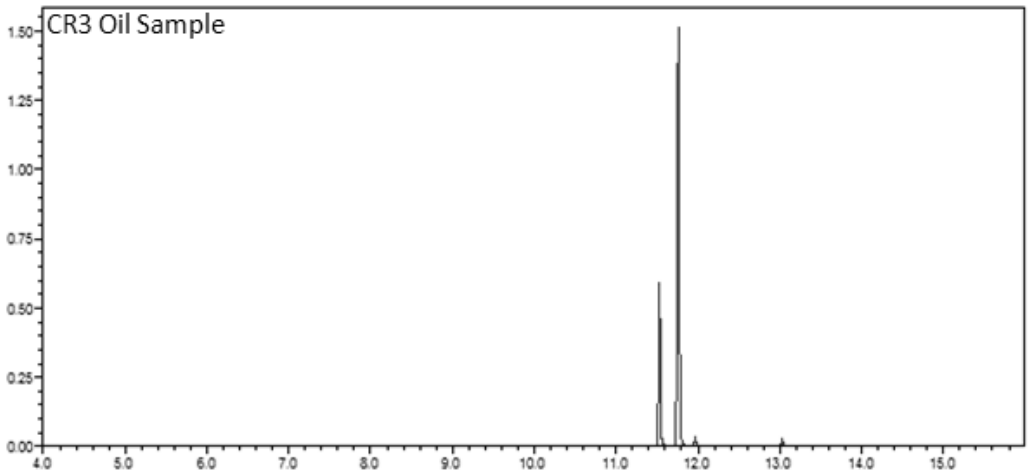

Figure 3-7: Gas chromatogram of the essential oil from catnip cultivar CR3 (*Nepeta cataria*) illustrating both nepetalactone peaks

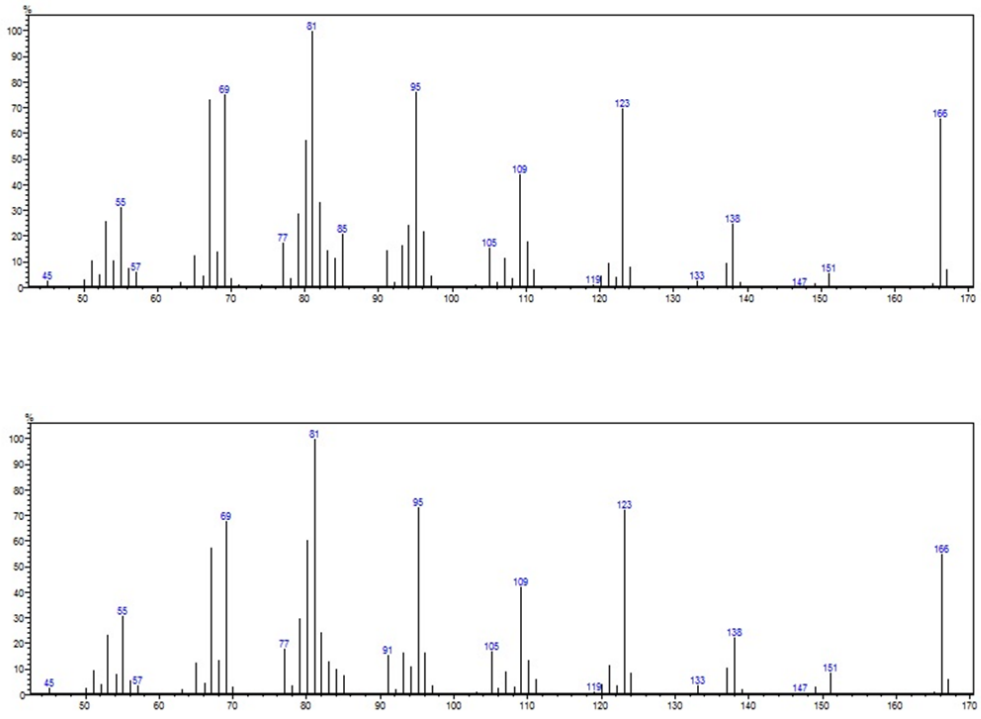

Figure 3-8: Two mass spectral results of the main peaks from the CR3 essential oil sample. *Top*: Z, E nepetalactone. *Bottom*: E, Z nepetalactone.

3.2.4 Performance

‘CR3’ performed better than each of the commercial seed companies to which it was compared **(Table 3-2, page 86)**. ‘CR3’ has a mean dry weight per plant of 135.7g per plant on the first harvest and 152.0g on the second harvest for a total of 287.7g per year with a 6% improvement over the closest commercial line. The essential oil yield per plant is 1.7g per plant on the first harvest and 0.9g per plant on the second harvest for a total of 2.6g per year with a 26% improvement over the closest commercial line. E, Z nepetalactone yield was 1.0g per plant on the first harvest and 0.59g on the second harvest for a total of 1.59g per year with a 34% improvement over the closest commercial line. This new cultivar survived winter conditions and exhibited the least winter injury and die-back compared to the commercial catnips that were evaluated.

2013 1H w/ All Lines	Plant Height (cm)	Plant Spread (cm)	Leaf Length (cm)	Leaf Width (cm)	Dry Weight Per Plant (g)	Oil Yield (g) Per Plant	Z,E Nepetalactone Yield (g) per Plant	E,Z Nepetalactone Yield (g) per Plant
CR3	63.3 A[y]	95.3 AB	5.1 A	4.0 A	135.7 A	1.7 A	0.5 B	1.0 A
JON	56.5 B	98.0 AB	4.7 AB	3.5 B	115.0 A	1.1 B	0.7 A	0.3 D
RICH	55.4 B	100.9 A	4.8 AB	3.6 AB	113.3 AB	0.9 BC	0.7 A	0.1 F
STOKES	53.3 BC	94.0 AB	4.9 AB	3.7 AB	127.7 A	1.2 AB	0.2 C	0.9 B
TERR	55.9 B	101.3 A	4.4 BC	3.3 B	112.3 AB	1.2 AB	0.2 C	0.8 C
CFM	50.4 C	87.5 B	4.0 C	3.4 B	88.0 B	0.5 C	0.1 C	0.3 E

2013 2H w/ All Lines	Plant Height (cm)	Plant Spread (cm)	Leaf Length (cm)	Leaf Width (cm)	Dry Weight Per Plant (g)	Oil Yield (g) Per Plant	Z,E Nepetalactone Yield (g) per Plant	E,Z Nepetalactone Yield (g) per Plant
CR3	45.6 A	82.9 AB	5.5 A	3.7 A	152.0 A	0.9 A	0.24 A	0.59 A
CFM	46.3 A	89.6 A	5.7 A	3.7 A	136.0 A	0.8 AB	0.21 AB	0.52 AB
STOKES	43.2 AB	80.6 AB	5.7 A	3.6 A	115.6 A	0.72 ABC	0.18 ABC	0.45 ABC
RICH	43.2 AB	82.6 AB	5.4 A	3.6 A	157.3 A	0.62 BCD	0.16 BCD	0.39 BCD
TERR	43.2 AB	77.4 B	5.9 A	3.9 A	101.6 A	0.50 CD	0.13 CD	0.32 CD
JON	39.4 B	79.7 AB	5.7 A	3.8 A	125.0 A	0.41 D	0.10 D	0.26 D

Table 3-2: Morphological and essential oil characteristics of the new catnip cultivar 'CR3' compared to commercial catnip varieties over two harvests, 2013[Z].

[z]CR9=Rutgers new cultivar release; CFM=Ferry Morse Seeds, Norton, MA; JON=Johnny's Selected Seeds, Albion, ME; RICH=Richters Herbs, Goodwood, Ontario, Canada; STOKES= Stokes Seeds, Buffalo, NY; TERR= Territorial Seed Company, Cottage Grove, OR;)

[y]Values within columns followed by the different letters are significantly different according to Duncan's test at $P \leq 0.05$.

3.2.5 Discussion

As a garden herb, 'CR3's progeny can live for many additional years on the landscape and could be considered aesthetically attractive with light green soft leaves and a highly pleasant spice-like aroma., This new cultivar, with its taller height and more upright growth habit lends itself more to mechanical harvesting as is required for larger-scale essential oil production and was developed for this purpose. Because of the increased essential oil, it makes the commercialization of this catnip cultivar as an essential oil crop more promising than currently available catnip lines. Producers of catnip essential oils and extracts designed for the insect repellent industry could increase their income significantly by planting this new cultivar given it was developed as an improved source of essential oil of catnip for larger growers with steam distillation facilities.

3.3 References

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Chapter 4 Non-Volatile Analysis and Anti-Inflammatory Activity of *Nepeta cataria* Accessions

Many members of the Lamiaceae family produce non-volatile medicinally active natural products in parallel to their volatile essential oils. While the volatile essential oils of the Lamiaceae family are well documented and the focus of public perception, non-volatile compounds, some of which are known as polyphenols, are a broad class of secondary metabolites biosynthesized by plants for numerous physiological purposes. The metabolites produced within this family have long been utilized for their medicinal activity and are often consumed by extracting dried plant material with boiling water to form an herbal tea. Due to prior investigations into the Lamiaceae family as a source of naturally derived polyphenols and due to the use of *N. cataria* as natural product, research within this dissertation focused on identifying polyphenols produced by *N. cataria*. Since little research exists on the sampling of different tissues within the *N. cataria* plant for the identification of differential polyphenol production, this investigation focused on sampling ten different *N. cataria* lines developed by Rutgers University to identify the polyphenols produced, quantify their production and assess their anti-inflammatory activity in the flowers, upper leaves and lower leaves. HPLC/UV/MS will be used to identify and quantify polyphenol production while anti-inflammatory will be determined by treating murine RAW 264.7 cells will LPS with methanol extracts of the various *N. cataria* extracts to quantify nitric oxide concentration, a signaling molecule involved in the pathogenesis of inflammation[1]. The results gained from this investigation will lead to the development of harvesting standards and product formulation guidelines for *N. cataria* natural products.

4.1 Introduction

Catnip, *(Nepeta cataria*; Family, Lamiaceae), has long been recognized for its phytochemical and medicinal properties[2,3,4]. Members of this plant family are highly aromatic with many species renown for culinary uses such as basil, mints, oregano, rosemary, sage and thyme while several such as peppermint, spearmint, clary sage and others are grown to distill their essential oils for use in foods, flavors, personal hygiene and industrial products[2,5]. While catnip is well known for producing volatile monoterpene iridoid nepetalactones, the compounds responsible for eliciting euphoria in felines, members of this genus have been reported to produce a wide array of non-volatile medicinally bioactive polyphenols that are often overlooked[6,7]. Less recognized is that catnip is consumed as an herbal tea purported in traditional cultures to be useful for treating medical disorders such as inflammation, digestive ailments, infantile colic, toothaches, used as a cold remedy, anxiety and as a blood depurative[2,8,9]. Native American tribes would make catnip tea and drink it multiple times a day to help treat their ailments even after the introduction of western medicine[10]. African American slaves as well as their owners used catnip for the same purposes in the southern Appalachian Mountains and today it is still used by African Americans to treat infantile colic and digestive issues[11]. Europeans and the Chinese have previously used catnip leaves in their cooking and herbal infusions[12,13]. Catnip while rich in aromatic volatile oils may also contain other water soluble bioactive compounds as other members of the Lamiaceae do.

Polyphenols are characterized by the presence of more than one aromatic hydroxyl group and are commonly consumed from natural sources such as herbs[14]. Individuals that frequently consume polyphenol rich foods have shown to have a reduced efficacy towards

getting cancer, cardiovascular diseases, neurodegenerative diseases and inflammation[15,16]. Polyphenols act by reducing free electrons that arise in an organism capitulating its ability to cause damage through oxidation[17]. Polyphenols biosynthesized in members of the Lamiaceae family have shown to exhibit medicinal bioactivity for a broad range of medical ailments such as having acetocholinesterase inhibitory activity indicating that they could be useful in enhancing the chlorogenic transmission of messages for people suffering from Alzheimers Disease[18]. Polyphenols from oregano and other Lamiaceae species inhibited DPP-IV, which is responsible for insulin secretion, warranting further investigation into Lamiaceae derived polyphenols for diabetes management[19]. Anti-oxidant activity is well reported within the Lamiaceae plant family with lavender, mint, oregano and lemon balm being just some of the species reported to contain high concentrations of bioactive polyphenols and other compounds exhibiting radical scavenging capabilities[20,21,22]. Anti-inflammatory activity has also been confirmed in many members of this family by measuring the production of NO after the addition of an extract to lipopolysaccharide (LPS)-induced RAW 264.7 cells to examine whether the extract inhibits the activity of inducible nitric oxide synthase (iNOS), a main indicator for an inflammatory response within an organism[23]. Mint and oregano polyphenols also inhibit iNOS and sequential NO production[20,21]. Aqueous extracts from *Schizonepeta tenuifolia* (Japanese catnip) reduced inflammation *in vivo* in carrageenan induced paw edema mice further inciting a need for the investigation of Lamiaceae species with respect to anti-inflammatory activity[24].

A subclass of polyphenols known as flavonoids can be further characterized with one group called flavones. Flavones extracted from plant material across the kingdom have been shown to be medicinally active[25]. Aqueous extracts containing flavones of chamomile

inhibited NO production and iNOS gene expression[26]. Members of the Lamiaceae plant family also produce medicinally bioactive flavones with apigenin, luteolin, and pebrellin commonly being found in the plant tissues[27,28]. Apigenin is an inhibitor of the transcriptional activator of COX-2 and iNOS resulting in reduced inflammation in LPS-induced RAW 264.7 murine macrophages[29]. Apigenin reduced TNF-α-induced monocyte adhesion to endothelial cells suggesting anti-inflammatory activity in the human vascular system[30]. Luteolins extracted from dandelion flowers have also been shown to inhibit the transcriptional activation of COX-2 and iNOS in LPS-induced RAW 264.7 murine macrophages resulting in a reduction of inflammation[31]. Another *in vivo* murine study showed that luteolin reduced vascular inflammation in mice and that it also suppressed the IKBα/NF-κB signaling pathway resulting in protected endothelial cells to TNF-α- induced monocyte adhesion[32].

Research supporting that *N. cataria* may have health and nutritional or medicinal applications is materializing. Medicinally bioactive phenolic acids such as rosmarinic acid, caffeic acid, p-coumaric acid, chlorogenic acid and cinnamic acid have been detected in *N. cataria*[33]. Glucuronidated and glycosylated apigenins and luteolins have been identified in *N. cataria* var. *citriodora*[34]. Anti-oxidant capabilities essential for reducing inflammation and hazardous free radical formations have been confirmed in *N. cataria* with one study showing three separate assays (ABTS, DPPH radical scavenging, ferric reducing power) to confirm the activity of ethanol extracts containing luteolin and apigenins[33]. Lamiuside A and verbascoside were isolated from *N. cataria* and they inhibited calcineurin, a regulator of T-cell mediated inflammation[35]. Methanol extracts containing the luteolin

aglycone and its derivatives from *N. sibthorpii* inhibited carrageenan-induced paw edema in rats, showing significant anti-inflammatory activity[36].

Growing usage of flavonoids such as luteolin and apigenin in herbal products and dietary supplements and recognizing the role catnip plays as a medicinal herb in the United States led us to investigate several genetic lines of *N. cataria* for their polyphenol profiles. Plant material that is highly concentrated in polyphenols as well as the presence of a diverse array in the qualitative presence of them could both partially explain the possible health benefits and traditional uses as well as have applications in the nutraceutical market due to the broad arrangement of biologically active polyphenols. Florae that are members of the same family that produce secondary metabolites may contain the same or often have different chemical profiles and bioactivity due to different genetic predispositions, environmental influences, growth stages and the timing of harvest[37,38,39,40]. Lavender, mint, oregano and thyme, other Lamiaceae family members, have shown broad chemical diversity in the production of their non-volatile compounds which has resulted in non-uniform marketable products[20,21,22,41]. Evaluating the uniformity in the production of secondary metabolites within a population of plants is crucial when first examining the chemical characteristics of a plant product and more so in introducing a new natural product since so much environmental and inherent variability can alter the product. When such variation exists in plants, it provides an opportunity to improve and enrich targeted phytochemicals that are responsible for the medicinal bioactivity. For these reasons we screened a population of *N. cataria* plants for polyphenol quantification, anti-oxidant capacity and anti-inflammatory activity to evaluate the medicinal potential and phytochemical variability with respect to an intraspecies population.

4.2 Materials and Methods

4.2.1 Chemicals and Cell Lines

HPLC grade acetonitrile (ACN), methanol (MeOH), ethanol (EtOH), formic acid (FA), acetic acid (AcOH), hydrochloric acid (HCl) and HPLC grade water (H_2O) were obtained from Fisher Scientific Co. (Fair Lawn, NJ). Trolox® (a water soluble vitamin E derivative), ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)), potassium persulfate ($K_2S_2O_8$) and sodium carbonate (Na_2CO_3) were obtained from Sigma Aldrich (St. Louis, MO). Commercial standards for caffeic acid, rosmarinic acid, luteolin and apigenin were obtained from Sigma Aldrich (St. Louis, MO). RAW 264.7 cells were procured from the American Type Culture Collection (Rockville, MD). RPMI-1640 cell culture medium, penicillin, streptomycin, dimethyl sulfoxide (DMSO), lipopolysaccharide (LPS) and the Griess Reagent was obtained from GIBCO (Grand Island, NY).

4.2.2 *Nepeta cataria* Cultivation

The clonal *N. cataria* populations used in this study included six Rutgers University breeding lines originally selected from the USDA germplasm, as well as four seeded commercial catnip sources that were initially grown at the Rutgers University Research Greenhouses[42]. The vegetative clones were made from the 10 different lines where freshly cut terminal node cuttings were briefly dipped with Hormodin 2® 0.3% indole-3-butyric acid (IBA) to induce rooting and placed under a mister until roots developed 14 days later[43]. The clones were then transplanted into single rows on raised beds into black plastic mulch with drip irrigation. The field study was conducted as a randomized complete block design with three blocks at the Rutgers University Clifford E. & Melda C. Snyder Research and Extension Farm in Pittstown, NJ. Individual plants within the 10 different clonal lines

showing desired phenotypic growth characteristics such as fully developed flowers, and broad disease free leaves were harvested independently of one another and sampled in three separate physical locations on the plant. The flowers, the leaves closest to the shoot apical meristem but before the flowers and the leaves near the basal portion of the plant were harvested separately and dried at 37^{o}C until no moisture was present with an onsite Powell walk-in forced air heat dryer. After the leaves and flowers had dried and lost no further weight, the plant samples were carefully manually separated from the stems for further analysis.

4.2.3 Analytical Equipment

Sample separation was performed on a Prodigy Phenomenex ODS3 5µm, 150 x 3.2 mm, 5 micro column (Phenomenex Inc., Torrance, CA). For all LC/ESI-MS analysis, a Hewlett Packard Agilent 1100 Series LC/MSD (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler, quaternary pump system, diode array detector, thermostated column compartment, degasser, MSD trap with an electrospray ion source (ESI) and HP ChemStation, Data Analysis 4.2 was used for data analysis.

4.2.4 HPLC/UV/MS Conditions for the Identification and Quantification of Polyphenols.

HPLC separation was performed with the mobile phase containing solvent A (0.1% FA in H_2O) and B (0.1% FA in ACN) in gradient: 0-30 min, linear gradient from 10% B to 40% B. The flow rate was set at 1.0 mL/min. The eluent was monitored by an electrospray ion mass spectrometer (ESI-MS) and scanned from *m/z* 100 to 1200. ESI was conducted in negative mode by using a needle voltage of -3.5 KV. High purity nitrogen (99.999%) was used as dry gas at a flow rate of 12 L/min and capillary temperature was at 350°C. Nitrogen

was used as nebulizer at 60 psi, and helium as collision gas set to 80% collision energy. For UV detection, phenolic acids were detected at 320 nm and flavonoids at 370 nm.

4.2.5 Sample Preparation

Nepeta cataria samples were extracted from ca. 200 mg of dried ground plant material that was sonicated for 10 minutes in 25 mL of 70% MeOH in H_2O with 0.1% AcOH. Extracts were then conditioned at room temperature overnight, after which they were filtered through a 0.45-µm filter for HPLC separation and oxygen radical absorbing capacity determination. The samples for the anti-inflammatory analysis were prepared by extracting the plant materials using 70% MeOH in H_2O with 0.1% AcOH. These extracts were allowed to condition for 24 hours. Once the MeOH was removed by rotary evaporation, the samples were cooled to -50˚C, lyophilized and prepared with DMSO at different concentrations.

Hydrolysis of apigenin and luteolin glycosides was done by weighing ca. 60 mg of *N. cataria* powder and placing it in a volumetric flask with 2 ml of a 2.7 M HCl solution in a water bath at 90°C for 60 min. After hydrolysis, the volume was filled up to 5 ml and filtered through a 0.45-µm filter before injection into the HPLC.

All standards were measured on an analytical balance and dissolved in 70% MeOH in H_2O with 0.1% AcOH to prepare the stock solution. The stock solution was serially diluted with 70% MeOH in H_2O with 0.1% AcOH. The range of quantifiable concentrations for each standard as well as their calibration curve and determination coefficient are as follows: caffeic acid (0.80-51.50 µg/mL, $y = 30.03x + 2.6422$, $R^2 = 0.9999$), rosmarinic acid (0.78-50.00 µg/mL, $y = 13.145x + 0.7892$, $R^2 = 0.9999$), luteolin (0.39-25.00 µg/mL, y =

19.204x- 0.5329, R^2 = 0.9999), apigenin (0.41-26.00 µg/mL, y = 13.022x + 0.5061, R^2 = 0.9999).

4.2.6 Trolox Equivalent Absorption Capacities

The method for the determination of total anti-oxidants was used as described with minor modifications, based on the capacity of a sample to inhibit the ABTS radical compared with to Trolox®[44]. The ABTS radical was generated by single electron oxidation by $K_2S_2O_8$, accomplished by preparing a solution containing 7.46 mM ABTS and 2.44 mM $K_2S_2O_8$, in deionized H_2O. This solution was allowed to stand in darkness at room temperature for 12–16 h (the time required for formation of the radical). The working solution was prepared by taking a volume of the previous solution and diluting it in EtOH until its absorbance at λ = 734 nm was 0.70 ± 0.02. The measuring was done using an Agilent G1111AA UV-Vis spectrophotometer. For this purpose, 990 µl of the ABTS radical was added to the cuvette; the absorbance was measured, and 10 µl of sample or standard solution were added immediately, followed by a 20 min reaction time in which the final absorbance was measured. There is a quantitative relationship between the reduction of the absorbance and the concentration of anti-oxidants present in the sample against a blank solution of ethanol. A calibration curve was prepared using Trolox at a range of 0.0359 mM - 1.65 mM in EtOH. Trolox Equivalent Anti-oxidant Capacity (TEAC) was expressed as the content in µM Trolox / g sample required to achieve the same response as measured in the sample.

4.2.7 Anti-inflammatory Conditions

As described, RAW 264.7 cells, derived from murine macrophages, were cultured in RPMI-1640 (without phenol red) supplemented with 10% endotoxin-free, heat-inactivated

fetal calf serum, 100 mg/mL penicillin, and 100 mg/mL streptomycin[45]. Once the cells reached a density of (2-3) x 10^6 cells/mL, they were activated by *E. coli* LPS at 100 ng/mL to signal the production of nitric oxide to destroy bacterial cells. Catnip extracts dissolved in DMSO at concentrations of 20, 40 and 80 µg/mL were combined together with LPS. The cells were cultured in 100 mm tissue culture dishes and incubated with 100 ng/mL of LPS for 12 h. The cells were treated with 0.05% DMSO without LPS as vehicle control. The cells were then harvested and plated in a 24-well plate and treated with LPS only or with different concentrations of test compounds for a further 12 h. Indomethacin was defined as 100% inhibition and used as positive control for the experiment due to its ability to completely inhibit NO production.

At the end of incubation time, 100 µL of the culture medium was collected for the nitrite assay. The amount of nitrite, an indicator of NO synthesis and subsequent iNOS expression, was measured using the Griess reaction. The supernatants (100 µL) were mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenedia- mine dihydrochloride in H_2O) in duplicate on 96-well plates. After incubation at room temperature for 10 min, absorbance at 570 nm was measured with a 96-well plate reader (Thermo Labsystems, Multiskan Ascent, Finland). The values are expressed as means (standard error of triplicate tests).

4.2.8 Statistical Analysis

Values are presented as means ± standard deviation (SD). Data was analyzed by an unpaired, two-tailed student's t-test to identify significant differences ($P < 0.05$).

4.3 Results

4.3.1 Qualitative Identification of Polyphenols

Using HPLC/UV/MS under negative ion mode, a total of 10 major polyphenols were identified based on retention time, UV absorption spectrum, mass spectrum and by comparison to co-injected authenticated standards. The aglycones of flavonoid glycosides were further confirmed by comparison with the authenticated standards of luteolin and apigenin in acidic hydrolyzed material. The retention time and detected mass ions under negative mode for each compound are summarized in **(Table 4-1, page 100)**. Representative UV chromatograms are shown in **(Figure 4-1, page 101)** and the mass spectra of the major compounds in **(Figure 4-2, page 102)**. Chemical profiling of the different tissues of catnip revealed that the aglycones were present largely in the flowers. The leaves did not produce the aglycone apigenin but three lines upper leaf samples produced the luteolin aglycone. The other eight compounds were frequently present in the flowers, upper leaves and lower leaves though their concentrations differed significantly statistically.

Compound ID #	t_R (min)	Identities	$[M-H]^-$ (*m/z*)
1	7.2	Caffeic Acid*	179
2	7.9	Luteolin diglucuronide	637
3	9.7	Apigenin diglucuronide	621
4	10.7	Luteolin glucoside	447
5	11.3	Luteolin glucuronide	461
6	13.0	Apigenin glucoside	431
7	13.9	Apigenin glucuronide	445
8	14.2	Rosmarinic Acid*	359
9	18.6	Luteolin*	285
10	22.3	Apigenin*	269

Table 4-1: Polyphenols identified in catnip (*Nepeta cataria*). *Compared with authenticated standards

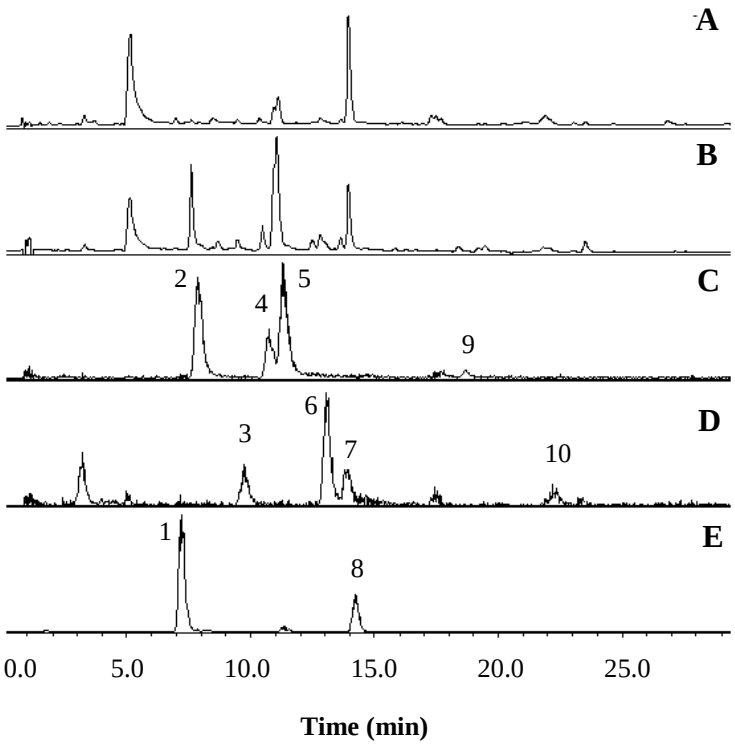

Figure 4-1: Representative LC/UV/MS chromatograms of a *Nepeta cataria* extract. (A) UV chromatogram at 370 nm for detection of flavonoids; (B) UV chromatogram at 320 nm for detection of phenolic acids; (C) Extracted ion chromatogram for luteolin and its derivatives; (D) Extracted ion chromatogram for apigenin and its derivatives. (E) Extracted ion chromatogram for caffeic acid and rosmarinic acid. The identities, retention time (tR) and MS of each peak are listed in Table 1.

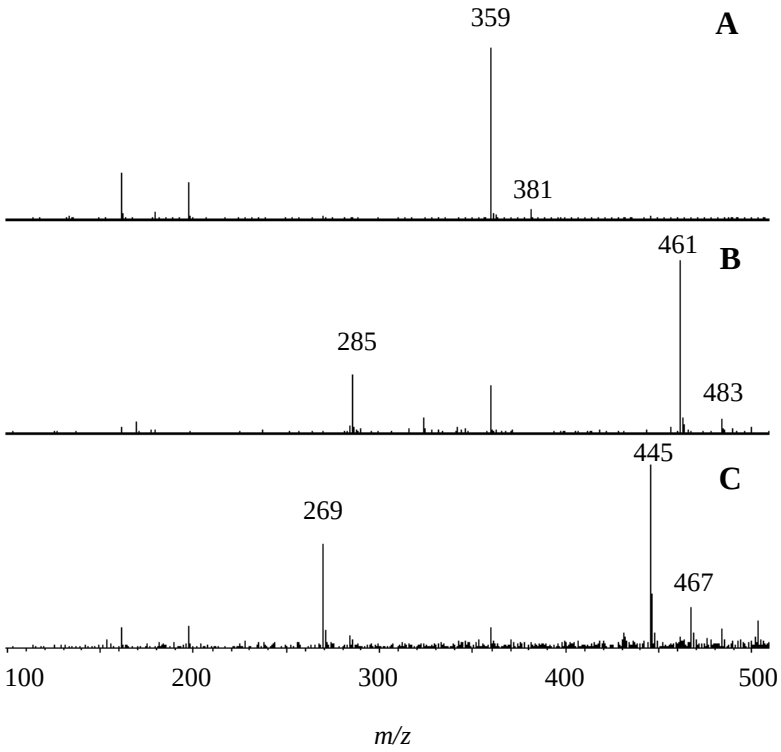

Figure 4-2: Mass spectra of major peaks identified in *Nepeta cataria*. (A) MS spectrum of rosmarinic acid; (B) MS spectrum of luteolin glucuronide; (C) MS spectrum of apigenin glucuronide.

4.3.2 Quantitative Analysis of Polyphenols

Quantification of polyphenols was performed on the HPLC with UV detection at 370 nm and 320 nm for flavonoids and phenolic acids, respectively. The individual glycosides of the flavones were estimated against the respective aglycones of apigenin and luteolin using the correction factor of molecular weight ratio. The results indicated that individual compound concentrations differed statistically within plant tissues: caffeic acid and luteolin diglucuronide were more concentrated in the leaves of the plants while all of the other compounds were more concentrated in the flowers. ($P < 0.05$) **(Table 4-2, page 104)**. Total polyphenols averaged 11.32 ± 3.95 mg/g for the flowers, 12.31 ± 2.5 for the upper leaves and 11.29 ± 2.88 for the lower leaves. There was no significant difference in the production of polyphenols between the different plant tissues **(Figure 4-3, page 105)**. Total flavonoids averaged 6.48 ± 2.98 mg/g for the flowers, 5.39 ± 1.40 mg/g for the upper leaves and 4.60 ± 1.26 mg/g for the lower leaves; their levels of production did not differ statistically by plant tissues ($P > 0.05$). Total phenolic acid content averaged 4.84 ± 1.22 mg/g for the flowers, 6.92 ± 1.18 mg/g for the upper leaves and 6.70 ± 1.72 mg/g for the lower leaves; these compounds did differ statistically with the upper and lower leaves having significantly more phenolic acid production than the flowers ($P < 0.05$). The total content of the apigenin aglycone and its derivatives differed significantly between each plant organ ($P < 0.05$). On average, the concentration for apigenins and the derived analogues in the flowers, upper and lower leaves was 1.35 ± 0.73 mg/g, 0.57 ± 0.18 mg/g and 0.40 ± 0.14 mg/g, respectively. For the luteolins, the average concentration of the aglycone and the derived analogues in the flowers, upper leaves and lower leaves was 5.13 ± 2.27 mg/g, 4.82 ± 1.33 mg/g and 4.20 ± 1.19 mg/g respectively and they did not

significantly differ (P > 0.05). Total flavonoids in our samples ranged from 0.23% to 1.10% of dry mass while similar members of this species were reported to produce under 0.50% of dry mass[34]. Total phenols ranged from 0.26% to 0.88% of dry matter which is lower than what was reported in a separate *N. cataria* study using different genetic lines that reported up to 1.4% of dry matter to be phenolic acids[34]. The presence of flavonoids within the flowers of the plant is considered to be related to biological or ecological functions as plants use polyphenols for pollination, defense, for normal physiological processes such as stress relief by reducing reactive oxygen species (ROS)[46].

Quantified Compounds (mg/g)	**Flowers**	**Upper Leaves**	**Lower Leaves**
Caffeic acid	3.18 ± 1.44 B*	6.62 ± 1.24 A	6.37 ± 1.82 A
Luteolin diglucuronide	0.82 ± 0.40 B	2.87 ± 1.32 A	2.60 ± 1.03 A
Apigenin diglucuronide	0.36 ± 0.28 A	0.28 ± 0.08 A	0.22 ± 0.08 A
Luteolin glucoside	0.63 ± 0.40 A	0.09 ± 0.06 B	0.03 ± 0.04 B
Luteloin glucuronide	3.57 ± 1.82 A	1.84 ± 0.66 B	1.56 ± 0.69 B
Apigenin glucoside	0.63 ± 0.36 A	0.14 ± 0.07 B	0.07 ± 0.05 B
Apigenin glucuronide	0.32 ± 0.18 A	0.14 ± 0.06 B	0.10 ± 0.04 B
Rosmarinic acid	1.65 ± 0.93 A	0.29 ± 0.20 B	0.32 ± 0.30 B
Luteolin	0.09 ± 0.06 A	0.01 ± 0.12 B	0.00 ± 0.00 B
Apigenin	0.03 ± 0.04 A	0.00 ± 0.00 B	0.00 ± 0.00 B
Bioactivity Data			
TEAC (µM Trolox® Equivalent/g)	30.35 ± 3.56 A	30.56 ± 4.71 A	29.14 ± 4.61 A
NO inhibition IC50 Value (µg/ml)	81.96 ± 37.49 A	95.99 ± 85.55 A	91.28 ± 47.71 A

Table 4-2: Quantification and bioactivity data in *N. cataria* flowers, upper leaves and lower leaves. *Values within rows followed by the different letters are significantly different according to unpaired, two-tailed Student's T-test (P < 0.05).

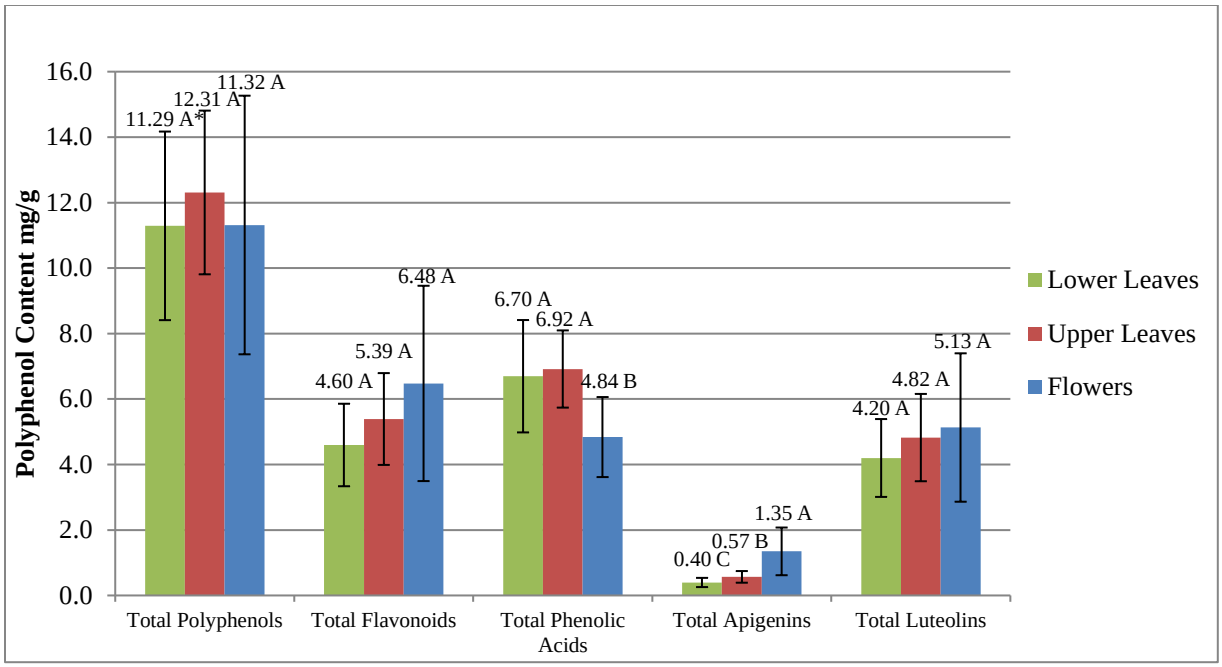

Figure 4-3: Polyphenol content (mg/g) of catnip (*Nepeta cataria*) in the flowers, upper leaves and lower leaves by HPLC. *Values within totals followed by the different letters are significantly different according to the unpaired, two-tailed Student's T-test ($P < 0.05$).

4.3.3 Anti-oxidant Capacities

Free radical absorption capacity was detected in every sample and the tissues were not statistically different from one another in radical reduction signifying that the bioactivity with respect to anti-oxidant activity did not differ significantly within the different plant tissues ($P > 0.05$). For the flowers, TEAC averaged 30.35 ± 3.56 µM trolox equivalent / g extract. Upper leaves average TEAC was 30.56 ± 4.71 µM trolox equivalent / g extract. Lower leaf TEAC averaged 29.7 ± 4.61 µM trolox equivalent / g extract. The anti-oxidant activity of the *N. cataria* samples was higher than numerous members of unrelated plant

species such as pears, apples, melons and bananas with two samples being more active than red grapes[47].

4.3.4 Anti-inflammatory Activity

Inhibition of NO production was measured by evaluating LPS-induced cells with the MeOH extracts of *N. cataria.* The inhibition of NO production for a representative sample of *N. cataria* is shown in **(Figure 4-4, page 107)**. All of the samples from the three *N. cataria* tissues exhibited signs of dose-dependent activity in the inhibition of the NO production. There was no statistically significant difference in the plant organ treatment to IC_{50} value ($P > 0.05$) indicating that the compounds essential for anti-inflammatory activity are biosynthesized throughout the plant. The IC_{50} value for the flowers averaged 81.96 ± 37.49 µg/mL. The IC_{50} value from the upper leaves averaged 95.99 ± 85.55 µg/mL. The IC_{50} value from the lower leaves averaged 91.28 ± 47.71µg/mL. Pure compounds made from H_2O and EtOH extracts of the above-ground biomass in oregano showed similar activity to all *N. cataria* tissues with respect to the inhibition of NO production in LPS-induced RAW 264.7 murine macrophages[20]. Catnip showed better inhibition in NO production than leaf MeOH extracts from Apple mint (*Mentha villosa*) and peppermint (*Mentha piperita*) in LPS-induced RAW 264.7 murine macrophages[21].

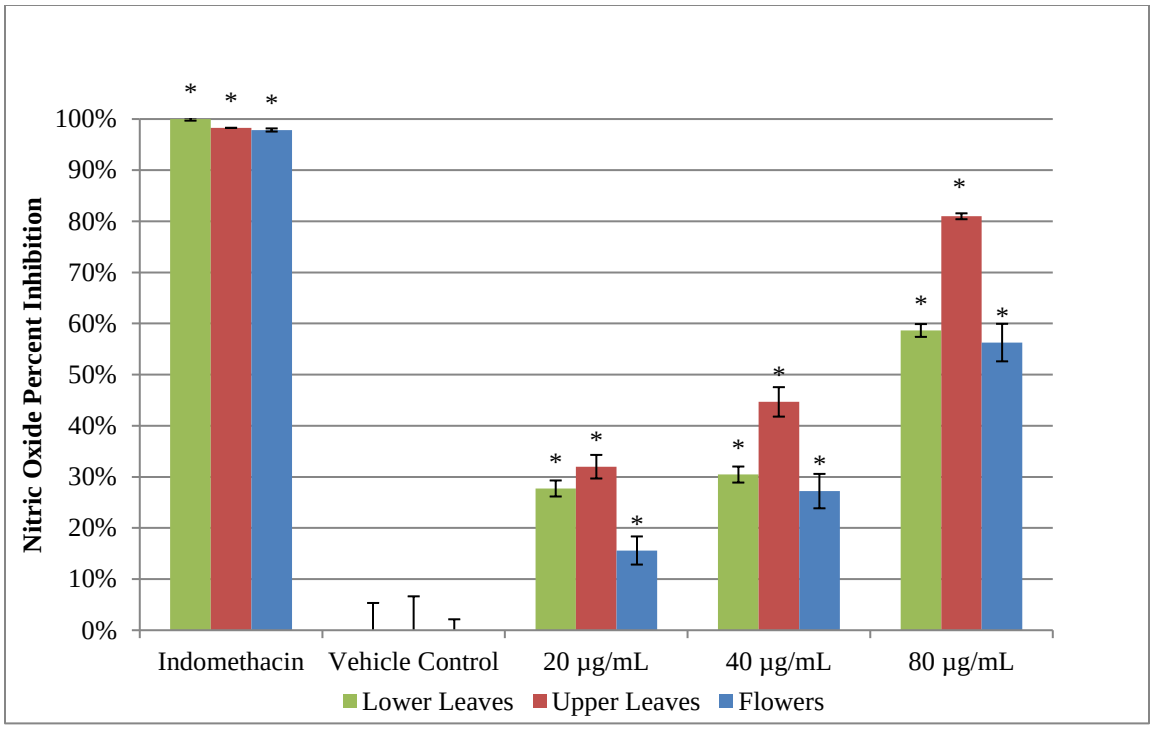

Figure 4-4: Inhibition of nitric oxide production in LPS-induced RAW264.7 murine macrophages by ethanol extracts of *Nepeta cataria* flowers, upper leaves and lower leaves. *Differed significantly from vehicle control (unpaired, two tailed Student's T-test, P < 0.05)

4.3.5 Discussion

All the non-glucuronidated compounds contained in the catnip extracts have been identified as being medicinally active *in vivo* with one example being caffeic acid, as it effects inflammatory cell recruitment and subsequent expression of pro-inflammatory chemokines and cytokines in ApoE KO mice[16]. In DBA/1 mice, rosmarinic acid reduced the arthritic index and COX-2 expression in the mice exhibiting anti-inflammatory activity[18]. Apigenin consumption also reduced LPS-induced expression of miR-155, a main construct in inflammation initiation[48]. *In vivo* studies of rats treated intradermally with radical generating enzymes showed that apigenin glucoside can reduce the overall

inflammation in the skin in a dose-dependent manner and in other murine models, inhibited IL-6 and TNF-α-production[49,50]. *In vivo* studies of luteolin as an anti-inflammatory agent were conducted on mice and they were protected from LPS-induced inflammation by luteolin[51,52]. Luteolin glucoside inhibited paw edema in the carrageenan and hind induced murine models[47].

These results indicate that catnip is a rich source of bioactive polyphenols including both apigenin and luteolin glycosylated and glurcuronidated derivatives. The *N. cataria* plants analyzed in this study were more effective at inhibiting NO production than Apple mint and peppermint and showed comparable activity to oregano. As the polyphenols accumulate in each of the three plant tissues, all parts of the plant can be harvested for polyphenols. While the inherent antioxidant and anti-inflammatory activity of *N. cataria* could not be deduced to a single compound, the wide array of flavones and phenolic acids present likely contribute to the associated bioactivity of the plant. Since *N. cataria* is being used as an herbal supplement and as an herbal tea, these results show that both the leaves and flowers should be included in dried products and extracts in order to provide the full battery of bioactive polyphenols. Breeding efforts to increase the yields and concentration of total and selected polyphenols appears most promising given the plant is still largely undomesticated varying in its essential oil composition and polyphenols. Catnip demonstrated strong bioactivity and the holistic use of it as a home remedy appears to be at least partially justified due to the high concentrations of apigenin and luteolin derivatives.

4.4 References

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Chapter 5 Repellency Assessment of *Nepeta cataria* Essential Oils and Isolated Nepetalactones on *Aedes aegypti*

Mosquitoes and other arthropods vector some of the deadliest diseases on the planet affecting the public health of human populations globally. Rural communities that do not have access to synthetic repellents have to rely on management techniques and natural products to protect themselves from contracting these deadly diseases. These communities are at the highest risk of contracting an infection and when they do there is often no specialized medical treatment for them making their immune system solely responsible for combatting the infection. Complicating things even more, if individuals in rural communities are fortunate enough to be around medical care, the similarities in symptoms between mosquito vectored diseases make if difficult to diagnose a patient resulting in patient mortality[1]. While different arthropods vector different diseases, certain insects have a higher impact on global health than others. Arthropod vectored diseases kill 1.5 million people annually and there are 500 million new cases of malaria each year alone[2]. While various tick and fly species vector many diseases, mosquitoes are by far the largest contributor to arthropod vectored diseases[2]. Mosquitoes inhabit almost every continent on the planet and their modes of reproduction make them perfect for reproducing in mass numbers in almost every climate[3]. Mosquitoes efficiency in breeding, reproduction and feeding has left them relatively unchanged for millions of years and is why they still present a real danger to public health[4]. Due to the deadly effect mosquito populations have on rural communities and the previous research showing that *N. cataria* is an efficient insect repellent led us to investigate the repellency efficacy of the two new cultivars ‘CR3’ and ‘CR9’.

5.1 Introduction

Mosquitoes vector the deadliest diseases on the planet killing an estimated half a million people annually by Malaria, Yellow fever and the Dengue virus alone[5,6,7]. Recently, a newer threat known as the Zika virus reemerged and is being rapidly spread by *Aedes aegypti* mosquitoes, as well as from a viremic mother to her newborn and by sexual intercourse throughout the western hemisphere, potentially causing neurological disorders and microcephaly[8]. Mosquitoes seek out hosts in search of a blood meal for reproduction providing an opportunity for the infectious agent to enter the host while feeding[9]. The common symptoms of all these diseases are rashes, a high fever and chills that all complicate emergency room diagnosis due to the similarities, resulting in a missed or delayed diagnosis that could result in mortality[1]. Some diseases such as the West Nile virus show very little symptoms and can go undiagnosed until movement loss or neurological illness[10]. Disease vectoring mosquitoes cover the world and efforts to control the spread of diseases, identify new repellents and to educate people lowering the risk of infection are currently being implemented on a multinational level[11,12,13]. DDT (1,1'-(2,2,2-Trichloroethane-1,1-diyl) bis (4-chlorobenzene)) is a pesticide that was used globally in the past to kill mosquitoes, but its usage has been severely curtailed due to its negative environmental impacts[14]. Educating individuals about mosquito control in regions of the globe where there is a high rate of disease incidence on how to manage mosquito populations, has been effective at reducing infections[15,16].

Insect repellents developed by government and private industries are very effective at deterring mosquitoes and protecting the users from contracting these diseases. DEET (N, N-Diethyl-3-methylbenzamide) has been the benchmark of insect repellents since its

development for the United States Army to use in tropical regions where there is a high incidence of insect transmitted diseases[17]. Picaradin, developed by Bayer, is very effective at repelling mosquitoes as well[18]. While DEET is extremely effective, it is not as volatile as other insect repellents and leads to a limited range of efficacy in repelling mosquitoes and other insects[19]. Whereas another critical concern associated with the consistent use of DEET is its potential toxicity. Studies have shown DEET inhibits human acetyl cholinesterase, modulates GPCRs and inhibits ion channels[20,21,22,23,24]. Numerous publications exist urging caution in its use and claim that DEET is unsuitable for young children and pregnant females, though the Centers for Disease Control and Protection (CDC; Atlanta, GA) still recommends the use of it for vector protection[25,26,27]. DEET is also absorbed through the skin at a high rate and special formulations are required to reduce transdermal absorption[28]. However, other reviews suggest that it does not cause adverse health effects[29]. The costs of DEET have made it unaffordable to many of those living in regions affected by disease vectoring mosquitoes such as sub-Saharan Africa, China and India. Between the costs, access, and public concern about the dangers of DEET individuals and families are preferring locally sourced natural insect repellants derived from eucalyptus, mints, cloves, basils and neem leaves over DEET[30].

While many regions of the world have access to DEET and other effective insect repellents, natural products still serve as a primary source of repellents in China, India and sub-Saharan Africa where people cannot afford or do not have access to them[30]. Specialty crops are cultivated to produce a wide array of insect repellents in China, India and sub-Saharan Africa to repel disease-causing insects from natural sources have been found dating back to before the Common Era[30,31]. Ethnobotanical resources have led to the identification of

plants that can be used to repel insects[30,32]. Volatizing citronella and geraniol from lemongrass oil as well as neem oil are the most common natural sources of mosquito repellents[33,34]. In 2005, the CDC endorsed para-Menthane-3, 8-diol (PMD), a steam distillate product from the leaves of the Australian lemon-scented gum tree as a mosquito repellent[35]. Insect repellents derived from natural sources such as volatile essential oils have been shown to be as efficient as DEET at repelling mosquitoes for up to an hour, however these volatiles in their non-encapsulated forms can only supplement the current use of DEET due to their limited duration of acceptable repellency[36,37,38].

Natural insect repellent formulations using essential oils from aromatic plants in the Lamiaceae family are sourced from the glandular trichomes on the epidermis of leaves and flowers. Plants biosynthesize multiple compounds in their essential oil, not just a single desirable chemical and breeding programs have increased the bioactive compounds concentration within the essential oil of multiple species of plants across the kingdom[39,40,41,42,43]. Botanicals from the Lamiaceae family have also demonstrated that their essential oils can act as a mosquito repellent comparable to DEET[44,45]. One member of this family, *Nepeta cataria* (catnip), has had its essential oils containing various nepetalactone stereoisomers tested and the results showed that it is comparable to DEET at repelling mosquitoes, while offering better spatial repellency[46,47,48]. Nepetalactones are the distinguishing natural compounds associated with catnip and are the euphoria inducing agent in felines responsible for their characteristic behavior[49]. Like many genera of the Lamiaceae plant family, the *Nepeta* genus contains species that produce a wide array of volatile compounds in their essential oil including nepetalactones, β-caryophyllene, nerol, citronellol and geraniol[50,51,52].

Insects can often discriminate different isomers, perceiving them differently from one another and the stereochemistry of an insect repellent compound can alter its efficacy as a repellent due to the mechanics of the repellent, receptor perception[53,54]. Members of the *Nepeta* genus produce many different stereoisomers of nepetalactone within their essential oils **(Figure 5-1, page 117)**[55,56,57]. Efforts to investigate the differences in the two main crude oil chemotypes (Z, E-nepetalactone dominated or E, Z-nepetalactone dominated) and the isomers of nepetalactone with respect to repellency in mosquitoes have been investigated[58,59]. A World Health Organization (WHO) approved topical application bioassay showed the different essential oil chemotypes were comparable to DEET at repelling *Anopheles gambiae* in a forearm assay from a steam distilled product[59]. The Z, E- and E, Z-nepetalactones showed similar efficacy at repelling *A. gambiae* in another WHO approved topical application bioassay where the crude oils and purified compounds were not statistically different from one another at repelling the mosquitoes[59]. A biting deterrent assay in *A. aegypti* showed no difference in the crude oils and purified nepetalactones in the ability to stop feeding, however a dose response curve was not generated and the duration of repellency was not investigated[58]. The *N. cataria* essential oil was tested for acute oral, dermal and inhalation toxicity and the results showed that *N. cataria* is safe for human use according to the United States Environmental Protection Agency but may cause mild skin irritation[60].

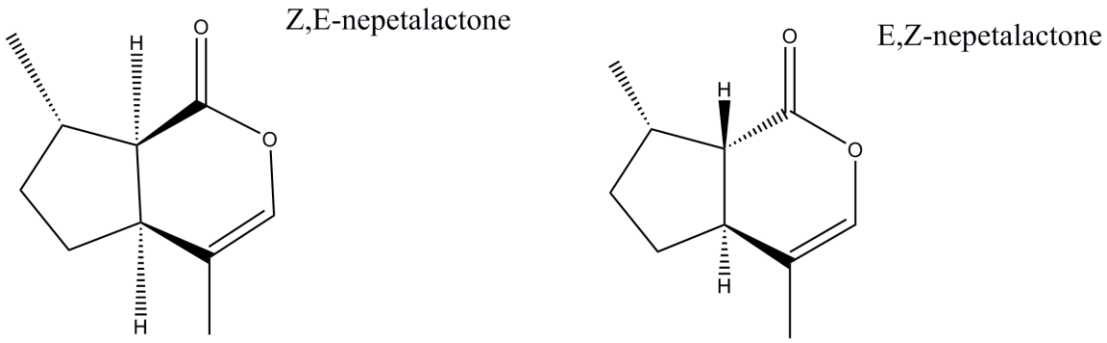

Figure 5-1: Chemical structures of the two main nepetalactones in *Nepeta cataria*. The structures of Z, E-nepetalactone and E, Z-nepetalactone; the two main nepetalactone isomers produced by *N. cataria*.

Due to the need for effective safe insect repellents and since the essential oils and purified compounds of *N. cataria* have shown to be as effective as DEET at repelling mosquitoes, we investigated several different established nepetalactone based chemotypes of *N. cataria*, their respective purified isomers and compared them to DEET to generate a landing reduction dose response repellency curve. We also conducted a time course analysis of a crude essential oil extract compared to DEET over a 24hr period to assess repellency.

5.2 Materials and Methods

5.2.1 Equipment and Chemical Reagents

Bug Dorm insect rearing cages (29.9 cm^3) were obtained from BioQuip Products (Rancho Dominguez, CA). A HotHands hand warmer was used as the heat source and these were procured from Kobayashi LLC (Dalton, GA). Crude commercial catnip oil was obtained from Plant Therapy Essential Oils (Twin Falls, ID). TetraMin Tropical Tablets used for

mosquito rearing were purchased from Tetra (Blacksburg, VA). FisherBrand filter paper circles (18.5cm; 09-795G) were obtained from Fisher Scientific Co. (Fair Lawn, NJ).

Chromatographic grade methyl tert-butyl ether (MTBE), as well as reagent grade acetone and DEET at 97% purity were obtained from Sigma Aldrich (St. Louis, MO). Anhydrous sodium sulfate (Na_2SO_4) and chromatographic grade hexane and ethyl acetate (EtOAc) were purchased from Fisher Scientific Co. (Fair Lawn, NJ). Chromatographic grade helium was obtained from Airgas, Inc. (Radnor, PA).

5.2.2 *Nepeta cataria* Cultivation and Essential Oil Preparation

The clonal populations serving as source material for the essential oils includes two new and unique Rutgers University catnip cultivars *N. cataria* cv. 'CR3' and *N. cataria* cv. 'CR9' both stable in unique essential oil chemistry, promising growth characteristics and high essential oil yields[43]. The CR3 essential oil chemotype is mainly comprised of the E, Z-nepetalactone isomer yet also produces Z, E-nepetalactone. The CR9 essential oil chemotype is dominated by the Z, E-nepetalactone isomer and produces little E, Z-nepetalactone. The hydro-distilled essential oil from these two populations served as the source for the crude essential oil treatments and partitioned for subsequent fractionation and nepetalactone purification.

These two genetically distinct populations were propagated at the Rutgers University Research Greenhouses (New Brunswick, NJ) where vegetative clones were made by making cuttings at the terminal nodes and briefly dipping them in Hormodin 2, 0.3% indole-3-butyric acid (IBA) to induce root formation and placed in a mist house until roots

developed. The clones were then transplanted on June 6th, 2015 to the Rutgers University Clifford E. & Melda C. Snyder Research and Extension Farm in Pittstown, NJ. Just prior to when the plants were in full flower, they were harvested and dried at 37°C with an onsite Powell walk-in forced air heat dryer. Once the plants had dried moisture, the leaves and flowers were separated from the stems before hydro-distillation. Essential oils were extracted by hydro-distilling 60 g of dried *N. cataria* leaves and flowers in a 2 L round bottom flask for 3 hours in 1 L of water and the essential oil was collected in a Clevenger-type trap.

5.2.3 GC/MS Sample Preparation and Separation Conditions

Essential oil samples were prepared by the extraction of 10 µL of crude *N. cataria* essential oil with 1.5 ml of MTBE which was then dried with Na_2SO_4 and centrifuged at 13 Krpm. The supernatant was transferred to a sampling vial for analysis. Essential oil separation was performed on a Shimadzu 2010 Plus gas chromatograph equipped with an AOC-6000 auto-sampler. The analysis of the relative abundance of compound fragments was performed on a Shimadzu TQ8040 MS.

An injection volume of 1µL was separated using chromatographic grade helium on a H-Rxi-5Sil MS column held at 35°C for 4 min then heated to 250°C at 20°C/min then held for 1.25 min at 250°C. The inlet temperature was 250°C with a splitless injection. The ion source temperature was set at 200°C, the interface temperature was set at 250°C, the solvent cut time was 3.5min, and the detector voltage was set to 0.2 kV with a threshold of 1000. Peak integration percentages were generated using the GCMSsolution v4.3© software from Shimadzu Corporation. Individual identities were determined by comparing

the mass spectral results to current literature and screening them in the NIST05.LIB, NIST05s.LIB, W10N14.lib and the W10N14R.lib mass spectral libraries. The CR3 chemotype contained 68.2% E, Z-nepetalactone and 25.6% Z, E-nepetalactone while CR9 contained 90.1% Z, E-nepetalactone and less than 0.5% E, Z-nepetalactone and the commercial *N. cataria* essential oil contained 24.4% Z, E-nepetalactone and 62.8% E, Z-nepetalactone **(Table 5-1, page 120; Figure 5-2, page 121)**.

ID #	Essential oil Constituent	Mass	R_T (min)	CR3 Peak Area %	CR9 Peak Area %	CO Peak Area %
1	α-Pinene	136	7.70	ND	ND	0.52
2	β-Pinene	136	8.22	T	0.58	0.57
3	Carvone	150	10.63	ND	ND	0.67
4	Z, E Nepetalactone	166	11.53	27.45	85.33	24.34
5	E, Z Nepetalactone	166	11.75	69.37	1.68	62.62
6	Carophyllene	204	11.96	1.57	4.72	8.11
7	Humulene	189	12.20	T	0.51	0.58
8	Caryophyllene Oxide	187	13.02	1.27	5.79	1.56

Table 5-1: Chemical profile of *Nepeta cataria* crude essential oil treatments. The eight essential oil constituents that represent > 98% of the overall peak area detected in the three essential oil treatments (catnip line CR3, cultivar CR9 and a commercial *N. cataria* essential oil) including their mass, retention time and peak area percentages. T: Trace level of compound where overall concentration was less than 0.5%. ND: Compounds not detected in the sample.

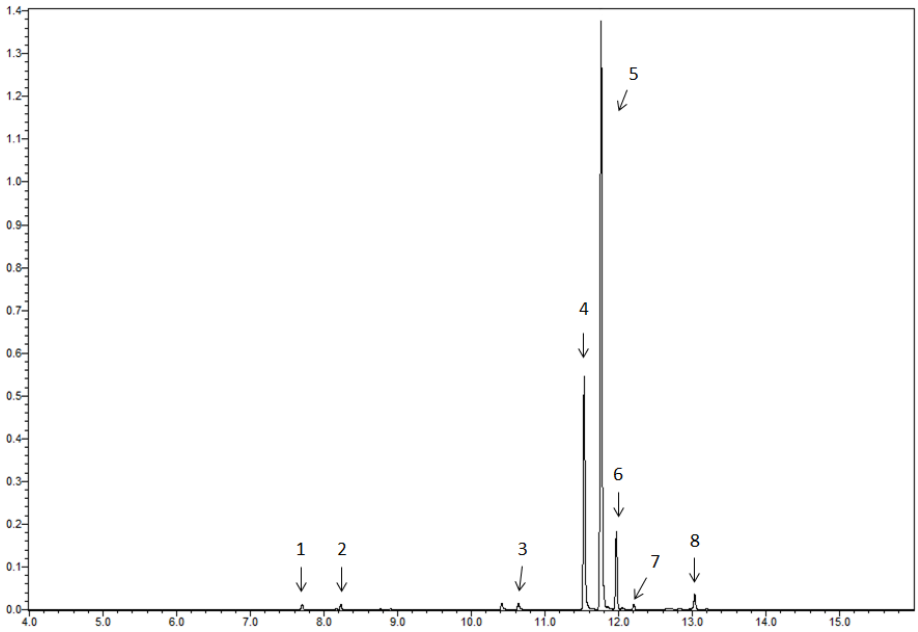

Figure 5-2: Representative gas chromatogram of *Nepeta cataria* essential oil. Commercial *N. cataria* essential oil chromatogram showing the separated compounds contained in all the crude essential oil treatments. Compound identification numbers correspond to Table 1.

5.2.4 Purification of Nepetalactones

Catnip essential oil was repeatedly chromatographed on a silica gel column using a stepwise-gradient of hexane-EtOAc. CR9 crude essential oil was used for Z, E-nepetalactone purification and CR3 crude essential oil was used for E, Z-nepetalactone purification. Achieved fractions containing the target components were monitored by TLC and LC–MS obtaining Z, E-nepetalactone (800 mg) and E, Z-nepetalactone (200 mg). The structures of these two purified compounds were determined and verified by UV, MS and NMR spectrometric methods and in comparison with references[61].

5.2.5 Mosquito Rearing

Aedes aegypti (*wt*. Orlando) eggs were reared in water at 27ºC with 80% humidity under a 12-hour day/night light cycle and were transferred during the rearing process with an eye dropper. General fish food tablets were used as the energy source for the maturing mosquitoes. The *A. aegypti* eggs were placed in a container holding water and once hatched and formed into larvae, they were separated from the unhatched eggs and placed into fresh water. As the mosquitoes began to form into pupae, they were separated from the smaller less developed larvae and placed into fresh water. This container was then placed into a rearing cage where the pupae were allowed to mature into adults. Mature females were visually identified, then separated out of the population by aspirating them into a separate rearing cage where they were given a 10% sucrose solution as an energy source. Mature females were kept at these conditions until experimentation.

5.2.6 Dose Dependent Curve Generation

Repellency was determined by a one-choice landing assay that uses the amount of mosquito landings to calculate the overall effectiveness of a candidate repellent when compared to a control. Twenty, adult female *A. aegypti* mosquitoes were aspirated into a rearing cage and starved for 1 day. Testing was performed between 10:00 am and 4:00 pm PST during *A. aegypti's* active hours. A 37ºC heat pack was used to as the heat source to attract the mosquitoes in the upper region of the back panel of the rearing cage. The different treatments were extracted in acetone and 500µL applied to the filter paper used to wrap the heat source. A control of acetone was applied to filter paper before and after each treatment to ensure reproducibility in mosquito behavior where percent repellencies for each treatment represented the percent reduction in mosquito landings from the prior control.

Six repetitions we performed with twenty mosquitoes for each treatment and at each concentration along with an acetone control. The dose response curve was generated from identifying a concentration of the treatments that exhibited complete repellency and then working in reverse logarithmically with respect to the concentration of the treatment. Initial tests showed that few enough landings were observed at 1.0% generating a 95% reduction in mosquito landings and was defined as complete repellency. Time-lapse photography recorded one image every five seconds for five minutes where a custom macro named "Final Mosquito Counter" generated a Z stack of the images and counted the number of mosquitoes on the filter paper wrapped heat pack automatically through the open source image processing software ImageJ[62].

5.2.7 Time Course Assay

This assay was performed the same way as the dose response curve, except we were evaluating how long the essential oils can be an effective repellent. During a 24hr period, the efficacy of a 10% CR9 essential oil and DEET treatment was monitored at 0hr, 1hr, 2hr, 4hr, 8hr and 24hr intervals. Six repetitions were done for each treatment where the mosquito landings were counted and analyzed similarly to the dose response curve method and at each time point along with an acetone control before and after to ensure mosquito behavior reproducibility where percent repellencies for each treatment represented the percent reduction in mosquito landings from the prior control. After the acetone treatment was applied to the filter paper and the 0hr treatment sample was done, the filter paper was collected and used in the subsequent time intervals.

5.2.8 Statistical Analysis

Values are presented as means ± standard deviation (SD). Data was analyzed by an unpaired, two-tailed student's t-test to identify significant differences ($P < 0.05$).

5.3 Results

5.3.1 Dose Dependent Results

In the dose response curve, DEET and all of the catnip treatments at 1.00%, decreased the landings of mosquitoes by > 95% and grouped together statistically for repellency **(Table 5-2, page 125; Figure 5-3, page 125; Figure 5-4, page 126)**. At 0.10%, Z, E-nepetalactone generated the highest repellency percentage and was significantly different from the CR3 essential oil treatment and purified E, Z-nepetalactone at the same concentration. The rest of the treatments were not significantly different from one another at 0.10%. At the lowest concentration, all of the catnip extracts were significantly higher than DEET at reducing landings. At concentrations of 0.01%, 0.10 % and 1.00% DEET repelled 31.7% ± 13.5, 80.0% ± 12.6 and 98.0% ± 1.6 of the mosquitoes respectively. The CR3 crude essential oil repelled 66.3% ± 12.7, 74.1% ± 17.3 and 97.2% ± 6.9 of the mosquitoes respectively. The CR9 crude essential oil repelled 65.9% ± 18.1, 80.6% ± 6.6 and 99.8% ± 0.2 respectively. The commercial essential oil repelled 58.7% ± 16.0, 83.1% ± 5.9 and 99.8% ± 0.3 respectively. The purified Z, E isomer repelled 53.1% ± 11.1, 90.9% ± 5.7 and 99.7% ± 0.3 of the *A. aegypti* mosquitoes respectively and the purified E, Z isomer repelled 55.7% ± 14.4, 74.2% ± 14.7 and 96.8% ± 3.3 respectively.

Aedes aegypti Dose Response Curve Repellency Percentages			
Treatment	Concentrations		
	0.01%	0.10%	1.00%
DEET	31.7% ± 13.5 F	80.0% ± 12.6 BC	98.0% ± 1.6 A
CR3	66.3% ± 12.7 ED	74.1% ± 17.3 CD	97.2% ± 6.9 A
CR9	65.9% ± 18.1 ED	80.6% ± 6.6 BC	99.8% ± 0.2 A
Commercial	58.7% ± 16.0 E	83.1% ± 5.9 BC	99.8% ± 0.3 A
Z, E Nep	53.1% ± 11.1 E	90.9% ± 5.7 AB	99.7% ± 0.3 A
E, Z Nep	55.7% ± 14.4 E	74.2% ± 14.7 CD	96.8% ± 3.3 A

Table 5-2: *Aedes aegypti* Dose Response Curve Repellency Percentages. Dose response landing reduction percentages for *Aedes aegypti* for DEET, *N. cataria* CR3 crude essential oil, *N. cataria* cv. 'CR9' crude essential oil, a commercial *N. cataria* essential oil, purified Z, E-nepetalactone and E, Z-nepetalactone treatments at 0.01%, 0.10% and 1.00%. Values show ± standard deviations where different letters after the treatments are significantly different according to Duncan's test at P ≤ 0.05.

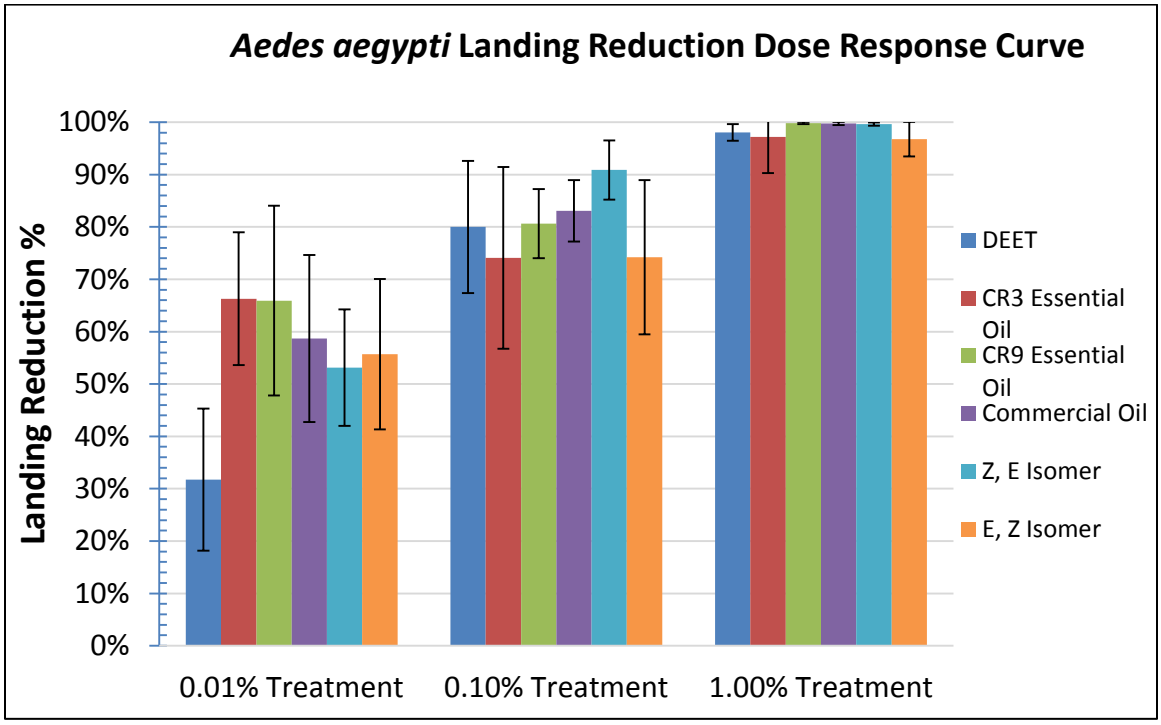

Figure 5-3: Landing reduction dose response curve for Aedes aegypti mosquitoes. Dose response landing reduction curve for *Aedes aegypti* for DEET, *N. cataria* CR3 crude essential oil, *N. cataria* cv. 'CR9' crude essential oil, a commercial *N. cataria* essential oil, purified Z, E-nepetalactone and E, Z-nepetalactone treatments at 0.01%, 0.10% and 1.00%. Different letters above treatments are significantly different according to Duncan's test at P ≤ 0.05.

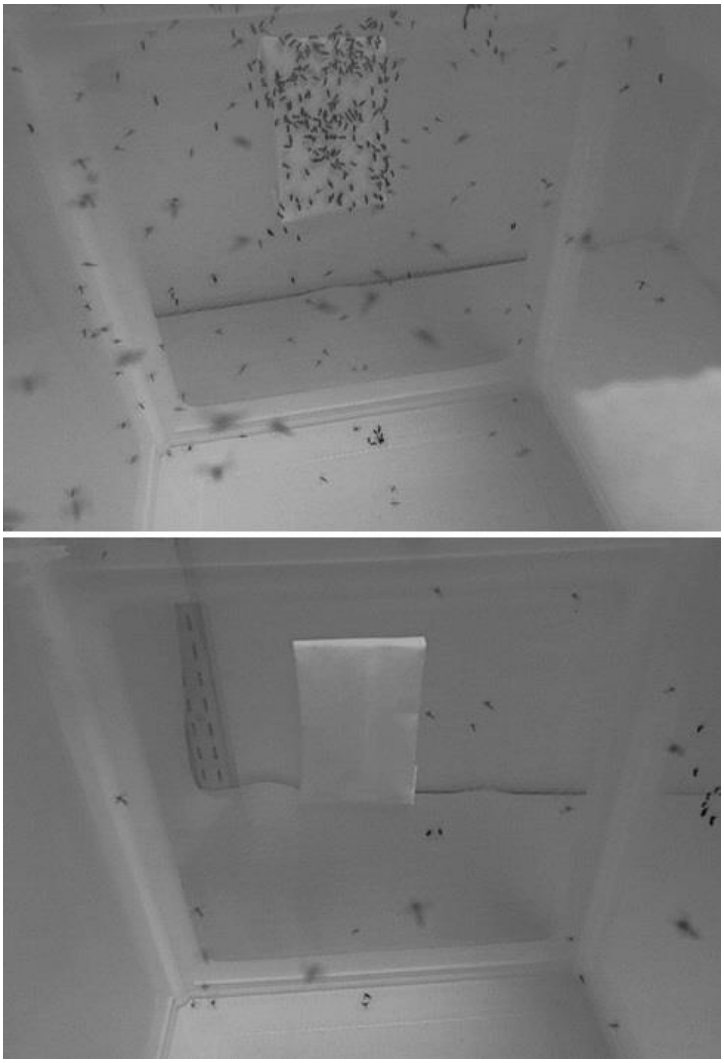

Figure 5-4: Representative heat packs showing landing areas before (top) and after (bottom) *Nepeta cataria* treatments. Z stacks consisting of 60 images taken at 5 second intervals over 5 minutes showing the inside of a rearing cage and the heat source

5.3.2 Time Course Results

In the time course assay, the 10% CR9 essential oil treatment generated > 95% repellency for the first two hours and was statistically similar to DEET **(Table 5-3, page 127; Figure 5-5, page 128)**. Between 2 and 4 hours, this repellency diminished and did not stop 95% of the landings and was no longer statistically similar to DEET. After 24 hours, the essential oil present on the filter paper was still able to generate a repellent effect but was much lower than 95%. The DEET treatment was able to maintain the > 95% repellency rate throughout the 24hr period. At the 0hr, 1hr, 2hr, 4hr, 8hr and 24 hour time intervals after treatment, DEET repelled 99.8% ± 0.3, 99.1% ± 1.0, 99.8% ± 0.3, 99.1% ± 1.0, 94.1% ± 7.0 and 97.5% ± 2.3 mosquitoes respectively. At the 0hr, 1hr, 2hr, 4hr, 8hr and 24 hour time intervals after treatment, CR9 essential oil repelled 99.7% ± 0.2, 98.7% ± 1.1, 97.2% ± 3.2, 87.8% ± 12.6, 81.0% ± 10.7, and 76.2% ± 18.3 mosquitoes respectively.

Aedes aegypti Landing Reduction Time Course Repellency Percentages						
Treatment	Time Intervals					
	0hr	1hr	2hr	4hr	8hr	24hr
10% DEET	99.8% ± 0.3 A	99.1% ± 1.0 A	99.8% ± 0.3 A	99.1% ± 1.0 A	94.1% ± 7.0 AB	97.5% ± 2.3 A
10% CR9	99.7% ± 0.2 A	98.7% ± 1.1 A	97.2% ± 3.2 A	87.8% ± 12.6 B	81.0% ± 10.7 C	76.2% ± 18.3 C

Table 5-3: Aedes aegypti Landing Reduction Time Course Repellency Percentages. Landing reduction time course analysis repellency percentages of A. aegypti mosquitoes with 10% DEET and crude CR9 essential oil treatments over 24 hours. The samples were tested at 0 hour, 1 hour, 2 hours, 4 hours, 8 hours and 24 hours after application of the treatments. Values show ± standard deviations where different letters after the treatments are significantly different according to Duncan's test at $P \leq 0.05$.

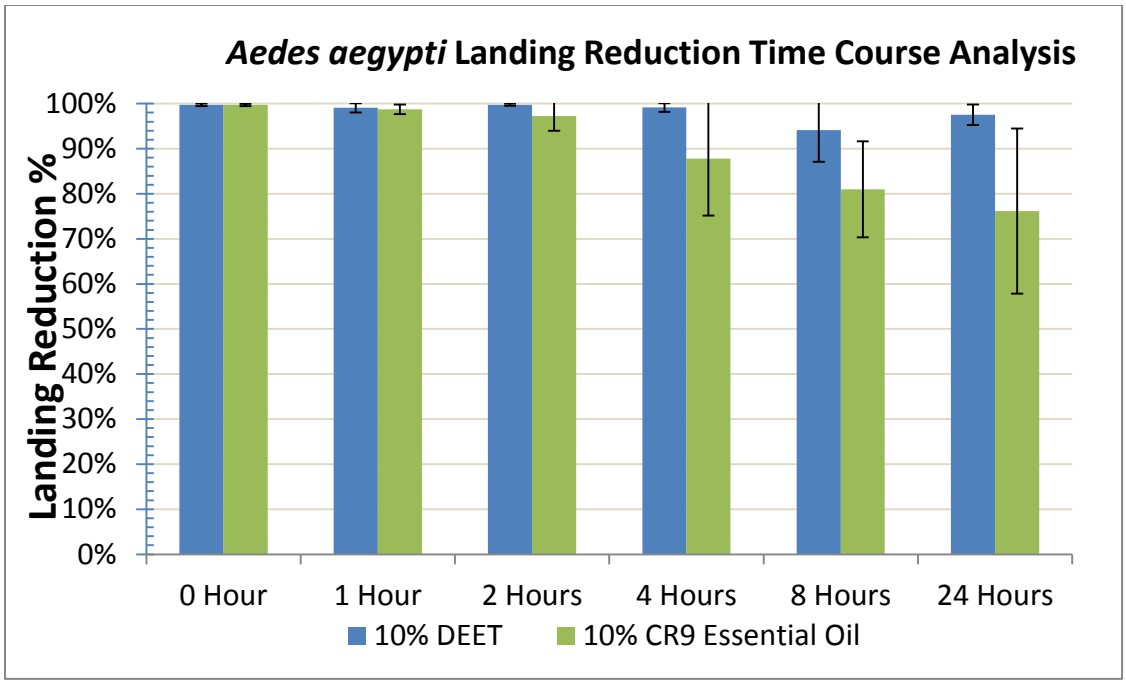

Figure 5-5: Landing reduction time course analysis for Aedes aegypti mosquitoes. Time course analysis comparing the landing reduction percentages of Aedes aegypti mosquitoes with 10% DEET and crude CR9 essential oil treatments over 24 hours. The samples were tested at 0 hour, 1 hour, 2 hours, 4 hours, 8 hours and 24 hours after application of the treatments. Different letters above treatments are significantly different according to Duncan's test at $P \leq 0.05$.

5.4 Discussion

The data presented herein demonstrates that the essential oils of *N. cataria* can serve as a natural source for an effective *A. aegypti* repellent that is comparable to DEET for the first two hours after application but needs to be reapplied to maintain complete repellency from *A. aegypti* mosquitoes. Since the two main chemotypes of the essential oils (CR3, CR9) were not statistically different from one another and DEET at 1.00%, individuals who need an immediately effective insect repellent could use either *N. cataria* essential oil chemotype for protection. The data from the 0.01% treatment in the dose response curve

is in contrast to a biting deterrent study in which DEET was shown to be more effective than either of the nepetalactones and can be attributed to the differences in the tests and/or mosquitoes, however in the time course assay demonstrates that the CR9 essential oil is as effective as DEET for the first two hours after application in the landing reduction assay[58]. The two isomers were equally effective at repelling mosquitoes so repellents made from these will be effective as long as it has a high enough concentration of either nepetalactone isomer. While the 10.00% solution of the CR9 chemotype is just as effective as DEET for the first 2hrs, a reapplication would be required to keep the numbers of landings reduced by 95% or greater. Efforts to increase the effective repellency duration of the catnip essential oils through formulation development should be considered so that the repellent maintains the > 95% landing reductions for over 2hrs so reapplication would be less frequent.

Nepeta species producing nepetalactones are found throughout the globe and their essential oil can be distilled to repel mosquitoes that inhabit the same regions[52]. The processing of raw plants to yield essential oils is accomplished using a variety of extraction or distillation technologies including solvent, super critical CO_2 or water and steam respectively. In rural communities, the same processing technologies now in commercial use that produce a wide range of essential oils can be used to procure the aromatic volatile oils from *N. cataria* in rural regions. These communities can be protected from disease vectoring insects from the plantings of *Nepeta sp*. that contain high amounts nepetalactones in their already established essential oil crop fields. While many catnip and catmint plants produce essential oils and of these many have nepetalactones as a component in their essential oil, overall functional agricultural yields of the total essential oil and/or the concentration of

the nepetalactones is low, leading to a commercialization bottleneck. These results are exciting because these two newly developed genetic varieties produce high yields of total essential oil, and of the oil, each are rich in the bioactive nepetalactones overcoming two of the major constraints in developing a new bioactive ingredient, the cost and availability of the raw material. Individuals in rural communities can overcome the costs of purchasing *N. cataria* essential oil by cultivating the plant and processing the essential oils themselves for protection. In this case, both CR9 and CR3 have overcome those two constraints and a 500µL 1.00% solution of their essential oil will immediately remove > 95% of *A. aegypti* mosquitoes from an area.

5.5 References

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Chapter 6 Formulation Development and Arthropod Repellency Efficacy with *Nepeta cataria* Essential Oils

As demonstrated in the previous chapter, *Nepeta cataria* essential oil is an effective *A. aegypti* repellent and comparable to DEET when applied at 0.1% and 1.0% concentrations. At 0.01% *N. cataria* 'CR3' and 'CR9' essential oil outperformed DEET at repelling the disease vectoring mosquitoes. However, despite its efficacy, *N. cataria* essential oil volatizes much faster than DEET and does not provide acceptable repellency efficacy after 2 hours while DEET maintained this standard throughout the 24-hour period. Previous research has shown that essential oils from members of the Lamiaceae family can be incorporated into formulations that are polyethylene glycol, paraffin, wax, and cyclodextrins to slow down the rate of their volatilization[1,2]. Decreasing the rate of volatilization of *N. cataria* essential oil should increase the duration of acceptable efficacy at repelling disease vectoring insects and provide individuals using the essential oil a longer period of safety. This chapter focuses on incorporating 'CR9' and 'CR3' essential oils into liquid sprays, a gel and a stick using the previously different molecular weight polyethylene glycols, a carbomer and beeswax respectively, by modifying previously developed formulations[3]. The essential oil from *N. cataria* will also be cyclodextrated by β-cyclodextrin to form the raw materials to be incorporated into future formulations to extend the duration of acceptable repellency efficacy. These formulations were tested against *A. aegypti* mosquitoes and the top performing formulation tested against *I. scapularis* ticks in a time course assay to determine the duration in which the formulation provided acceptable repellent efficacy.

6.1 Introduction

Mosquito species cover an increasing part of the globe and vector some of the deadliest diseases on the planet leading to over a million deaths per year due to their transfer of pathogens to individuals while feeding[4,5,6,7]. Malaria, one of the most notorious deadly diseases is vectored by *Anopheles gambiae* and is present throughout the world where mosquitos live, where few treatment methods exist and when treatments are available it is often too expensive. Malaria be mistaken for other mosquito vectored diseases such as Yellow Fever vectored by the *Aedes aegypti* mosquitoes complicating treatment by a misdiagnosis due to symptom similarities[8,9]. These mosquitoes represent a clear and present danger to the public health of individuals around the globe and efforts to minimize the reproduction and spread of them have been accomplished by the application of insecticides, reduction of stagnant water and sources where the mosquitoes bred and by insecticide impregnated bed nets, cloths and other such interventions. However, insecticides themselves can have larger ecological implications than eradicating mosquitoes and negative human health implications have been seen in their use even including the use of bed nets as fishing nets and the unintentional spread of insecticides into local water systems[10]. Proactive approaches to controlling the spread of the mosquitoes and subsequently the diseases they vector have been done in the form of educating the population on management techniques to reduce the amount of mosquitoes in an area and the application of effective insect repellents instead of spraying entire areas eradicating mosquito populations[11,12].

Insect repellents are at the forefront of the effort to reduce infection by disease vectoring insects. While many effective repellents have been developed by government and private

entities such as DEET (N, N-Diethyl-3-methylbenzamide) and picaridin respectively, rural populations from around the globe do not have access to these repellents and must find alternative solutions including but not limited to the natural product alternatives to available to them[12,13,14]. Locally sourced natural product based insect repellants derived from the essential oils eucalyptus, mints, cloves, basils and the burning of neem leaves have been identified ethnobotanically with eucalyptus oil and the bioactive repellent PMD (*para*-Menthane-3,8-diol), the main compound in the essential oil, being so effective it was endorsed in 2005 by the Centers for Disease Control and Protection (CDC; Atlanta, GA) for vector protection[15,16]. Essential oils are a matrix of volatile compounds and efforts to increase the overall yield of essential oils and the bioactive or desired compounds within the essential oil have been taken in plants across the kingdom including mint and basil species[17,18,19,20,21,22,23]. While essential oils can provide great efficacy at deterring disease vectoring insects, one of the main concerns associated with them is the duration of acceptable repellency since essential oils distilled from plants are inherently volatile and do not provide long term protection in their crude forms[24,25].

There are many ways to increase the duration of acceptable repellency of an essential oil by decreasing the volatility of it and controlling the release of the bioactive compounds including using liquid paraffins and polyethylene glycol's varying in molecular weight, waxes and encapsulation techniques[1,2]. Most of these formulations incorporate hydrophobic stabilizers to regulate the delivery of the essential oils in a more controlled manner and are generally recognized as safe. Liquid paraffins that can solidify at room temperature consist entirely of carbon and hydrogen denoting them as hydrocarbons, their physical properties of interest (solubility, stabilization of bioactive compound) depend on

their chemical structure and have been introduced into formulations up to 20% v/v[1]. Polyethylene glycols are polymers made of ethylene glycol monomers and like liquid paraffins, their physical properties (solid or liquid, stabilization of bioactive compound) depend on the number of oligomers that make up the polymer. Essential oils have also been incorporated into various types of paraffin based waxes for controlled releases and the animal natural product beeswax however, the chemical composition of beeswax can change due to the species of bee that is making it, making the standardization of beeswax products more difficult than synthetics waxes[26].

Natural insect repellent formulations using essential oils from aromatic plants in the Lamiaceae family are sourced from the glandular trichomes on the epidermis of leaves and flowers and have also demonstrated that their essential oils can act as a mosquito repellent comparable to DEET[27,28]. While normally cultivated for the pet toy industry as a safe attractant to cats and for ornamental applications, recent research has shown that essential oils from catnip (*Nepeta cataria*) are an efficient insect repellent and are at least comparable to repelling insects to the industry standard repellent DEET with far less toxicity[29,30,31,32,33]. Other tests showed that *Nepeta cataria* (catnip), has had its essential oils containing various nepetalactone stereoisomers tested and the results showed that it is comparable to DEET at repelling mosquitoes, while offering better spatial repellency[33,34,35]. Catnips volatile oil effectively repels mosquitoes, including the females that carry the plasmodium that causes malaria and those that transmit yellow fever, filariasis, the West Nile virus and encephalitis for a total of six different mosquito species repelled[33,24,36,37]. In one study, 41 different plant species were tested for repellency toward three species of mosquitos that carry pathogens and *N. cataria* was one of the top five

plants whose oil exhibited repellency[24]. Like many genera of the Lamiaceae plant family, the *Nepeta* genus contains species that produce a wide array of non-volatile and volatile compounds in their essential oil with nepetalactones, β-caryophyllene, nerol, citronellol and geraniol present[38,39,40]. Insects can often discriminate different isomers, perceiving them differently from one another and the stereochemistry of an insect repellent compound can alter its efficacy as a repellent[41,42]. Members of the *Nepeta* genus produce many different stereoisomers of nepetalactone wit in their essential oils[43, 44].

Various formulations were produced by modifying the bioactive ingredient to *N. cataria* 'CR9' and *N. cataria* 'CR3' essential oils from previously published insect repellent formulations[3]. These formulations consisted of varying degrees of polyethylene glycols, a gel with carbomer and stick made with beeswax. Preliminary data using the newly developed *N. cataria* 'CR9' cultivar to investigate the duration of acceptable repellency of the *N. cataria* 'CR9' crude essential oil treatment was compared to various *N. cataria* 'CR9' essential oil based formulations over four hours against *A. aegypti* mosquitoes. The best performing *N. cataria* 'CR9' formulations will then compared to the same *N. cataria* 'CR3' formulations to investigate nepetalactone stereoisomer differences in repellency over 8 hours.

6.2 Materials and Methods

6.2.1 Insects and Chemical Reagents

The *A. aegypti* eggs were sourced Benzon Research (Carlisle, PA). Glycerin, acetone, isopropanol, 200MW polyethylene glycol, 1000MW polyethylene glycol, 10000MW polyethylene glycol, butylated hydroxytoluene, molecular grade water and carbomer 940

were purchased from Fisher Scientific (Hampton, NH). DEET was procured from Sigma Aldrich (St. Louis, MO). Stearyl alcohol was obtained from VWR (Radnor, PA). Beeswax was obtained from Lavender Lane Inc. (Rome City, IN.)

6.2.2 *Nepeta cataria* Cultivation and Essential Oil Extraction

The clonal *N. cataria* 'CR9' and 'CR3' populations used in were initially grown at the Rutgers University Research Greenhouses. Seeds were planted from the selfed cultivars of 'CR9' and 'CR3' and upon germination were watered as needed. One month later after the plants were established, they were transplanted into single rows on raised beds into black plastic mulch with drip irrigation at the Rutgers University Clifford E. & Melda C. Snyder Research and Extension Farm in Pittstown, NJ. Once the plants were in full flower, the two populations were harvested separately and dried at 37^{o}C until no further moisture loss using an onsite Powell walk-in forced air heat dryer. Essential oils were extracted by the hydrodistillation of the above ground biomass of the plant using a Clevenger-type distillation unit with 60g of dried plant matter. GC/MS analysis was conducted on the essential oil samples and the results were consistent with what was expected from each cultivar ('CR9' and 'CR3').

6.2.3 Liquid Spray Formulation Development

Pump sprays are the most common form of applying insect repellents as they can easily be carried, applied and are stable outside. Three *N. cataria* essential oil spray formulations **(Figure 6-1, page 140)** were developed to increase the duration of acceptable arthropod repellency. The formulations developed the same except they varied in the molecular weight of the polyethylene glycols in them. Each spray consisted of, by weight, 69.95% isopropanol, 15% *N. cataria* essential oil, 15% polyethylene glycol (200MW, 1000MW,

10000MW) and 0.05% butylated hydroxy toluene. The sprays were made by adding the butylated hydroxy toluene to isopropanol to act as a solvent. The polyethylene glycol was then added to the solution followed by the catnip essential oil. The solution was stirred on a magnetic plate spinner with a magnetic stir rod throughout development. After development, the higher molecular weight spray formulations (1000MW and 10000MW) solidified at room temperature and were no longer in their liquid form.

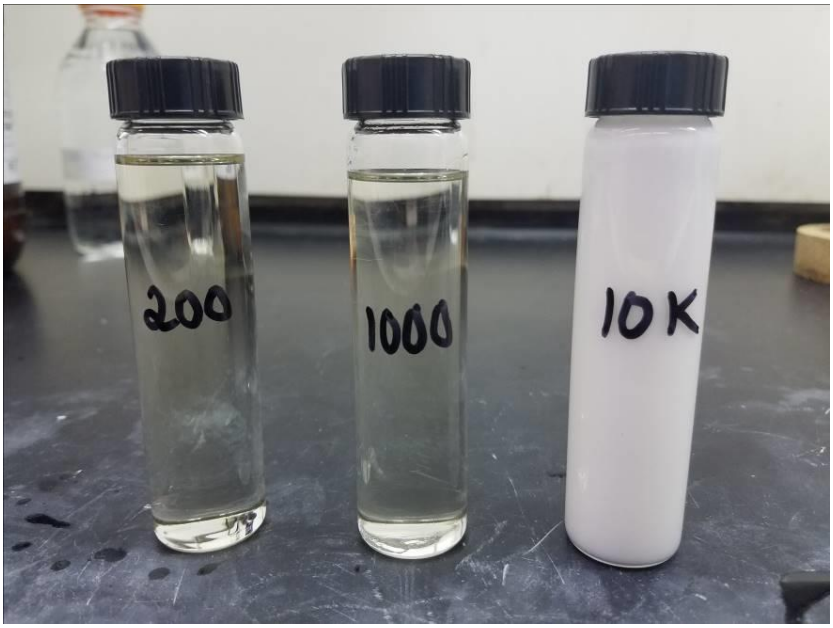

Figure 6-1: Three insect repellent spray formulations with *N. cataria* essential oil as the bioactive ingredient that differ in the molecular weight of the polyethylene glycol added.

6.2.4 Gel Formulation Development

Another commonly found method of applying insect repellents are gel formulations that suspend the active ingredient in a water solution. The gel formulation **(Figure 6-2, page**

141) was developed to increase the duration of acceptable arthropod repellency. The gel formulation consisted of, by weight, 40% water, 45% isopropanol, 13% *N. cataria* essential oil, and 2% carbomer 940. The formulation was made by adding *N. cataria* essential oil to the isopropanol followed by water. Immediately after the addition of the carbomer, the gel solution was homogenized with a bench top homogenizer at 25,000 rpm.

Figure 6-2: Gel insect repellent formulation with *N. cataria* essential oil as the bioactive ingredient.

6.2.5 Stick Formulation Development

The last formulation created to extend the duration of acceptable arthropod repellency is a stick formulation **(Figure 6-3, page 142)**. The stick formulation was made with 20% catnip oil, 55% isopropanol, 4% glycerin, 20% beeswax and 1% stearyl alcohol. Beeswax was melted on a heated, magnetic stir plate with a magnetic stir rod mixing the wax. The glycerin and stearyl alcohol were dissolved in the isopropanol then added to the melted

beeswax. The solution was allowed to cool and prior to solidigifying the *N. cataria* essential oil was added to the mixture.

Figure 6-3: Stick insect repellent formulation with *N. cataria* essential oil as the bioactive ingredient.

6.2.6 *A. aegypti* Rearing and Testing

Aedes aegypti eggs were hatched in water at 27ºC with 80% humidity under a 12-hour day/night light cycle and were transferred during the rearing process with an eye dropper. General fish food tablets were used as the energy source for the maturing mosquitoes. The *A. aegypti* eggs were placed in a container holding water and once hatched and formed into larvae, they were separated from the unhatched eggs and placed into fresh water. As the mosquitoes began to form into pupae, they were separated from the smaller less developed larvae and placed into fresh water. This container was then placed into a rearing cage where the pupae were allowed to mature into adults. Mature females were visually identified, then

separated out of the population by aspirating them into a separate rearing cage where they were given a 10% sucrose solution as an energy source. Mature females were kept at these conditions until experimentation.

Repellency was determined by a one-choice landing assay that uses the amount of mosquito landings to calculate the overall effectiveness of a candidate repellent when compared to a control. This assay evaluated how long the essential oils can be an effective repellent. During an 8hr period, the efficacy of the different formulations monitored at 0hr, 1hr, 2hr, 4hr, 8hr intervals. Twenty, adult female *A. aegypti* mosquitoes were aspirated into a rearing cage and starved for 1 day. Testing was performed between 10:00 am and 4:00 pm PST during *A. aegypti's* active hours. A 37^{o}C heat pack was used to as the heat source to attract the mosquitoes in the upper region of the back panel of the rearing cage. 5g of the different treatments were applied to the filter paper used to wrap the heat source. A control of non-treated filter paper before and after each treatment to ensure reproducibility in mosquito behavior. Six repetitions were performed with twenty mosquitoes for each treatment and at each concentration along with an acetone control. Time-lapse photography recorded one image every five seconds for five minutes at each time interval where mosquitoes were manually counted after.

6.3 Results

6.3.1 Preliminary Testing

Early testing of the formulations was carried out using only 'CR9' essential oil to validate the formulas, assay and feasibility of using the different formulations on the filter paper. A 10% *N. cataria* 'CR9' crude essential oil (extracted in acetone) and five formulations, three

spray formulations (200MW, 1000MW and 10000MW polyethylene glycols), the gel formulations and the stick formulation were tested for repellency efficacy over 4hrs against *A. aegypti* mosquitoes **(Figure 6-4, page 144; Table 6-1, page 145)**.

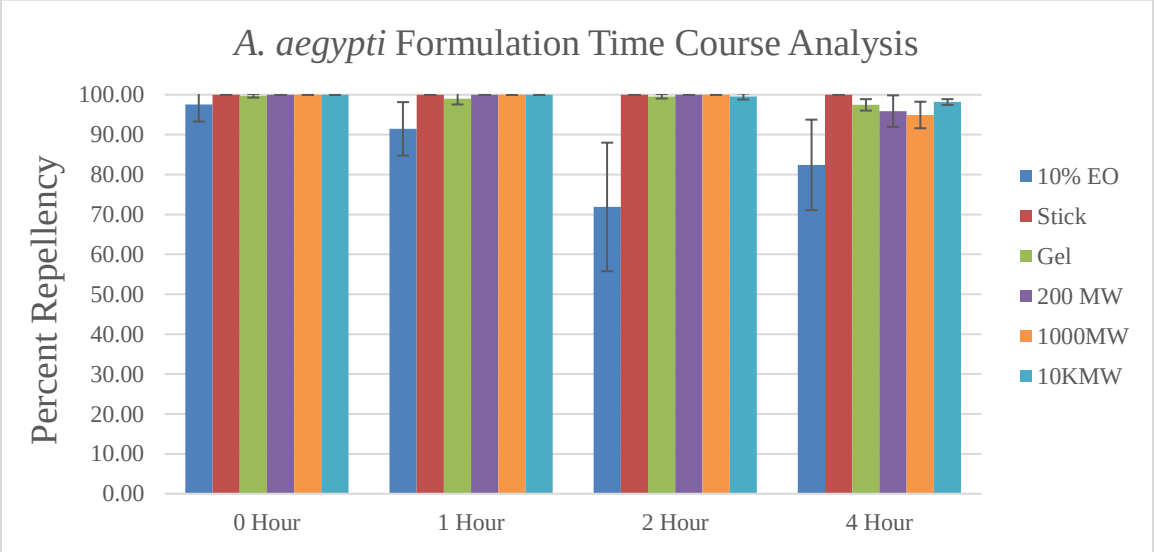

Figure 6-4: Bar graph showing the preliminary assessment of *N. cataria* 'CR9' essential oil and 'CR9' essential oil based formulations against *A. aegypti* mosquitoes over four hours.

	Time Intervals for Preliminary CR9 Formulation Data			
Treatment	0 hour	1 hour	2 hour	4 hour
10% 'CR9'	97.54 ± 4.26 A	91.44 ± 6.70 A	71.87 ± 16.11 C	82.41 ± 11.34 B
Stick	100.00 ± 0.00 A	100.00 ± 0.00 A	100.00 ± 0.00 A	100.00 ± 0.00 A
Gel	99.73 ± 0.45 A	99.00 ± 1.44 A	99.60 ± 0.56 A	97.46 ± 1.43 A
200 MW	100.00 ± 0.00 A	100.00 ± 0.00 A	100.00 ± 0.00 A	95.89 ± 3.95 A
1000MW	100.00 ± 0.00 A	100.00 ± 0.00 A	100.00 ± 0.00 A	94.92 ± 3.33 A
10KMW	100.00 ± 0.00 A	100.00 ± 0.00 A	99.56 ± 0.76 A	98.17 ± 0.73 A

Table 6-1: Repellency values for the preliminary data of the *N. cataria* 'CR9' essential oil and 'CR9' essential oil based insect repellent formulations against *A. aegypti* mosquitoes over four hours. Values show ± standard deviations where different letters after the treatments are significantly different according to Duncan's test at P ≤ 0.05.

The 10% 'CR9' crude essential oil treatment repelled 97.54% ± 4.26, 91.44% ± 6.70, 71.87% ± 16.11 and 82.41% ± 11.34 at 0 hour, 1 hour, 2 hour and 4 hour respectively against *A. aegypti* mosquitoes. The stick formulation treatment repelled 100.00% ± 0.00, 100.00% ± 0.00, 100.00% ± 0.00 and 100.00 ± 0.00 at 0 hour, 1 hour, 2 hour and 4 hour respectively against *A. aegypti* mosquitoes. The gel formulation treatment repelled 99.73 ± 0.45, 99.00 ± 1.44, 99.60 ± 0.56 and 97.46 ± 1.43 at 0 hour, 1 hour, 2 hour and 4 hour respectively against *A. aegypti* mosquitoes. The 200MW polyethylene glycol formulation treatment repelled 100.00 ± 0.00, 100.00 ± 0.00, 100.00 ± 0.00 and 95.89 ± 3.95 at 0 hour, 1 hour, 2 hour and 4 hour respectively against *A. aegypti* mosquitoes. The 1000MW polyethylene glycol formulation treatment repelled 100.00 ± 0.00, 100.00 ± 0.00, 100.00 ± 0.00 and 94.92 ± 3.33 at 0 hour, 1 hour, 2 hour and 4 hour respectively against *A. aegypti* mosquitoes. The 10000MW polyethylene glycol formulation treatment repelled 100.00 ± 0.00, 100.00 ± 0.00, 99.56 ± 0.76 and 98.17 ± 0.73 at 0 hour, 1 hour, 2 hour and 4 hour respectively against *A. aegypti* mosquitoes. The non-formulated crude *N. cataria* 'CR9' essential oil treatment decreased in repellency over the four hours tested.

The preliminary data shows that all of the formulations increased the duration of acceptable repellency significantly from the crude *N. cataria 'CR9'* essential oil formulation and were significantly similar to one another. All formulations except for the 1000MW polyethylene

glycol formulation exhibited complete repellency defined by reducing ≥95% of the mosquito landings. Only the stick formulation did not allow a single mosquito to land on the heat pack and maintained 100.00% ± 0.00 repellency throughout the four hours. Due to the stick and gel formulation performing the best and that the 10000MW spray formulation solidified at room temperature, the stick, gel and 200MW formulations will be further evaluated for repellency efficacy in which *N. cataria* 'CR9' essential oil based formulations will be compared to *N. cataria* 'CR3' formulation to assess any stereochemical differences in repellency.

6.3.2 *Nepeta cataria* 'CR9' and 'CR3' Best Performing Formulation Comparison

The three top performing formulations in terms of duration in acceptable repellency efficacy, had *N. cataria* 'CR3' and 'CR9' essential oil were next compared to one another to asses if stereochemical differences between nepetalactone isomers within their respective essential oil have differences in repellency efficacy. The three formulations tested included the 200MW polyethylene glycol, the gel and the stick formulation. While the 200MW polyethylene glycol formulation did not perform as well as the 10000 polyethylene glycol formulation, it was selected as the 10000MW formulation solidified at room temperature and was no longer a liquid spray. The three formulations were tested against *A. aegypti* mosquitoes over 8 hours.

6.3.2.1 *Nepeta cataria* 'CR9' and 'CR3' 200MW Spray Formulation Comparison

The *N. cataria* 'CR3' essential oil based 200MW spray formulation reduced *A. aegypti* mosquito landings by 100.00 ± 0.00, 100.00 ± 0.00, 99.88 ± 0.12, 85.68 ± 8.98 and 87.61 ± 3.97 at 0hr, 1hr, 2hr, 4hr and 8hr respectively **(Figure 6-5, page 147; Table 6-2, page 147)**. The *N. cataria* 'CR9' essential oil based 200MW spray formulation reduced *A.*

aegypti mosquito landings by 100.00 ± 0.00, 100.00 ± 0.00, 99.93 ± 0.12, 97.08 ± 2.25 and 89.28 ± 12.21 at 0hr, 1hr, 2hr, 4hr and 8hr respectively.

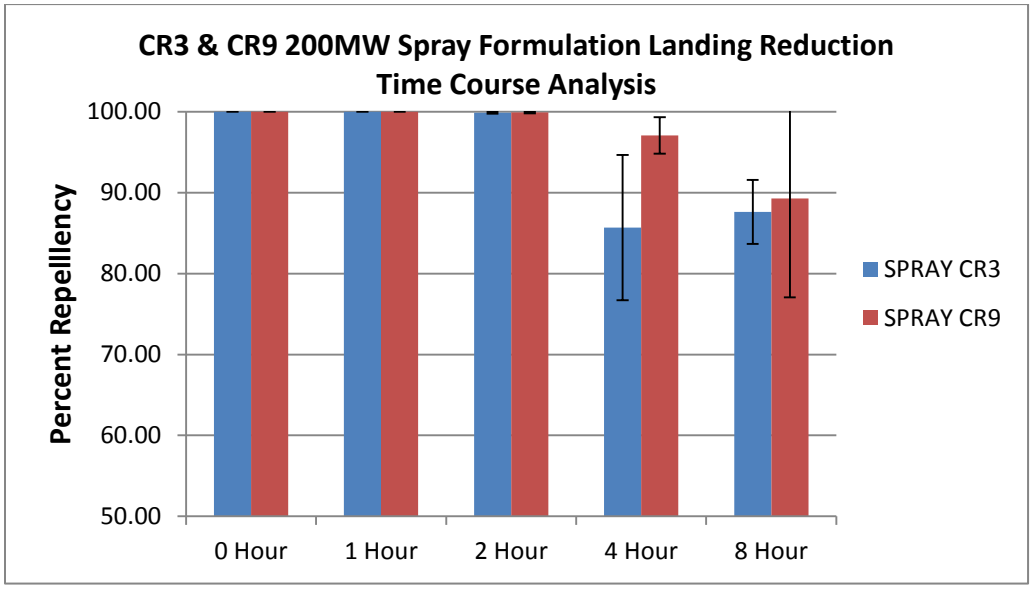

Figure 6-5: Comparison of *N. cataria* 'CR3' and 'CR9' essential oil based 200MW insect repellent formulation over 8 hours.

	Time Intervals for 200MW CR9, CR3 Formulations				
Treatment	0 Hour	1 Hour	2 Hour	4 Hour	8 Hour
CR3	100.00 ± 0.00 A	100.00 ± 0.00 A	99.88 ± 0.12 A	85.68 ± 8.98 C	87.61 ± 3.97 C
CR9	100.00 ± 0.00 A	100.00 ± 0.00 A	99.93 ± 0.12 A	97.08 ± 2.25 AB	89.28 ± 12.21 BC

Table 6-2: Repellency values for *N. cataria* 'CR3' and 'CR9' essential oil based 200MW insect repellent formulation over 8 hours. Values show ± standard deviations

where different letters after the treatments are significantly different according to Duncan's test at P ≤ 0.05.

In reducing the amount of mosquito landings on the heat pack, both formulations were significantly similar in repellency up to two hours. After two hours, the *N. cataria* 'CR9' essential oil 200MW formulation reduced mosquito landings significantly better than the *N. cataria* 'CR3' essential oil formulation. Between 4 and 8 hours, the *N. cataria* 'CR9' essential oil formulation lost the ability to exhibit complete repellency and did not reduce ≥95% of the mosquito landings. Between 2 and 4 hours, the *N. cataria* 'CR3' essential oil formulation lost the ability to exhibit complete repellency and did not reduce ≥95% of the mosquito landings.

6.3.2.2 *Nepeta cataria* 'CR9' and 'CR3' Gel Formulation Comparison

The *N. cataria* 'CR3' essential oil based gel formulation reduced *A. aegypti* mosquito landings by 100.00 ± 0.00, 100.00 ± 0.00, 99.65 ± 0.36, 99.48 ± 0.9 and 99.82 ± 0.17 at 0hr, 1hr, 2hr, 4hr and 8hr respectively **(Figure 6-6, page 149; Table 6-3, page 149)**. The *N. cataria* 'CR9' essential oil based gel formulation reduced *A. aegypti* mosquito landings by 100.00 ± 0.00, 100.00 ± 0.00, 97.45 ± 0.45, 98.35 ± 1.06 and 96.34 ± 1.48 at 0hr, 1hr, 2hr, 4hr and 8hr respectively.

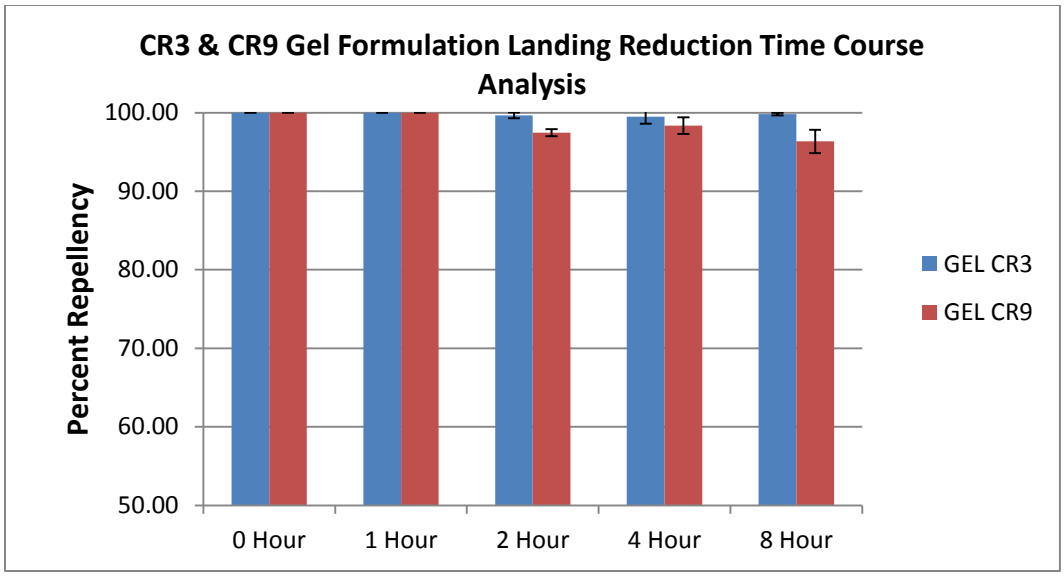

Figure 6-6: Comparison of *N. cataria* 'CR3' and 'CR9' essential oil based gel insect repellent formulation over 8 hours.

	Time Intervals				
Treatment	0 Hour	1 Hour	2 Hour	4 Hour	8 Hour
CR3	100.00 ± 0.00 A	100.00 ± 0.00 A	99.65 ± 0.36 A	99.48 ± 0.9AB	99.82 ± 0.17 A
CR9	100.00 ± 0.00 A	100.00 ± 0.00 A	97.45 ± 0.45 CD	98.35 ± 1.06 BC	96.34 ± 1.48 D

Table 6-3:Repellency values for *N. cataria* 'CR3' and 'CR9' essential oil based gel insect repellent formulation over 8 hours.

In reducing the amount of mosquito landings on the heat pack, both formulations were significantly similar in repellency up to one hour. After one hour, the *N. cataria* 'CR3' essential oil 200MW formulation reduced mosquito landings significantly better than the *N. cataria* 'CR9' essential oil formulation. Throughout the 8 hours, both formulations reduced ≥95% of the mosquito landings.

6.3.2.3 *Nepeta cataria* 'CR9' and 'CR3' Stick Formulation Comparison

The *N. cataria* 'CR3' essential oil based stick formulation reduced *A. aegypti* mosquito landings by 100.00 ± 0.00, 100.00 ± 0.00, 100.00 ± 0.00, 100.00 ± 0.00 and 100.00 ± 0.00 at 0hr, 1hr, 2hr, 4hr and 8hr respectively **(Figure 6-7, page 150; Table 6-4, page 150)**. The *N. cataria* 'CR9' essential oil based stick formulation reduced *A. aegypti* mosquito landings by 100.00 ± 0.00, 100.00 ± 0.00, 100.00 ± 0.00, 100.00 ± 0.00 and 99.92 ± 1.48 at 0hr, 1hr, 2hr, 4hr and 8hr respectively.

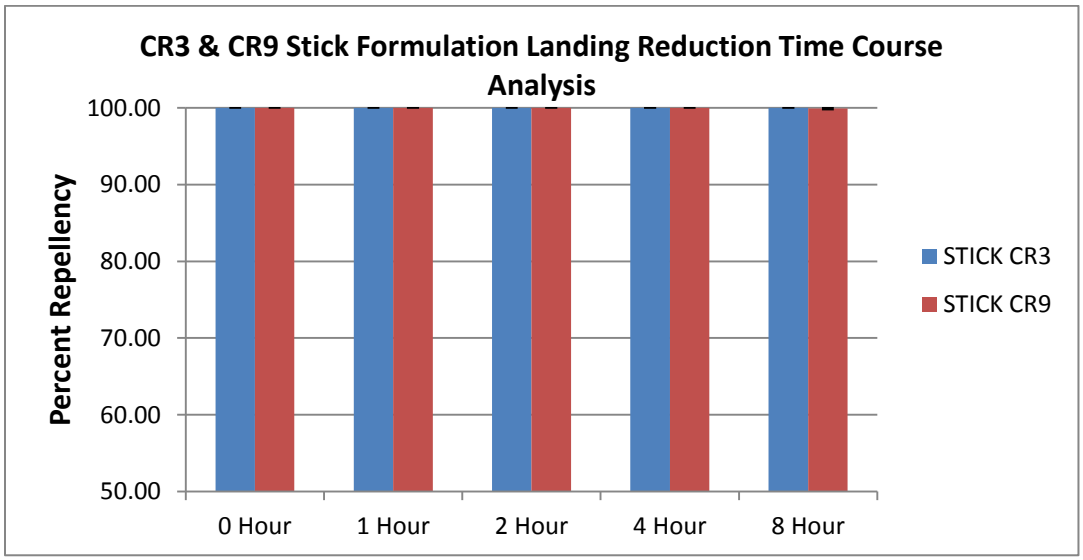

Figure 6-7: Comparison of *N. cataria* 'CR3' and 'CR9' essential oil based stick insect repellent formulation over 8 hours.

	Time Intervals				
Treatment	0 Hour	1 Hour	2 Hour	4 Hour	8 Hour
CR3	100.00 ± 0.00 A	100.00 ± 0.00 A	100.00 ± 0.00 A	100.00 ± 0.00 A	100.00 ± 0.00 A
CR9	100.00 ± 0.00 A	100.00 ± 0.00 A	100.00 ± 0.00 A	100.00 ± 0.00 A	99.92 ± 1.48 A

Table 6-4: Repellency values for *N. cataria* 'CR3' and 'CR9' essential oil based stick insect repellent formulation over 8 hours.

In reducing the amount of mosquito landings on the heat pack, both formulations were significantly similar for the 8 hour duration. Throughout the 8 hours, both formulations reduced ≥95% of the mosquito landings and with the exception of the *N. cataria* 'CR9' essential oil formulation at 8 hours, did not allow a single mosquito to land on the heat packs.

6.4 Discussion

These results show that *N. cataria* 'CR9' and *N. cataria* 'CR3' essential oils can be incorporated into various formulations to control the release of the nepetalactones responsible for repelling *A. aegypti* mosquitoes. With the exception of the 200MW formulation at 4 hours, there were no observable differences in nepetalactone isomers at reducing ≥95% of mosquito landings however, both 200MW essential oil formulations lost complete repellency between 4 and 8 hours. The stick and gel formulations exhibited complete repellency (reducing ≥95% of mosquito landings) up to 8 hours. The stick formulation made with 20% *N. cataria* 'CR3' essential oil, 55% isopropanol, 4% glycerin, 20% beeswax and 1% stearyl alcohol was the best performing formulation as it did not allow a single mosquito to land on the attractant for 8 hours. The 200MW and 1000MW spray formulations should be investigated in altering the ratio of inactive and active ingredients to increase the duration of repellency so that less mosquitoes land on the attractant. Overall, due to the success of the gel and stick formulations, each should be considered for human and animal use as *A. aegypti* insect repellents pending toxicological investigations.

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Chapter 7 Repellency efficacy *Nepeta cataria* formulations Against *Dermacentor variabilis* and *Ixodes scapularis* Ticks

While mosquitoes vector some of the deadliest diseases on the planet, leading to over a million human deaths annually throughout the globe by infecting people with malaria and yellow fever, other insects vector diseases as well that effect the overall public health of individuals[1]. Other examples of arthropods that vector diseases include the *Triamone* species that vector the *Trypanosoma cruzi* (Chagas) disease, a systemic parasitic infection that causes cardiomyopathy and digestive ailments vectored by *Triatominae* species[2]. Ticks and the diseases they vector cover the globe with numerous types of diseases they vector causing serious health concerns[3]. Livestock, pets and humans are all susceptible to tick borne diseases that can cause fevers, skin lesions, muscle cramping, inflammation of the brain, weakness and other flue like symptoms[4]. Individuals in rural farming communities throughout the globe are at a higher risk of contracting a tick borne disease due to the lack of management and control measures to combat them[5]. Due to the previous research that has been conducted on ticks utilizing *N. cataria* essential oil as a repellent, we created a dose response curve using *N. cataria* 'CR3' essential oil and DEET against two species of ticks, *Ixodes scapularis* and *Dermacentor variabilis*. We also conducted a two choice assay comparing DEET and *N. cataria* 'CR3' essential oil to determine which repellent is more effective to the ticks given two options. Finally, we evaluated our best performing *A. aegypti* formulation (stick formulation) for the duration of acceptable repellency over 8 hours. The results gained throughout this investigation will aid in the understanding of *N. cataria* essential oil as an insect repellent and assist in further

formulation development by identifying minimum concentrations needs for complete repellency.

7.1 Introduction

There are many types of disease vectoring insects around the world that cause medical illnesses and death to millions of people annually with Yellow Fever being transmitted by *Aedes aegypti* mosquitoes, malaria transmitted by the *Anopheles gambiae* mosquito, while other diseases such as Lyme disease spread by *Ixodes scapularis* ticks are not deadly, but has a serious negative health impact on those who contract it[1,6]. Not only can mortality result after contracting these diseases, but running a fever, skin lesions, muscle cramping, inflammation of the brain, weakness and other flu like symptoms are all common ailments associated with the diseases vectored by insects[1,4]. Tick species that can cause the previously mentioned medical ailments cover the United States and can vector additional diseases such as Relapsing Fever, Rocky Mountain Spotted Fever and Tick Paralysis[7]. These tick species can be invasive as well, *Haemaphysalis longicorni* can cause ecological concerns to native wildlife by infecting them with new deadly diseases[8].

While integrated pest management techniques such as removing still water to limit mosquito hatching and emergence to removing brush while constantly mowing your lawn is critical to reduce the chance of contracting diseases, insecticides such as DDT (1,1'-(2,2,2-Trichloroethane-1,1-diyl)bis(4-chlorobenzene)) have been very effective at eradicating the presence of these vectors though their use has been curtailed as evidence shows larger negative ecological impacts[9,10,11,12]. Insect repellents such as DEET (N, N-Diethyl-3-methylbenzamide), developed by government institutions, have been an

incredibly effective proactive approach at reducing the chance of transmission of deadly diseases from vectors around the world[13,14]. Picaridin developed by the Bayer chemical company utilizing molecular modeling to repel mosquitoes as an alternative to DEET, has since been integrated into the armed forces[15].

While insect repellents have been very effective, many people that live in rural locations around the globe have not been educated on vector control measures and do not have access to effective insect repellents are more susceptible to contracting an illness by disease vectoring mosquitoes[5,16]. Regions such as China, India and sub-Saharan Africa still use natural products as a primary source of plant-based repellents[17]. Plants known locally and regionally to repel insects are collected and cultivated in China, India and sub-Saharan Africa to repel disease-causing insects and ethnobotanical efforts have identified plants that can be used to repel insects [17,18,19]. In 2005, the CDC endorsed para-Menthane-3, 8-diol (PMD), a steam distillate product from the leaves of the Australian lemon-scented gum tree as a mosquito repellent[20]. Volatile essential oils identified as insect repellents have been shown to be as efficient as DEET at repelling disease vectoring insects for up to an hour, however these volatiles in their non-encapsulated forms can only supplement the current use of DEET due to their limited duration of acceptable repellency[21,22,23].

While known for the euphoric effect the essential oil has on felines, the essential oils contained in various *Nepeta* species have long been recognized for their insect repellent properties with efficacy similar to DEET[24,25,26]. In one study, 41 different plant species were tested for repellency toward three species of mosquitos that carry pathogens and *N. cataria* was one of the top five plants whose oil exhibited repellency[22]. Numerous species of disease vectoring mosquitoes, ticks and cockroaches have been repelled by the essential

oils in *Nepeta* species[26,27,28]. Common brown ticks and the deer tick that harbor the bacterium responsible for the Rocky Mountain Spotted Fever and Lyme disease respectively are repelled by the nepetalactones in *N. cataria* as well as the dihydronepetalactones[26,28].

Due to the need and demand for naturally produced insect repellents and since *Nepeta* species have previously shown promising activity towards repelling various species of ticks, an investigation was conducted to generate a dose response curve with *N. cataria* 'CR3' essential oil on *Dermacentor variabilis* and *Ixodes scapularis* ticks to identify concentrations of the essential oil that achieve complete repellency (repelling ≥95% of ticks). Once a concentration that exhibits complete repellency is determined, a two choice assay will also be conducted with *Dermacentor variabilis* and *Ixodes scapularis* ticks comparing the repellency of the concentration of *N. cataria* 'CR3' essential oil that exhibited complete repellency to the same concentration of DEET to determine which repellent is more effective at repelling ticks. Finally, a time course analysis comparing a *N. cataria* 'CR3' essential oil beeswax based formulation will be compared to the same DEET formulation over 8 hours to determine the duration of acceptable repellency. The results generated will inform individuals of the minimum concentration required of *N. cataria* 'CR3' essential oil to remove all ticks from an area. Also, this information will inform individuals how frequent they need to apply the beeswax formulation in order to remove all the ticks from an area.

7.2 Materials and Methods

7.2.1 Insects and Chemical Reagents

Adult, female *Ixodes scapularis* and *Dermacentor variabilis* ticks were sourced from Oklahoma State University (Stillwater, OK). Glycerin, isopropanol and acetone were purchased from Fisher Scientific (Hampton, NH). DEET was procured from Sigma Aldrich (St. Louis, MO). Stearyl alcohol was obtained from VWR (Radnor, PA). Beeswax was obtained from Lavender Lane Inc. (Rome City, IN)

7.2.2 *Nepeta cataria* Cultivation and Essential Oil Procurement

The clonal *N. cataria* 'CR3' population used was initially grown at the Rutgers University Research Greenhouses. Seeds were planted from the selfed cultivar 'CR3' and upon germination were watered as needed. One month later after the plants were established, they were transplanted into single rows on raised beds into black plastic mulch with drip irrigation at the Rutgers University Clifford E. & Melda C. Snyder Research and Extension Farm in Pittstown, NJ. Once the plants were in full flower, the population was harvested and dried at 37ºC until no further moisture loss was observed at this temperature and pressure using an onsite Powell walk-in forced air heat dryer. Essential oils were extracted by the hydrodistillation of the above ground biomass of the plant using a Clevenger-type distillation unit with 60g of dried plant matter. GC/MS analysis was conducted on the essential oil samples and the results were consistent with what was expected from the *N. cataria* 'CR3' cultivar.

7.2.3 Repellent Stick Formulation Development

The stick formulation was created to extend the duration of acceptable arthropod repellency. The stick formulation was made with 20% *N. cataria* 'CR3' essential oil, 55%

isopropanol, 4% glycerin, 20% beeswax and 1% stearyl alcohol. Beeswax was melted on a heated, magnetic stir plate with a magnetic stir rod mixing the wax. The glycerin and stearyl alcohol were dissolved in the isopropanol then added to the melted beeswax. The solution was allowed to cool and prior to solidigifying the *N. cataria* essential oil was added to the mixture. The DEET stick formulation was created the same way and was subsititued for *N. cataria* 'CR3' essential oil as the bioactive ingredient.

7.2.4 *Ixodes scapularis* Testing

Three different tests will be conducted to evaluate *N. cataria* essential oil as a repellent against two species of ticks. The first assay will be generating a one-choice dose response curve to determine the concentration of *N. cataria* 'CR3' essential oil required to achieve complete repellency against *D. variabilis* and *I. scapularis* ticks. The second test is a two-choice test in which the candidate repellent will be tested against DEET to determine which repellent is better a reducing tick positions. The third test used will evaluate a *N. cataria* 'CR3' essential oil based stick repellent to a DEET stick repellent formulation to evaluate how long the *N. cataria* 'CR3' essential oil based stick repellent maintains acceptable repellency efficacy.

7.2.4.1 Dose Response Curve

Crude *N. cataria* 'CR3' essential oil was used to develop a dose response curve to identify dose dependent activity of a candidate repellent against two species of ticks. An arena was constructed out of petri dishes to test the efficacy of a repellent on filter paper by monitoring the total amount of tick positions over 15 minutes (1 image every 15 sec) and calculating percent repellency. In the one-choice assay, the repellent is applied (extracted with acetone to desired concentrations) to half of the filter paper and placed into the petri

dish where the ticks are placed, the petri dish cover put back on and the number of ticks counted applied to the repellent zone.

Total Tick positions = (# ticks in petri dish) X (60 images)

Percent repellency = 1- [(# of ticks in the repellent zone) / (Total Tick positions)] * 100

7.2.5 Two Choice Repellency Test

Crude *N. cataria* 'CR3' essential oil was compare to DEET to develop to analyze the two-choice activity of a candidate repellent against DEET with two species of ticks. An arena was constructed out of petri dishes to test the efficacy of a repellent on filter paper by monitoring the total amount of tick positions over 15 minutes (1 image every 15 sec) and calculating percent repellency. In the two-choice repellency test, DEET and a candidate repellent are applied to the filter paper on either side and the ticks are counted referencing the test repellent, not DEET. Three replicates were performed for each treatment.

Total Tick positions = (# ticks in petri dish) X (60 images)

Percent repellency of *N. cataria* essential oil = 1- [(# of ticks in the *N. cataria* repellent zone) / (Total Tick positions)] * 100

7.2.5.1 Time Course Analysis

A *N. cataria* 'CR3' insect repellent stick formulation will be tested against a DEET insect repellent stick formulation over 8 hours. The time course assay is performed the same as the generation dose response curve except images will be taken at the 0hr, 1hr, 2hr, 4hr and 8 hour for 5 minutes (1 image every 15 sec) in which ticks will be manually counted from the images.

Total Tick positions = (# ticks in petri dish) X (60 images)

Percent repellency = 1- [(# of ticks in the repellent zone) / (Total Tick positions)] * 100

7.3 Results

7.3.1 Dose Response Curve Results

For *D. variabilis* when DEET was applied at concentrations 0.10% and 1.00%, the percent repellency was 86.06 ± 10.2 and 99.31 ± 1.00 respectively **(Figure 7-1, page 162)**. When hydrodistilled *N. cataria* 'CR3' essential oil was applied at concentrations 0.10% and 1.00%, the percent repellency was 84.07 ± 12.47 and 100.00 ± 0.00 respectively. At 1.00% DEET and *N. cataria* 'CR3' achieved complete repellency and were not significantly different from one another. At 0.10% neither DEET nor *N. cataria* 'CR3' achieved complete repellency. At 0.10% DEET was not significantly different from either DEET or *N. cataria* 'CR3' at 1.00% and was not significantly different from *N. cataria* 'CR3' essential oil at 0.10%. *Nepeta cataria* 'CR3' essential oil at 0.10% was significantly different from DEET and *N. cataria* 'CR3' essential oil at 1.00%.

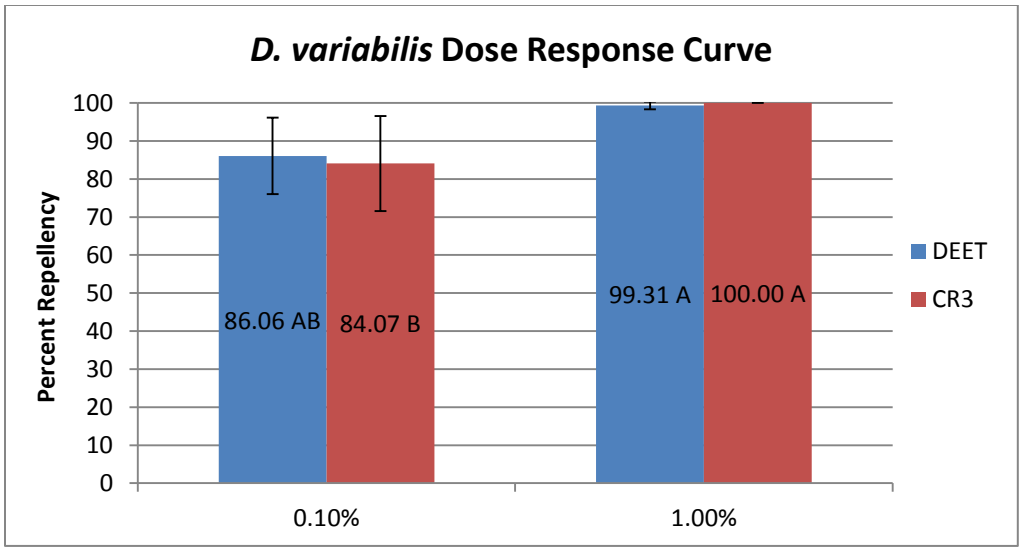

Figure 7-1: Dose response curve for *Dermacentor variabilis* with DEET and *N. cataria* 'CR3' essential oil.

For *I. scapularis* when DEET was applied at concentrations 0.10% and 1.00%, the percent repellency was 59.16 ± 8.94 and 99.79 ± 0.36 respectively **(Figure 7-2, page 163)**. In contrast, when hydrodistilled *N. cataria* 'CR3' essential oil was applied at concentrations of 0.10% and 1.00%, the percent repellency was 99.51 ± 0.43 and 99.79 ± 0.360 respectively. All of the treatments exhibited complete repellency and were not significantly different from one another exhibit when DEET was applied at the 1.00% concentration, yet the *N. cataria* 'CR3' would provide that same level of effective protection at the lower, 0.10% concentration whereas DEET did not repel the ticks sufficiently.

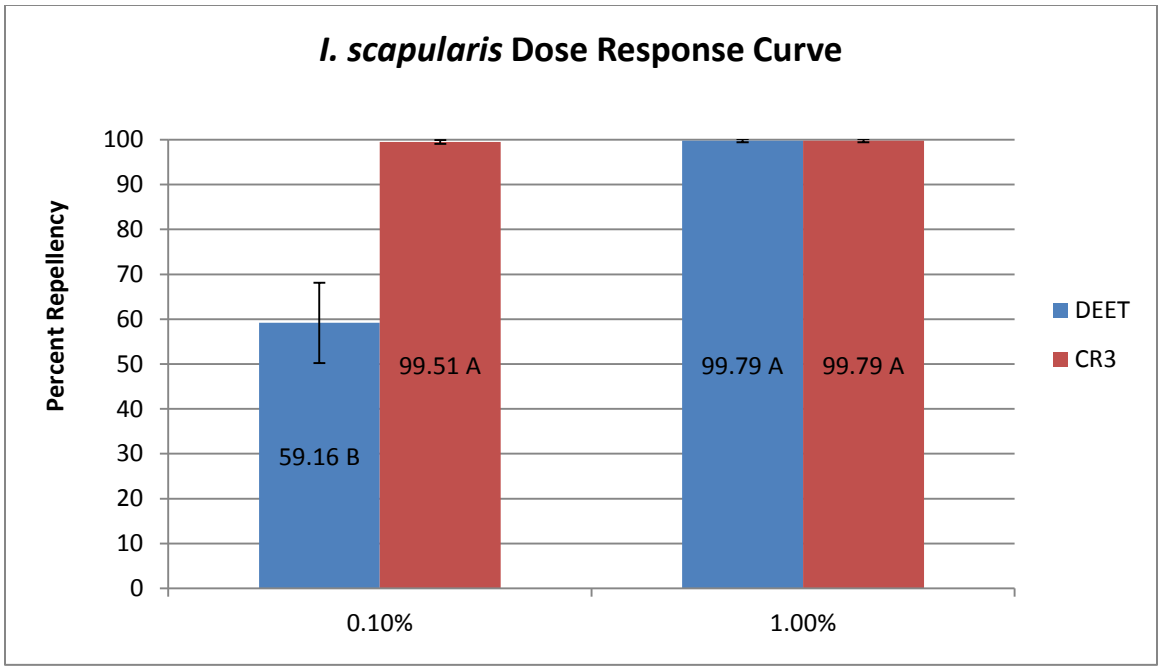

Figure 7-2: Dose response curve for *Ixodes scapularis* with DEET and *N. cataria* 'CR3' essential oil.

7.3.2 Two-Choice Repellency Testing Results

For the two choice assay **(Figure 7-3, page 164; Figure 7-4, page 164)**, *N. cataria* 'CR3' was able to repel both tick species better than DEET. For *D. variabilis* the CR3 essential oil reduced tick counts by 84.86% ±1.13 when compared to DEET. For *I. scapularis* the *N. cataria* 'CR3' essential oil reduced tick counts by 99.72% ± 0.32 when compared to DEET. The *N. cataria* 'CR3' essential oil treatments were significantly better than the DEET treatments at repelling the ticks in the two choice assay.

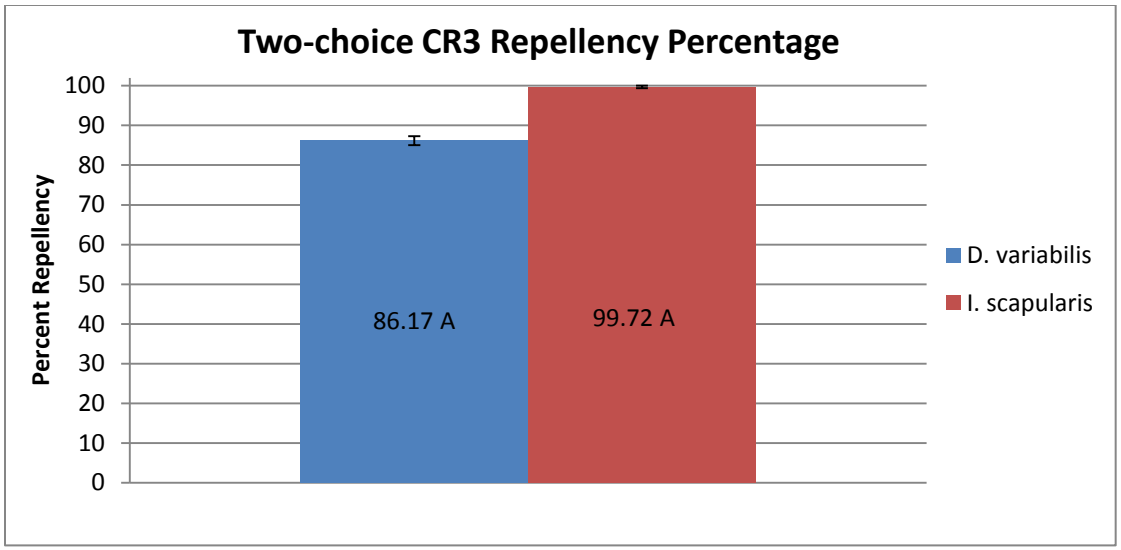

Figure 7-3: Two choice *N. cataria* 'CR3' repellency percentages compared to DEET against *Dermacentor variabilis* and *Ixodes scapularis* ticks. Values represent percentage of total tick positions on the DEET side of the filter paper.

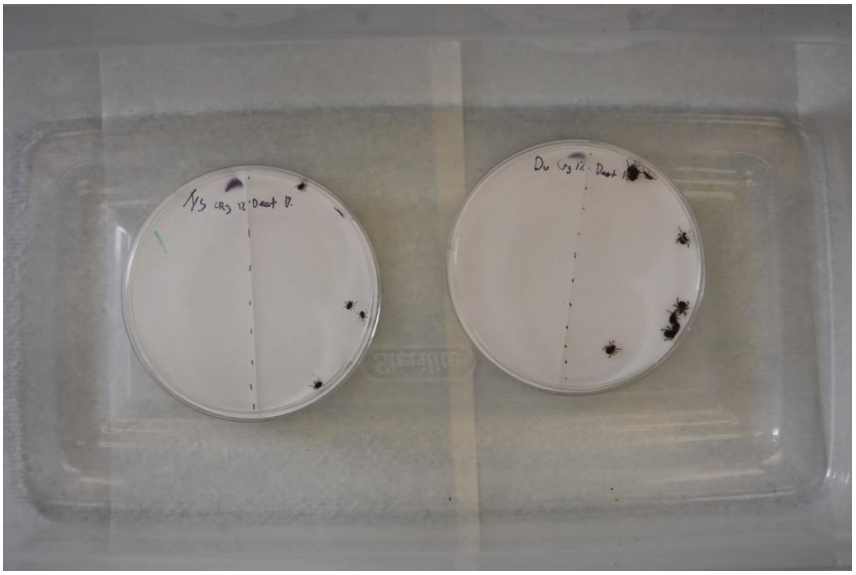

Figure 7-4: Image showing two-choice assay with both tick species with DEET and CR3 essential oil applied at 1.00%. *Ixodes scapularis* ticks are in the left petri dish, *Dermacentor variabilis* ticks are on the right side. *Nepeta cataria* 'CR3' essential oil is applied to each of the filter papers on the left side while DEET is on the right.

7.3.3 Time Course Analysis

Over the course of 8 hours **(Figure 7-5, page 165; Figure 7-6, page 165)**, the *N. cataria* 'CR3' essential oil based stick formulation repelled 100.00 ± 0.00, 99.86 ± 0.24, 99.92 ± 0.13, 99.76 ± 0.41 and 81.11 ± 17.83 at 0hr, 1hr, 2hr, 4hr and 8hr respectively. The DEET stick formulation repelled 99.96 ± 0.12, 100.00 ± 0.00, 100.00 ± 0.00, 99.16 ± 1.10 and 99.83 ± 0.14 at 0hr, 1hr, 2hr, 4hr and 8hr respectively.

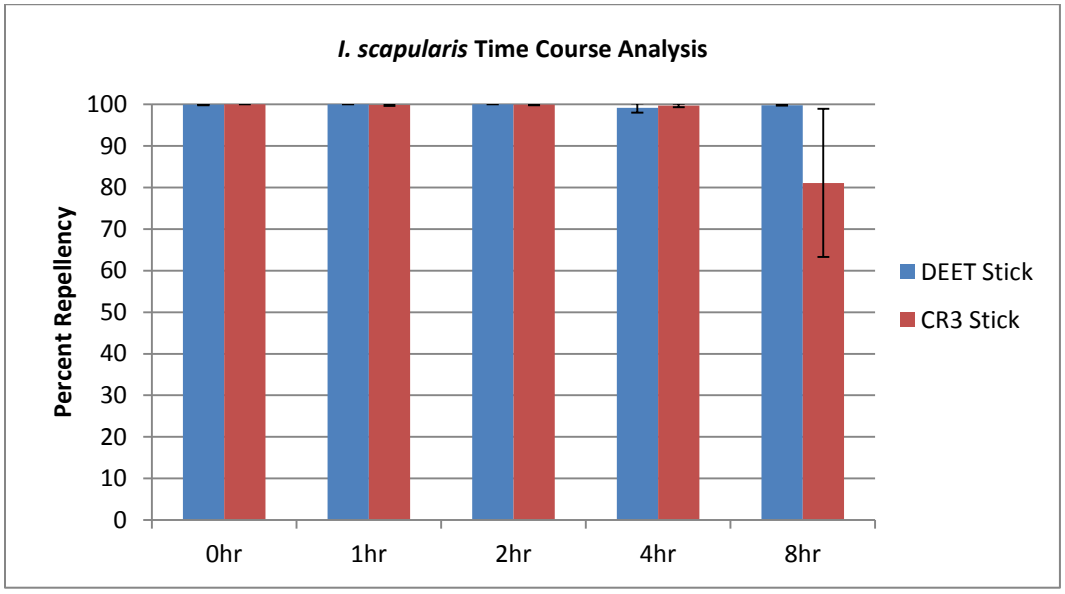

Figure 7-5: Time course analysis comparing stick formulations with bioactive ingredients of DEET and *N. cataria* 'CR3' essential oil over 8 hrs on *Ixodes scapularis* ticks.

	Time Intervals				
Treatment	0 Hour	1 Hour	2 Hour	4 Hour	8 Hour
CR3 Stick	100.00 ± 0.00 A	99.86 ± 0.24 A	99.92 ± 0.13 A	99.76 ± 0.41 A	81.11 ± 17.83 B
DEET Stick	99.96 ± 0.12 A	100.00 ± 0.00 A	100.00 ± 0.00 A	99.16 ± 1.10 A	99.83 ± 0.14 A

Figure 7-6: Repellency values for the time course analysis comparing stick formulations with bioactive ingredients of DEET and *N. cataria* 'CR3' essential oil over 8 hrs on *Ixodes scapularis* ticks.

Results show that the *N. cataria* ‘CR3’ essential oil stick formulation maintained complete repellency (repelling ≥ 95%) of each species of ticks for up to 4 hours and grouped statistically with DEET until then. Between 4 and 8 hours, the *N. cataria* ‘CR3’ essential oil stick formulation lost the ability to repel ≥ 95% of the ticks and DEET was significantly better at repelling them.

7.4 Discussion

Results of this study show that *N. cataria* ‘CR3’ essential oil that produces the Z, E and E, Z nepetalactone isomers showed significant dose response repellency against both the *Dermacentor variabilis* and *Ixodes scapularis* ticks when compared to DEET providing individuals who apply this essential oil to their clothing or skin protection from the disease vectoring insects. Individuals who are interested in finding a better repellent than DEET can use *N. cataria* ‘CR3’ essential oil since in the two choice assay when *N. cataria* ‘CR3’ essential oil was tested against DEET, both species of ticks spent a significant more amount of time on the DEET side of the filter paper signifying that they could tolerate the stress associated with that repellent more than they could of the *N. cataria* ‘CR3’ essential oil identifying it as the better repellent than DEET. Individuals that want to repel *I. scapularis* ticks for up to 4 hours can use the stick repellent as it offers complete protection (repelling ≥95% of ticks) for that amount of time and grouped statistically similar throughout that time. Efforts to increase the duration to match DEET’s efficacy at 8 hours could be done by further limiting the volatility of *N. cataria’s* ‘CR3’ essential oil by encapsulating the essential oil in various cyclodextrins or by changing the percentages of the inactive and active ingredients in the formulation. Future efforts on testing the stick formulation for

repellency in a human test should be done since the environment is different than that of the filter paper.

7.5 References

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Chapter 8 Skin Sensitization Evaluation of *Nepeta cataria* 'CR3' Stick Formulation

When evaluating candidate insect repellents, one must consider the toxicological implications that the new insect repellent will have on the end user. Insect repellents are made to deter disease vectoring insects by manipulating their biochemical pathways and the shared characteristics between the mode of actions between insects and humans could lead to negative health outcomes upon application by the user[1]. There is not a single mode of action for all repellents due to the structural differences of the repellents and the different ways disease vectoring insects sense their environments[2]. The public perception that natural product based insect repellents are safe due to their natural origins is fundamentally incorrect as the application of pure essential oils can induce an immune responses in humans causing hypersensitivity of the skin when applied to the epidermis and respiratory inflammation when inhaled and when natural products are sufficiently bioactive to induce pharmacological responses in human and animals, it can also lead to toxicities and adverse responses[3,4]. Essential oils have long been recognized as being irritants and having an essential oil based insect repellent that is applied to the skin and is unsafe to its user defeats the purpose of using it since it could cause an acute dermatological response[5]. In this chapter, we evaluated the most successful *N. cataria* insect repellent formulation (stick formulation) for skin sensitization capabilities. There is not a single assay to predict whether a compound or formulation is a skin sensitizer and according to the European Union Reference Laboratory for Alternative to Animal Testing (EURL-ECVAM) a compound must pass 2 out of 3 assays to be classified as a non-sensitizer. Here, the stick formulation was selected to be tested and shipped to an outside lab to test for skin

sensitization activity using the Direct Peptide Reactivity (DPRA), Human Cell Line Activation Test (h-CLAT) and the KeratinoSens assays that mimic the human response to allergens.

8.1 Introduction

Disease vectoring arthropods are estimated to kill over a million people per year by transmitting malaria, yellow fever and other diseases they are hosting to an individual after feeding[6]. Insect repellents created by public and private entities have become extremely effective at controlling the spread of diseases. DEET (N, N-Diethyl-3-methylbenzamide), originally developed by the United States Department of Agriculture as a pesticide and eventually sourced by the United States Army for infantry protection in areas that have a high incidence of diseases, has been paramount at repelling disease vectoring insects and is currently the most commonly used repellent[7]. Picaridin was developed by the Bayer chemical company utilizing molecular modeling to repel mosquitoes as an alternative to DEET and has since been integrated into the armed forces[8]. While insect repellents have become an effective means of repelling insects that vector diseases, concerns over the safety of using insect repellents has arisen and questions on the bioactive chemical's shared mode of actions between arthropods and mammals have been examined[1]. DEET has been claimed to inhibit acetylcholinesterase activity in mammalian cells, but conflicting research shows that DEET has a low affinity for it and is still recommended for use**Error! Bookmark not defined.**[9].

Essential oils have also been used to successfully repel disease vectoring insects including mosquitoes and ticks with repellency similar to DEET[10,11,12,13]. In 2005, the Center for

Disease Control (Atlanta, GA) endorsed para-Menthane-3, 8-diol (PMD), a steam distillate product from the leaves of the Australian lemon-scented gum tree as a mosquito repellent[14]. Insect repellents utilizing natural products as their bioactive compound have the public perception that they are safer than synthetic ones however, essential oils and other extracted natural products can cause poisonings from ingestion (due to adulteration and concentrated consumption), skin irritation (cytotoxicity and sensitization) as well as respiratory inflammation (direct contact with vascular system)[5]. Pennyroyal essential oil induced hepatoxicity and lung toxicity in mice after injecting them with the essential oil illustrating systemic ailments once entering the blood stream[15]. Essential oils from *Backhousia citriodora* (Lemon Myrtle Tree) showed necrosis and changes in cellular function in human epidermal tissue when applied in its crude form but the effects were limited upon essential oil formulation and subsequent dilution[16].

Insect repellents that are formulated into wax candles or coils to control the rate of dispersal, need to be investigated for toxicity to the respiratory system as individuals using them breathe in the repellent while increasing the spatial repellency of disease vectoring insects in an area. Insect repellents that are applied to the applied to the skin need to be investigated for epidermal cytotoxicity and sensitization of the of the skin before being used on an individual. Insect repellents applied to the skin have to undergo the same testing that new cosmetic formulations do to assess the toxicity of a formulation. Animal and non-animal models exist to address toxicity of a formulation and since the ban on all cosmetic testing on animals in the European Union in 2011, alternatives to skin toxicity evaluation have been developed to address the concerns of the toxic implications of the formula on the end user[17]. Natural product based insect repellents have to undergo the same testing as

any new repellent and essential oil based repellents that are applied to the skin need to be investigated for skin sensitization capabilities due to the inherent chemical reactivity of them. The most common method of using insect repellents is applying a liquid spray or stick formulation directly on exposed skin. However, when considering essential oil based insect repellents, one must address skin sensitization capabilities of the formulation on the skin due to the inherent reactivity of the essential oils. Essential oils applied in their crude form have shown epidermal skin cell necrosis and changes in the physiology of the cells after application and after dilution and subsequent formulation, the negative effects of the essential oil were minimized[18].

8.1.1 Skin Sensitization Pathway

Allergic Contact Dermatitis (ACD) is medical condition from the skin sensitization pathway and is a delayed response to an allergen that elicits an immune response by recruiting T-lymphocytes that induce hypersensitivity in epidermal cells following exposure to the allergen[19]. This condition is the result when repeated exposure to an allergen or hapten results in the hypersensitization and inflammation of epidermal skin cells[20]. Skin sensitization occurs in two phases, the sensitization or induction phase and the elicitation phase[19,21]. The sensitization phase starts with haptens gaining access to the skin cells and the binding of them to skin proteins[19,20]. Langerhans cells (dendritic cells of the skin) bind to this complex and differentiate into mature dendritic cells while migrating to the lymph node where they present the complex to T-lymphocytes[19,20]. Effector and memory T-cells then clonally expand before release into the blood stream[19,20]. The elicitation phase begins with T-lymphocytes being released into the blood stream causing sensitization and upon re-exposure of the haptens to the individual, epidermal cells release

proinflammatory cytokines (molecules that have effects on other cells) and chemokines (molecules that recruit T-cells to an area) drawing the T-cells into the epidermis[19,20]. Newly infiltrated T-cells into the epidermis release cytokines triggering keratinocytes to release chemokines resulting in increased T-cell recruitment to that area[19,20]. Allergic Contact Dermatitis then develops as the skin is inflamed due to repeat exposure[19,20].

8.1.2 European Union Standards for Non-Animal Skin Sensitization Evaluation

Since the ban on animal testing with respect to cosmetics in 2011 in the European Union, toxicologists have been developing *in vitro* assays to replace the murine Local Lymph Node Assay to evaluate candidate skin senstizers[17,22,23]. New assays to identify skin sensitizers have been developed that mimic the human skin sensitization pathway utilizing custom peptides and cultured cells[24]. The Direct Peptide Reactivity Assay (DPRA) simulates the initial sensitization phase in which a hapten binds to skin proteins[25]. The human Cell Line Activation Test is used to see if the formulation being tests induces the expression of epidermal cell membrane proteins that are activated when exposed to skin sensitizers and are quantified against controls[26]. The KeratinoSens assay is used to determine if test formulations activate keratinocyte Nrf2 dependent genes, genes that are upregulated when skin sensitizers are present and once activated, release cytokines and chemokines into the cell epidermis resulting in further T-cell recruitment and eventual inflammation[27]. Using these newly approved assays, formulations must pass two out of three of the tests to be classified as a non-sensitizer[28]. Here, descriptions of the initial skin protein binding and cell activation assays will be desired and explored for *N. cataria* ‘CR3’ insect repellent stick formulation.

8.1.2.1 Direct Peptide Reactivity Assay (DPRA)

Briefly, the Direct Peptide Reactivity Assay is used to simulate and allergen binding to a skin protein, the first event in the skin sensitization pathway[25]. In this initiating event mimicking assay, two custom peptides, cysteine (Ac-RFAACAA-COOH) and lysine (Ac-RFAAKAA-COOH) based oligopeptides, are incubated with a test formulation for 24 hours. Depending on the passing of the acceptance criteria **(Table 8-1, page 174)** percent depletion of the oligopeptides, formulations are classified as a skin sensitizer or not according to the prediction model **(Table 8-2, page 175)**. This is the first test done to assess the sensitization of a test chemical as it mimics the first step in the allergic response pathway and provides the benefit of being high throughput when assessing multiple samples.

Acceptance Criteria for Direct Peptide Reactivity Assay
Standard Curve must have an r2 value >0.99
The mean percent depletion value of the three replicates for the positive control cinnamic aldehyde should be between 60.8% and 100% for the Cysteine peptide and between 40.2% and 69.4% for the Lysine peptide
The mean percent depletion value of the three replicates for the positive control cinnamic aldehyde should be between 60.8% and 100% for the Cysteine peptide and between 40.2% and 69.4% for the Lysine peptide.
The standard deviation for the positive control replicates should be <14.9% for the percent Cysteine depletion and <11.6% for the percent Lysine depletion.
Mean peptide concentration of reference controls A should be 0.50 ± 0.05mM
The coefficient of variation (CV) of peptide peak areas for the 9 reference controls B and C should be <15.0%
Standard deviation for test item replicates should be <14.9% for the percent Cysteine depletion and <11.6% for the percent Lysine depletion
Mean peptide concentration of the three reference controls C should be 0.50 ± 0.05mM

Table 8-1: Acceptance Criteria for Direct Peptide Reactivity Assay

Prediction Model for Direct Peptide Reactivity Assay		
Mean of Cysteine and Lysine % Depletion	**Reactivity Class**	**DPRA Prediction**
0%≤ mean % depletion ≤ 6.38%	No or minimal reactivity	Non-Sensitizer
6.38% < mean % depletion ≤ 22.62%	Low Reactivity	Sensitizer
22.62% < mean % depletion ≤ 42.47%	Moderate Reactivity	Sensitizer
42.47% < mean % depletion ≤ 100.00%	High Reactivity	Sensitizer

Table 8-2: Prediction Model for Direct Peptide Reactivity Assay.

8.1.2.2 Human Cell Line Activation Test

Briefly, the human Cell Line Activation Test (h-CLAT) is used to see if the formulation being tested induces the expression of cell membrane proteins (CD54/CD86) that are known to be activated when exposed to skin sensitizers[26]. This is measured by a flow cytometer and cytotoxicity is concurrently measured to establish sensitization classification. The cells being tested are transgenic human monocytic leukemia cells where membrane proteins are tagged with fluorochrome antibodies. The relative fluorescence intensity of the surface proteins are compared to the solvent and vehicle control which are used in a prediction model to identify the formulation as either a sensitizer or not. The criteria for predicting sensitization of a formula using the h-CLAT assay comes is in three objectives; cell viability, the acceptance of the expression of the CD54/CD86 membrane proteins in the run validated by controls and the acceptance of the expression of the CD54/CD86 membrane proteins in the data **(Table 8-3, page 176)** and the prediction model **(Table 8-4, page 176)**.

Human Cell Line Activation Test Prediction Criteria
Cell Viability:
Cell viability must be ≥ 75% at the lowest dose.
The highest test item concentration should produce cytotoxicity (< 90% cell viability) unless 5mg/ml in medium, 1mg/ml in DMSO or the highest soluble concentration is used as the maximal test concentration of a test item.
CD54/CD86 Expression Run Acceptance Criteria:
Cell viability of medium and DMSO controls should be greater than 90%
In the positive control (Nickel Sulphate; 100µg/ml), RFI values of both CD86 and CD54 should be over the positive criteria (CD86 ≥ 150 and CD54 ≥ 200) and cell viability should be > 50%
In the DMSO solvent control, RFI values compared to the medium control of both CD86 and CD54 should not exceed the positive criteria (CD86 ≥ 150 and CD54 ≥ 200)
For both medium and DMSO controls, the MFI ratio of both CD86 and CD54 to isotype control should be > 105%
CD54/CD86 Expression Data Acceptance Criteria:
For a test item resulting in a negative value (i.e. Non-Sensitiser), the cell viability at the 1.2 x CV75 value should be less than 90%
For a test item resulting in a positive value (i.e. Sensitiser), a cell viability at the 1.2 x CV75 value of more than 90% is considered acceptable
When test items in RPMI are tested at 5mg/ml or test items in DMSO are tested at 1mg/ml or the highest soluble concentration is used as the maximal dose instead of the CV75 based dose, the data for the test item are accepted regardless of the cell viability
Cell viability of at least 4 doses in each assay should be > 50%.

Table 8-3: Acceptance Criteria Human Cell Line Activation Test.

Prediction Model for Human Cell Line Activation Test
Sensitizer: RFI of CD86 ≥ 150% at any dose (≥ 50% cell viability) in two runs and/or RFI of CD54 150% at any dose (≥ 50% cell viability).
Non-sensitizer: RFI values of CD86/CD54 fall outside of sensitizer range.

Table 8-4: Prediction Model for Human Cell Line Activation Test

8.1.2.3 KeratinoSens Activation Test

Briefly, the Keratinosens assay is used to determine if test formulations activate keratinocyte cell Nrf2 dependent genes, genes that are upregulated when skin sensitizers are present and once activated, release cytokines and chemokines into the cell epidermis resulting in inflammation and further T-cell recruitment. This assay uses cultured, luciferase transfected HaCAT human keratinocytes that are under the control of the Nrf2 transcription factor. Luciferase production allows for quantitative measurements to assess skin sensitization. Sensitization is determined by producing positive results using the same solvent the test sample is soluble in and identifying the molar range in which the test samples generated cinnamic aldehyde equivalents. Samples are considered non-sensitizers if the acceptance criteria **(Table 8-5, page 177)** and prediction models are met **(Table 8-6, page 178)**.

KeratinoSsens Activation Test Acceptance Criteria
The positive control (cinnamic aldehyde) produces positive results, i.e. the luciferase gene induction by this control is statistically above the threshold of 1.5 in at least one of the tested concentrations.
The lmax and the EC1.5 for cinnamic aldehyde is calculated and meet the following targets:
average induction in the three replicates for cinnamic aldehyde at 32 µM is between the XCellR8 historical range (currently 1.6 and 3)
EC1.5 value for cinnamic aldehyde is between the XCellR8 historical range (currently 6 µM and 39 µM).
Note: At least one of these criteria must be met, otherwise the run is discarded. If only one criterion is met, it is recommended to check the dose-response curve of cinnamic aldehyde in order to decide on acceptability.
CV% of blank values < 20%

Table 8-5: Acceptance Criteria for Keratinosens Assay.

KeratinoSens Assay Prediction Model
Test items are sensitizers if the following conditions are met in 2/3 repetitions
The IMAX is higher than 1.5 fold and statistically significantly different as compared to the solvent (negative) control (as determined by a two-tailed, unpaired Student's T-test).
The cellular viability is higher than 70% at the lowest concentration with induction of luciferase activity above 1.5 fold (i.e. at the EC1.5 determining concentration). Test items that only induce the gene activity at cytotoxic levels are not rated positive, as in the case for some non-sensitizing skin irritants.
The EC1.5 value is less than (<) 1000 µM (or < 200 µg/mL for test chemicals with no defined MW).
There is an apparent overall dose-response for luciferase induction (or a biphasic response).

Table 8-6: Prediction Model for KeratinoSens Assay.

8.2 Materials and Methods

8.2.1 *Nepeta cataria* 'CR3' Stick Insect Repellent Formulation

The *N. cataria* 'CR3' stick formulation was created to extend the duration of acceptable arthropod repellency and has previously shown acceptable efficacy to 8 hours. The stick formulation was made with 20% *N. cataria* 'CR3' essential oil, 55% isopropanol, 4% glycerin, 20% beeswax and 1% stearyl alcohol. Beeswax was melted on a heated, magnetic stir plate with a magnetic stir rod mixing the wax. The glycerin and stearyl alcohol were dissolved in the isopropanol then added to the melted beeswax. The solution was allowed to cool and prior to solidifying the *N. cataria* essential oil was added to the mixture.

8.2.2 *Nepeta cataria* 'CR3' Stick Formulation Molecular Weight Estimation

To calculate the molar mass of the stick formulation for the KeratinoSens assay, the molecular weight of the stick formulation was estimated by averaging the molecular weight

contribution of the percent of each individual component in the formulation. Essential oil molecular weight was estimated by averaging the molecular weight contribution of the percent of each individual component in the essential oil. Due to the variability in the production of fatty acids in beeswax by different species of bees and their environments, gel permeation chromatography was used to estimate the average molecular weight of the beeswax sample in the formulation[29]. Gel permeation chromatography was performed by following the application note provided by Agilent Technologies in which the beeswax sample's molecular weight was calculated to be 885 g/mol[30].

8.2.3 Direct Peptide Reactivity Assay (DPRA)

The procedure for this assay was followed according to the OECD protocol[25]. The formulation was incubated for 24hrs (±2hrs) at 25 ±2.5˚C in solution at 100mM in combination with either Cysteine or Lysine containing peptides and then run on an HPLC system (20-minute run-time) using gradient elution and UV detection at 220nm to measure peptide concentration. The formulation was compared to reference controls containing the test item solvent in combination with either Cysteine or Lysine peptide in order to determine the relative percent peptide depletion. Relative percent peptide depletion values were used in a prediction model that assigns test items to one of four reactivity classes.

Data analysis for this study was performed using the validated EURL-ECVAM analysis template (DPRA validated study template) available from the EURL-ECVAM website: https://ecvam-dbalm.jrc.ec.europa.eu/methods-and-protocols/search/dpra. The template is a Microsoft Excel workbook (version 5) containing formula to process the raw data as per OECD TG 442C. The final data output is a percentage peptide depletion value for the Cysteine and Lysine peptides after exposure to the test item. The validated template also

assesses adherence to the acceptance criteria. Acceptance criteria and prediction models for the assay were previously listed.

8.2.4 Human Cell Line Activation Test

The procedure for this assay was followed according to the OECD protocol[26]. Solubility was first determined for the test item using either culture medium (RPMI 1640) or DMSO. Note that for this method, test items with a Log Kow (octanol/water partition coefficient) of up to 3.5 have been tested successfully. However, test items with a Log Kow of greater than 3.5 can still be tested at lower soluble concentrations. In such a case, a negative result (non-sensitizer) should be considered inconclusive, whereas a positive result (sensitizer) could still be considered valid.

THP-1 cells were pre-cultured for 48 hrs. Following this, the cells were dosed with the test item over an 8 dose range and incubated for 24 ±0.5hrs. The cells were then washed and stained with propidium iodide which allows for discrimination of live/dead cells by flow cytometry. The dose of test item that yields 75% cell viability (CV75) was calculated and taken forward for the next stage of testing. This dose finding assay was carried out over two independent runs.

THP-1 cells were then pre-cultured for 72hrs. Once the CV75 was determined, a narrower dilution series based around the CV75 value was produced for the test item. This dilution range was used to dose the cells again for 24 ±0.5hrs. The cells were then washed and stained with propidium iodide and also with antibodies that detect CD54 and CD86 expression as well as a negative control antibody. This allowed for discrimination of live/dead cells and also changes in CD54 and CD86 marker expression by flow cytometry.

Data analysis for this study was performed using the following validated Microsoft Excel workbooks containing formulae to process the raw data as per OECD TG 442E. The templates: CV75 Analysis Template v.3 - Determines the dose of test item that yields 75% viability. h-CLAT Analysis Template v.7 - Determines the extent of CD54 and CD86 expression in the system in response to the test item. EC Value Calculator (not used as test item was classified as a non-sensitizer) - Determines the Effective Concentration (EC) of test item that induces a relative fluorescence Intensity (RFI) of either 200 for CD54 (EC200) or 150 for CD86 (EC150). RFI is a measure of CD54 and CD86 expression that is calculated from the experimental data as follows:

RFI = (MFI of test item treated cells - MFI of test item treated isotype cells) / (MFI of solvent treated cells - MFI of solvent treated isotype cells)

MFI = Mean Fluorescence Intensity (geometric mean) of the fluorescence data generated for 10,000 cells in each test condition.

Acceptance criteria and prediction model for the assay was previously listed.

8.2.5 KeratinoSens Assay

The procedure for this assay was followed according to the OECD protocol and cells cultured and sourced according to the Givaudan (Switzerland) protocol [27,31]. Preliminary testing to determine top concentration by solubility was performed first. On day one, Seeding cells (3 x 96-well plates for Luminescence; 2 x 96-well plate for MTT); 10,000 cells per well, passage number p21 (reps 1 and 2) and p13 (rep 3). On day two, 24h after seeding the test and control items were applied and plates were incubated at 37oC, 5% CO2, ≥ 95% relative humidity for 48 ± 2h. On day four, Evaluation of luciferase activity

by luminescence (3 plates) and cell viability by MTT testing (2 plates). For reference items, per plate, single application of 5 concentrations of the positive control was applied in cell culture medium (dilution factor of 2) with a final concentration of Isopropanol of 1% and single application of culture medium with 1% Isopropanol was applied as the negative control (6 wells per plate). One well per plate is left empty (no cells). Cells were then incubated with the test or reference items for 48 ± 2h before endpoint measurements. Three repetitions (runs) were performed. Each repetition consisted of 3 x 96-well plates for luminescence and 2 x 96-well plates for MTT. The validity of each repetition was assessed following acceptance criteria.

Data Analysis for KeratinoSens were used to analyse data and hese forms are Microsoft Excel workbooks containing formulae to process the raw data as described in SOP L0057. Parameters calculated in the report included the maximal average fold induction of luciferase activity (IMAX) value observed at any concentration of the test item and positive control. Also calculated was the the EC1.5 value representing the concentration for which induction of luciferase activity is above the 1.5- fold threshold (i.e. 50% enhanced luciferase activity). For each concentration showing > 1.5-fold luciferase activity induction, statistical significance is calculated (e.g. by a two-tailed Student's t-test), comparing the luminescence values for the three replicate samples with the luminescence values in the solvent (negative) control wells to determine whether the luciferase activity induction is statistically significant ($p < 0.05$). The lowest concentration with > 1.5-fold luciferase activity induction is the value determining the EC1.5 value. It is checked in each case whether this value is below the IC30 value, indicating that there is less than 30% reduction in cellular viability at the EC1.5 determining concentration. The percentage of

viability was compared to the negative control. Acceptance criteria and the prediction model for the assay was previously listed.

8.3 Results

8.3.1 Direct Peptide Reactivity Assay (DPRA)

Prior to the main test, the test stick was assessed for solubility and was found to be soluble in Isopropanol at 100mM. Acceptance criteria for all controls and the test item were met in both runs with the exception of the PC for Cysteine Run 1 (highlighted in orange), where the cysteine depletion was marginally below the range (57.750%; range = 60.8 - 100%) and the RefC concentration (highlighted in orange) for Lysine Run 2 where the value was marginally above the range (0.557mM; range = 0.45 - 0.55mM) **(Table 8-7, page 184)**. Both these values were borderline and were accepted by the SD as appropriate. The data from the run was considered valid and these values did not impact the final outcome of the study.

Criterion	Run 1 (Cysteine)	Run 2 (Lysine)	Outcome
Std Curve r^2 >0.99	0.994	0.993	**PASS**
PC 60.8% to 100% depletion Cys	57.750	N/A	**ACCEPT/PASS**
PC 40.2% to 69.0% depletion Lys	N/A	56.484	**PASS**
SD Cys Depletion PC <14.9%	1.896	N/A	**PASS**
SD Lys Depletion PC <11.6%	N/A	0.426	**PASS**
RefA Mean Conc 0.50 ± 0.05mM	0.523	0.548	**PASS**
Peak Area CV RefB <15.0%	2.444	1.666	**PASS**
Peak Area CV RefC <15.0%	4.619	2.411	**PASS**

Criterion	Run 1 (Cysteine)	Run 2 (Lysine)	Outcome
SD Cys Depletion Test Item <14.9%	3.495	N/A	**PASS**
SD Lys Depletion Test Item <11.6%	N/A	0.083	**PASS**
RefC Mean Conc 0.50 ± 0.05mM	0.527	0.557	**PASS/ACCEPT**

Table 8-7: Direct Peptide Reactivity Assay Acceptance Criteria Results

The test item produced 3.612% mean Cysteine and Lysine peptide depletion, therefore, using the Cysteine 1:10 / Lysine 1:50 prediction model, the test item was classified as a Non-Sensitizer with No or Minimal Reactivity. A single HPLC analysis for both the Cysteine and the Lysine peptide was considered sufficient for the test item as the result was conclusive. Minimal co-elution was observed for this test item in the Lysine peptide buffer. The co-elution peak area value for lysine was removed from the overall peak area for each test item replicate.

8.3.2 Human Cell Line Activation Test

Prior to the CV75 determination, the test item was assessed for solubility and was found to be soluble in Isopropanol at 10 mg/ml. The stick formulation was found to be non-cytotoxic at the highest soluble concentration using the CV75 assay and therefore the maximum dose (20 µg/ml) was taken forward for the CD54/86 expression measurement. Acceptance criteria for all controls and the test item were met in both runs for the CV75 determination **(Table 8-8, page 186)**. During the measurement of CD54 and CD86 expression, all acceptance criteria were met with the exception of the positive control for CD86 in Run 1 which was borderline (RFI = 133, threshold = 150). However, as the test item was a clear Non-Sensitizer in both runs the positive control value in Run 1 was accepted and the data/run is considered to be valid.

CV75 Determination			
Criterion	**Run 1**	**Run 2**	**Outcome**
Cell viability must be ≥ 75% at the lowest dose.	97.86	96.92	**PASS**
The highest test item concentration should produce cytotoxicity (< 90% cell viability) unless 5mg/ml in medium, 1mg/ml in DMSO or the highest soluble concentration is used as the maximal test concentration of a test item.	97.11 but HSC was used	96.96 but HSC was used	**PASS**
Measurement of CD54 and CD86 Expression			
Criterion	**Run 1**	**Run 2**	**Outcome**
Cell viabilities of medium and solvent controls should be higher than 90%	Medium: 96.73 Solvent: 97.54	Medium: 97.37 Solvent: 97.68	**PASS**
In the solvent control, RFI values of both CD86 and CD54 should not exceed the positive criteria (CD86 RFI ≥ 150% and CD54 RFI ≥ 200%) compared to the medium control	CD54: 98 CD86: 100	CD54: 103 CD86: 106	**PASS**
For both medium and solvent controls, the MFI ratio of both CD86 and CD54 to isotype control should be > 105%	CD54: 171.89% CD86: 164.33% Solvent CD54: 172.65% CD86: 166.24%	CD54: 140.75% CD86: 132.97% Solvent CD54: 139.28% CD86: 132.49%	**PASS**
In the positive control (Nickel Sulphate), RFI values of both CD86 and CD54 should meet the positive criteria (CD86 RFI ≥ 150 and CD54	CD54 RFI: 210 CD86 RFI: 133	CD54 RFI: 240 CD86 RFI: 151	**ACCEPT/PASS**

RFI ≥ 200) and cell viability should be greater than 50%.	CD54 Via: 89.57% CD86 Via: 89.82%	CD54 Via: 89.82% CD86 Via: 89.52%	
For each test item, the cell viability should be greater than 50% in at least four tested concentrations in each run	8/8	8/8	**PASS**
Negative results are acceptable only for test items exhibiting a cell viability of less than 90% at the highest concentration tested, unless 5mg/ml in medium, 1mg/ml in DMSO or the highest soluble concentration is used as the maximal test concentration of a test item.	97.84 (HSC used)	97.34 (HSC used)	**PASS**

Table 8-8: Human Cell Line Activation Test Acceptance Criteria Results. RFI - Relative Fluorescence Intensity, MFI - Mean Fluorescence Intensity, DMSO - Dimethyl Sulphoxide.

The CV75 dose informs the dosing range selected for the CD54/86 expression assay. Stick was found to be non-cytotoxic at the highest soluble concentration using the CV75 assay and therefore the maximum dose (20 µg/ml) was taken forward for the CD54/86 expression measurement. The following tables show the expression of CD54 and CD86 against test item dose with concurrent cytotoxicity measurement.

Run 1						
Test Item Dose (µg/ml)	**Cell Viability (%)**			**Average Cell Viability (%)**	**CD54 RFI**	**CD86 RFI**
	Isotype	**CD54**	**CD86**			
20.00	97.76	97.59	98.18	97.84	98	78
16.67	97.97	97.92	97.95	97.95	93	62

13.89	98.20	97.83	98.00	98.01	92	69
11.57	97.90	97.33	97.35	97.53	93	76
9.65	97.68	97.44	96.36	97.16	86	71
8.04	97.11	97.57	97.36	97.35	89	75
6.70	97.00	96.83	97.64	97.16	85	75
5.58	96.98	97.48	96.14	96.87	72	74
Run 2						
Test Item Dose (µg/ml)	**Cell Viability (%)**			**Average Cell Viability (%)**	**CD54 RFI**	**CD86 RFI**
	Isotype	**CD54**	**CD86**			
20.00	97.47	97.35	97.21	97.34	92	82
16.67	97.82	97.67	97.17	97.55	92	64
13.89	97.18	97.55	96.34	97.02	76	75
11.57	96.36	97.49	96.84	96.90	78	85
9.65	96.66	97.03	97.13	96.94	87	94
8.04	96.76	96.56	97.24	96.85	88	93
6.70	96.93	97.30	96.91	97.04	100	94
5.58	97.38	96.77	97.11	97.08	66	88

Table 8-9: Human Cell Line Activation Test Prediction Model Results

As can be seen from the data **(Table 8-9, page 187)**, the expression of CD54 as measured by the RFI did not cross the threshold (RFI ≥200) at any of the doses tested. The expression of CD86 as measured by the RFI did not cross the threshold (RFI ≥150) at any of the doses tested. As the CD54/CD86 expression did not cross the threshold the test item is classified as a Non-Sensitizer. Cell viability did not fall below 50% at any of the test item concentrations.

8.3.3 KeratinoSens Assay

The catnip stick formulation was found to be insoluble in the standard solvents for the method (culture medium, DMSO, ethanol). Therefore, solubility in Isopropanol was attempted. Solubility of Stick in Isopropanol was confirmed up to 2mg/ml; subsequent dilution in cell culture medium containing gave a top concentration of 20µg/ml that was further diluted upon addition of the cells to yield a final top concentration of 10µg/ml. Due to the insoluble nature of Stick in Culture Medium, DMSO and Ethanol, Isopropanol was used as the solvent for the KeratinoSens method and the individual results listed **(Figure 8-1, page 188; Figure 8-2, page 189; Figure 8-3, page 190; Figure 8-4, page 191 and Table 8-10, page 189; Table 8-11, page 190; Table 8-12, page 191; Table 8-13, page 192; Table 8-14, page 192)**.

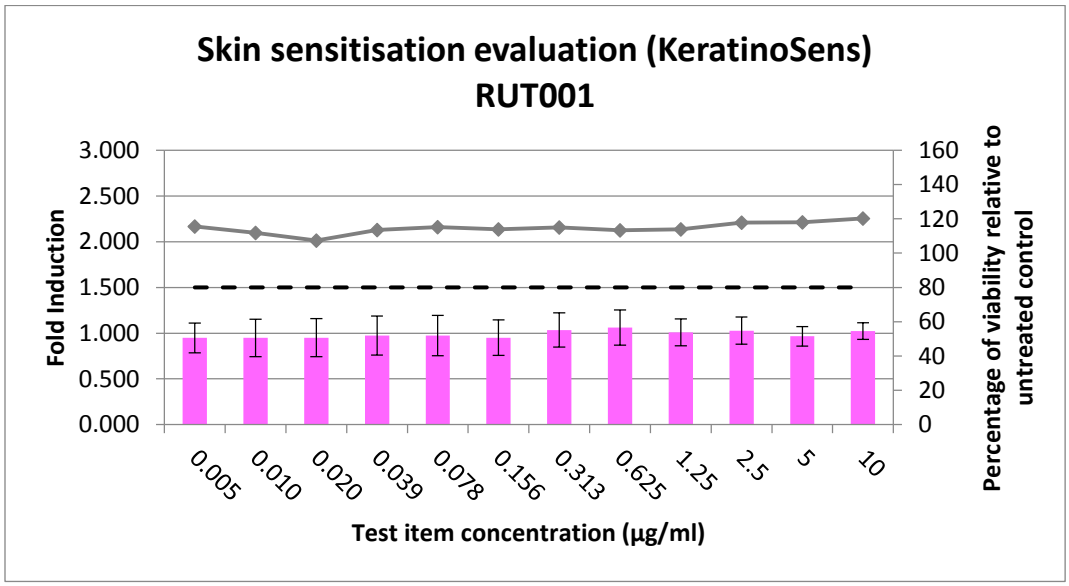

Figure 8-1: Rep 1 Graph: Dose Dependent Nrf2 Fold Induction Values, the dotted line shows the 1.5 induction value threshold.

REP 1	Test item concentration (µg/ml)											
	0.005	0.010	0.020	0.039	0.078	0.156	0.313	0.625	1.25	2.5	5	10
Mean fold induction	0.948	0.948	0.951	0.974	0.973	0.950	1.035	1.060	1.010	1.027	0.966	1.022
SD	0.112	0.101	0.097	0.051	0.059	0.035	0.103	0.155	0.113	0.160	0.099	0.165
Viability %	115.58	111.74	107.34	113.53	115.15	113.95	115.00	113.29	113.81	117.71	118.01	120.28
I_{MAX}	1.060 at 0.625 µg/ml											
EC1.5	N/A											
IC_{50}	N/A											
IC_{30}	N/A											

Table 8-10: Rep 1: Dose Dependent Nrf2 Fold Induction Values.

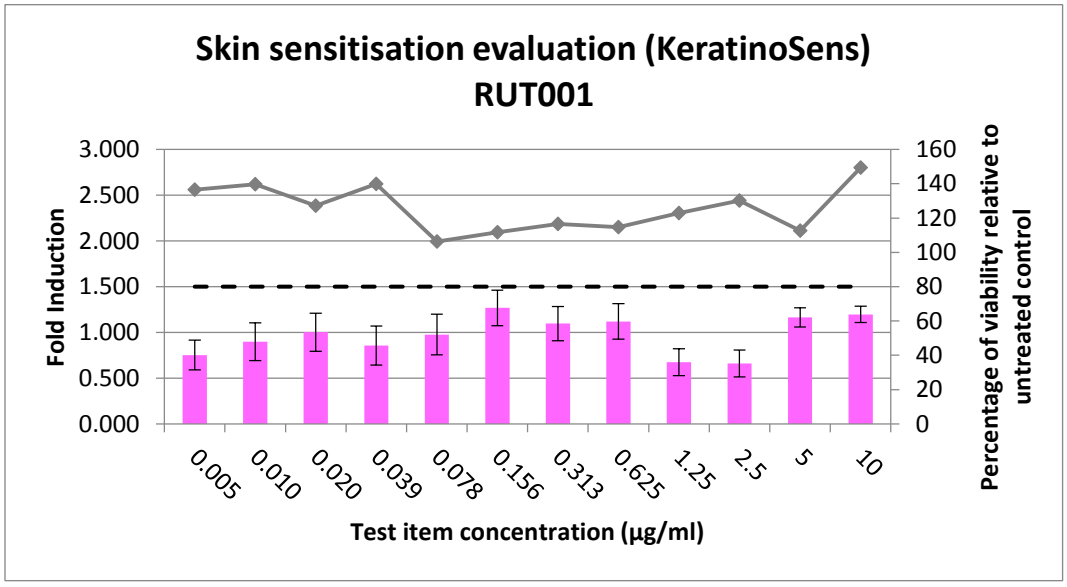

Figure 8-2: Rep 2 Graph: Dose Dependent Nrf2 Fold Induction Values, the dotted line shows the 1.5 induction value threshold.

REP 2	Test item concentration (µg/ml)											
	0.005	0.010	0.020	0.039	0.078	0.156	0.313	0.625	1.25	2.5	5	10
Mean fold induction	0.752	0.898	1.002	0.857	0.976	1.268	1.096	1.120	0.673	0.660	1.165	1.197
SD	0.000	0.267	0.051	0.108	0.201	0.077	0.250	0.000	0.000	0.000	0.215	0.082
Viability %	136.46	139.72	127.15	139.91	106.26	111.70	116.48	114.77	122.97	130.25	112.60	149.30
I_{MAX}	1.268 at 0.156 µg/ml											
EC1.5	N/A											
IC_{50}	N/A											
IC_{30}	N/A											

Table 8-11: Rep 2: Dose Dependent Nrf2 Fold Induction Values.

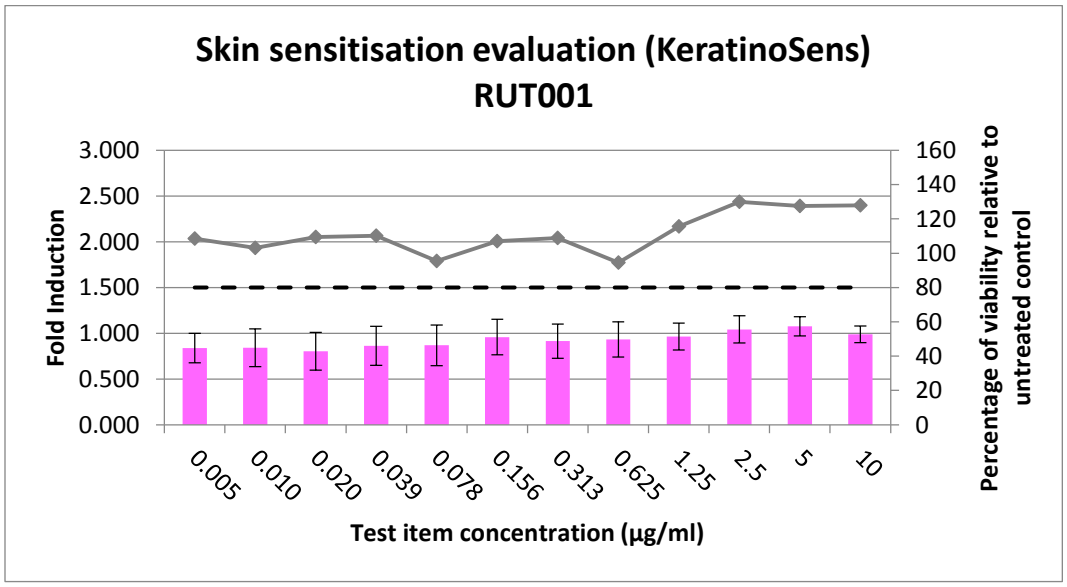

Figure 8-3: Rep 3 Graph: Dose Dependent Nrf2 Fold Induction Values, the dotted line shows the 1.5 induction value threshold.

REP 3	Test item concentration (µg/ml)											
	0.005	0.010	0.020	0.039	0.078	0.156	0.313	0.625	1.25	2.5	5	10
Mean fold induction	0.838	0.841	0.804	0.863	0.868	0.959	0.913	0.933	0.964	1.042	1.076	0.988
SD	0.026	0.026	0.060	0.057	0.068	0.168	0.060	0.116	0.156	0.036	0.244	0.211
Viability %	108.58	103.11	109.49	110.18	95.54	107.01	108.83	94.55	115.60	129.91	127.50	127.86
I_{MAX}	1.076 at 5 µg/ml											
EC1.5	N/A											
IC_{50}	N/A											
IC_{30}	N/A											

Table 8-12: Rep 3: Dose Dependent Nrf2 Fold Induction Values.

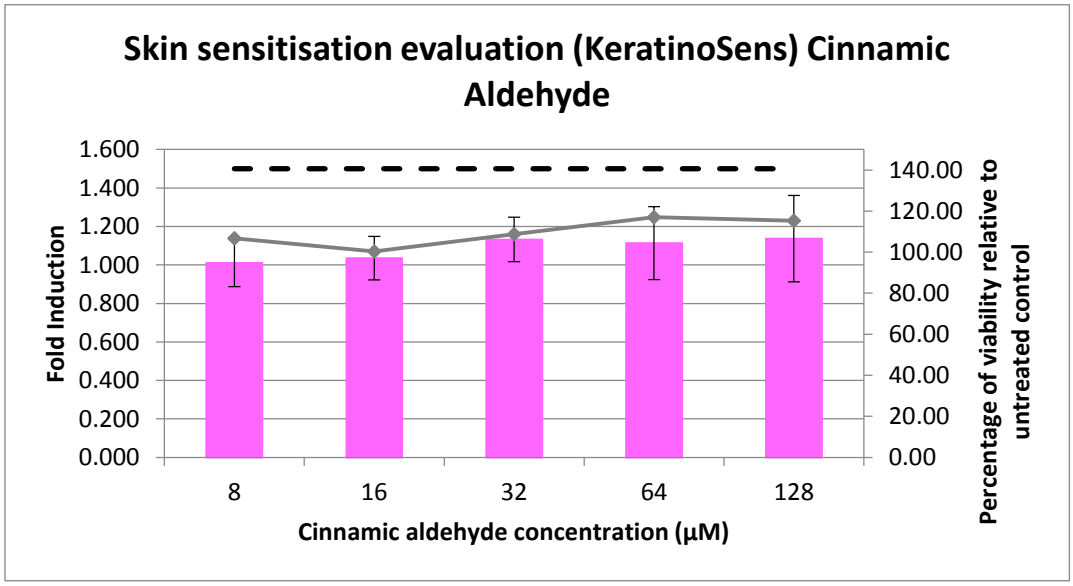

Figure 8-4: Positive Control Dose Dependent Nrf2 Fold Induction Values

The sensitization potential of Stick was quantified by calculating two parameters known as the EC1.5 and the IMAX value. The first, the EC1.5 value is the Effective Concentration (EC) of test item that caused an induction of luciferase activity of greater than 1.5-fold over untreated controls. The second, The IMAX value is the maximum-fold induction observed within the concentration range tested. As shown, only 1 out of 4 the formal acceptance criteria for the positive control for the test was met and therefore the results were considered invalid.

Criteria	Result	Pass or Fail
1-Positive Control (PC) (Cinnamic aldehyde) induction >1.5-fold in at least one concentration	No	Fail
2-Average induction of PC at 32µM is [1.6-3.0]	No (1.13)	Fail
3-EC1.5 value is [6-39µM]	No	Fail
4-CV% of blank values < 20%	Yes (10.76%)	Pass

Table 8-13: KeratinoSens Assay Acceptance Criteria Results.

Determination criteria for the skin sensitization potential of Stick			
	REP1	**REP2**	**REP3**
Does at least one concentration of Test Item induce luciferase activity ≥1.5-fold:	No	No	No
Does the first concentration inducing luciferase activity above 1.5, have a viability above 70%:	N/A	N/A	N/A
Does $EC_{1.5}$ value occur at a concentration <1000µM (or <200µg/ml)	N/A	N/A	N/A
Does the test item induce the luciferase in a dose-dependent manner	N/A	N/A	N/A
Classification	**Not Classified (Non-Sensitiser)**	**Not Classified (Non-Sensitiser)**	**Not Classified (Non-Sensitiser)**

Table 8-14: KeratinoSens Assay Prediction Model Results.

As shown, the maximum induction values observed were 1.060 at 0.625 µg/ml (Rep 1), 1.268 at 0.156 µg/ml (Rep 2) and 1.076 at 5 µg/ml (Rep 3). Although the test item did not induce any responses that crossed the sensitization threshold, the positive control did not yield the required response using Isopropanol as the solvent. Therefore, Stick could not be classified due to incompatibility of the test item/solvent combination with the test method.

8.4 Discussion

The *N. cataria* 'CR3' stick formulation did not meet any of the prediction criteria for sensitizing skin. However, in the KeratinoSens assay, the only solvent that was able to solubilize the stick formulation was not able to solubilize the positive control, so the acceptance criteria could not be validated and the stick formulation was not classified. However, following the OECD on skin sensitizer classification, the *N. cataria* 'CR3' stick formulation can be classified as a non-sensitizer due to the formulation meeting all of the acceptance criteria and passing all of the prediction models for two out of the three tests, including the Direct Peptide Reactivity Assay and the human Cell Line Activation Test[28]. This natural sourced formulation will provide users with an effective means of repellency against *A. aegypti* mosquitoes and *Ixodes scapularis* ticks for up to 8 and 4 hours respectively. This formulation is not cytotoxic to the skin and will not generate Allergic Contact Dermatitis on one's skin.

8.5 References

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Chapter 9 Conclusions

9.1 Dissertation Impact

This dissertation has focused on understanding *N. cataria's* ecological role as well as human and animal beneficial potential as an insect repellent and nutraceutical. The results of the breeding program outlined in this dissertation have increased the biomass, essential oil and nepetalactone yield of *N. cataria* to commercially relevant levels allowing for the production on these plants on larger scales for incorporation into natural insect repellents. Breeding programs focusing on further improving the nepetalactone concentration in *N. cataria* can rogue out populations that contain the recessive effective factors for expressing low nepetalactone concentrations in the essential oil. The seeds of these plants can be provided to rural communities throughout the globe that live in areas with incidences of diseases vectored by arthropods and that cannot afford or do not have access to the current standards of insect repellents. The success of the essential oils in repelling deadly arthropods demonstrated the potential of this research to directly impact the wellbeing of individuals that live in areas where disease vectors are a clear and present danger to the public health.

Distilling the essential oil of *N. cataria* will not only provide the user with an efficient arthropod repellent but boiling the plant material to extract the essential oils that are collected in the distillation unit, will also extract the non-volatile compounds contained in the leaves and will be collected in the distillation chamber. This dissertation shows that the bioactive relevant levels of the non-volatile polyphenols produced by *N. cataria* leaves and flowers are medicinally bioactive, exhibiting anti-oxidant and anti-inflammatory activity.

These compounds can be consumed by individuals after the distillation process and contribute to the health of the people consuming them.

The formulas in which the *N. cataria* essential oil was incorporated into not only increase the duration of acceptable repellency but do not induce the auto-immunogenic allergic response, contact dermatitis. Users of *N. cataria* essential oil-based insect repellent formulations will be able to protect themselves while damaging the epidermis of their skin. Modifications to the formulas can be made so rural communities can develop them on site while not having to purchase additional chemicals not easily acquirable.

9.2 Future Work Recommendations

This dissertation has set up the framework for further studies involving *N. cataria* in terms of breeding, physiology, applications of its essential oil toward disease vectoring insects. The breeding of *N. cataria* plants can be improved upon by developing molecular markers to assist in the development of high yielding nepetalactone populations. Molecular markers developed form the now known segregating populations with respect to nepetalactone concentration, could increase the yields even higher. Furthermore, markers developed for increased essential oil content should be investigating and populations segregating in terms of low and high essential oil yields could be developed to further enhance the production of these plants. Finally, in terms of the non-volatile polyphenols, populations can be developed that identify more medicinally bioactive compounds that can then be bred for increased concentrations within the plant tissues offering consumers of *N. cataria* nutraceuticals a higher quality consumable.

In terms of altering the physiology of the plant to increase the essential oil content of the plant, explorations into modifying the water content and nitrogen sources should be explored as these treatments are commonly associated with an increase in essential oil accumulations. Chemical elicitors such as jasmonic acid and other hormones such as gibberellins should be investigated as well as they are commonly associated with an increase in essential oil yield.

Finally, mosquitoes and ticks, while deadly in terms of the diseases the vector, are not the only arthropods that can negatively impact the public health. Additional disease vectoring arthropods need to be examined in terms of repellency from *N. cataria* essential oils to offer new solutions those that cannot afford or do not have access to currently offered repellents or for those that want to choose an alternative to current synthetic repellents. Efforts should also focus on investigating *N. cataria* essential oil to repel vectors that spread plant pathogens offering an organic alternative to producers of various crops. New delivery mechanisms can be formulated in addition to the sticks, sprays and gels to disperse *N. cataria* essential oil such as foliar sprays, and spatial repellents to be applied in areas before people are expected to be there or for crops in which there is a suspicion of future infestation by phytopathogens. The safety of these essential oils needs to be investigated in terms of pulmonary toxicity as many of the formulations are volatile and could impact individual's health negatively in that sense.

In conclusion, the research in this dissertation has helped develop two new *N. cataria* cultivars, producing commercially relevant amounts of essential oil capable of offering a safe, effective and accessible alternative to individuals needing disease vector repellents that endanger populations globally.