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Effects of Neem on Plasmatocyte Counts in Galleria Mellonella Larvae

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EFFECTS OF NEEM ON PLASMATOCYTE COUNTS IN *GALLERIA*

MELLONELLA LARVAE

A Master's Thesis

Presented to

The Graduate College of

Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Agriculture

By

Michael Fenske

August 2018

EFFECTS OF NEEM ON PLASMATOCYTE COUNTS IN *GALLERIA MELLONELLA* LARVAE

Agriculture

Missouri State University, August 2018

Master of Science

Michael Fenske

ABSTRACT

The neem tree (*Azadirachta indica*) has been called the tree of the 21st century, due to its many beneficial properties. This research studies the effects of neem products on plasmatocytes of *Galleria mellonella* larvae challenged by injections of Sumi ink. Counts of circulating plasmatocytes increased in response to the ink within 4 hours. Concurrent injections of neem essential oil reversed this process in a dose-dependent manner. Methanolic extracts of neem essential oil had similar effects on counts of circulating plasmatocytes mobilized by ink injections. Aqueous extracts from neem bark did not have effects on plasmatocyte counts. Comparative high-performance thin layer chromatography (HTPLC) showed a band of difference between chromatographic profiles of biologically active methanolic extracts of neem essential oil and biologically inactive aqueous extracts from neem bark. This band was stigmasterol. Stigmasterol alone reversed effects of ink injections on plasmatocyte counts with a dynamic like that exhibited by methanolic extracts of neem.

KEYWORDS: neem, *Galleria mellonella*, Sumi ink, plasmatocytes, HPTLC, stigmasterol

This abstract is approved as to form and content

_____
Dr. Maciej Pszczolkowski
Chairperson, Advisory Committee
Missouri State University

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Maciej Pszczolkowski, PhD

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INTRODUCTION

The global population is likely to grow at a rate of 70 million per year, with a projected 9.2 billion people by 2050. Food production will also have to escalate at a projected rate of 70%. Currently, a percentage of agriculture production focuses on biofuel and fiber (Popp, Pető, & Nagy, 2013). Combined with the limited and decreasing amount of agricultural land available, this is a monumental task.

Synthetic pesticide development after World War II has dramatically increased (Popp et al., 2013). This, with other practices, has led to the growth of agricultural productivity to all new highs. To accelerate the food production to new levels, pesticides are part of the answer. Global pesticide use is already at 3 million tons annually, at a cost of over 40 billion dollars. There is a continued dependence on the use of chemical pesticides, which entomologists have called the "pesticide treadmill" (Popp et al., 2103, p. 246). The treadmill, also called a trap, is when agriculture producers must use larger amounts of pesticides and/or ones that are increasingly stronger due to pest resistance. There are two determining factors involved. The first is to use less effective chemicals that need higher treatment rates coupled with a higher frequency of applications. The second is to develop new pesticides that are effective until the resistance of the target is met (Popp et al., 2013).

Continued use of chemical pesticides is having detrimental effects on the environment. Pesticides can enter bodies of water through infiltration or point-source inputs. Infiltration is the leaching of chemicals into ground water from land applications. Point-source inputs may come from several factors, including surface runoff, erosion,

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spray drift, tile drains, and volatilization (Reichenberger, Bach, Skitschak, & Frede, 2007).

Surface runoff can occur in practically every arable field, even ones without a measurable slope. During heavy rains or prolonged periods of moisture saturation, excess runoff occurs, and it is called *Hortonian flow*. This happens when the water table rises to the surface, causing any rainfall to run off (Reichenberger et al., 2007). The U.S. Geological Society estimated that over 90% of water and fish samples obtained from all streams contained one or more pesticides. In ground water, it has found over 143 different pesticides (Aktar, Sengupta, & Chowdhury, 2009).

Spray drift and volatilization also are problems with pesticides. Spray drift may account for up to 25% loss of chemicals, which can spread from short distances to hundreds of miles. Volatilization can account for up to a 90% loss of applied pesticides. Almost every pesticide has been detected in the atmosphere and precipitation, throughout the United States at various times of the year (Aktar et al., 2009).

Human exposure is especially problematic for agricultural laborers and chemical manufacturing. Globally, chemical pesticides are responsible for nearly one million chronic diseases and deaths annually. It is possible for organochlorine insecticides to contaminate the atmosphere, water, and tissues of all life forms on the planet (Aktar et al., 2009).

Pesticides affect every life form, from plants to soil microorganisms to wildlife. Many plants use mycorrhizal fungi, found on their roots, to increase nutrient uptake. Laboratory studies have shown that Roundup is toxic to this beneficial organism, even in slight amounts. Dolphins' high trophic level combined with their low ability to

metabolize chemicals is causing global poisoning. The Indus river dolphin (*Platanista minor*), found in China, is facing extinction due to pesticides (Aktar et al., 2009). In the United States alone, pesticides account for over 9.6 billion dollars annually in losses from public health, natural pollinators, pest resistance, wildlife, pets, and water contamination (Popp et al., 2013).

Biopesticides and integrated pest management (IPM) are essential for our future. Biopesticides come from natural substances that are ecofriendly, and from control pests with nontoxic processes. Biopesticides are less toxic than chemical pesticides, are effective in minute amounts, decompose quickly, and usually only target the desired pest or related organisms (Dutta, 2015).

Accordingly, the Environmental Protection Agency established the Biopesticides and Pollution Prevention Division in 1994. It promotes safer pesticides in conjunction with IPM (Dutta, 2015). IPM is using practices that have a negligible impact on the environment, while maintaining pests below the economic threshold. This is when the cost of the pest's damage exceeds the cost of control.

It usually takes a minimum of 3 years to register a conventional pesticide. Biopesticides normally require less than a year, due to the lower risks involved. As of 2014, there were over 430 registered active ingredients for biopesticides, and 1,320 registered products. The market for biopesticides was at 1.3 billion dollars globally in 2011, and it may soon top 3.2 billion dollars. The increased demand for organically produced foods is a key factor in this increased usage of biopesticides, along with faster certification by the Environmental Protection Agency (Dutta, 2015).

Many consider neem the most effective biopesticide, and it is ecofriendly (Dutta, 2015). The international scientific community includes neem in the top 10 list for the most promising plants for the "sustainable development of the planet and the health of living beings" (Nicoletti, Maccioni, Coccioletti, Mariana, & Vitali, 2012, p. 414). The World Health Organization and the United Nations Environment Programme, in their 1989 report, stated that neem is "one of the most promising trees of the 21st century for its enormous potential in pest management, environmental protection, and medicine" (Nicoletti et al., 2012, p. 414).

Neem (*Azadirachta indica* A. Juss) is a tropical evergreen tree, which can be deciduous in drier zones (Girish & Shankara, 2008). It is native to the Indo-Pakistan subcontinent, and it grows well in semiarid climates (Ahmed & Grainge, 1986). The neem tree is tolerant of elevated temperatures, but it cannot survive cold or frost. This tree grows well in nutrient-deficient soils, and it can extract any necessary nutrients from its deep root system (Koul, Isman, & Ketkar, 1990). The neem tree bark, leaves, fruit, and seeds have many uses (Ahmed & Grainge, 1986). Ayurvedic medicine has used neem for its properties for over 4,000 years, leading to the native Indian name "Village pharmacy" (Girish & Shankara, 2008, p. 108). For centuries, Indo-Pakistani farmers have soaked their grain storage sacks in a mixture of dried neem leaves and water overnight. They then dry these sacks and use them to prevent pest damage in their stored grain (Ahmed & Grainge, 1986).

Segregation and identification of neem constituents in India began in 1942. The first compounds researchers found were nimbin and nimbinin, with another bitter substance called nimbidin (Koul et al., 1990). Not until 1959 was the neem tree globally

recognized. It was then that noted entomologist, Dr. Heinrich Schmutterer observed that swarming locusts defoliated almost all plant life in Sudan, except for a few neem trees that others had introduced to the area (Ahmed & Grainge, 1986). Since the mid-20th century, neem research has intensified, with it being the most promising natural substance for biological control of pests (Biswas, Chattopadhyay, Banerjee, & Bandyopadhyay, 2002).

Research in subsequent years with neem extracts has shown that they disrupt insect reproduction and development. Researchers have isolated over 135 compounds from the neem tree (Biswas et al., 2002). In the leaves, bark, fruit, and seeds there are many biologically active compounds. "These include protolimonoids, limonoids, or tetranortriterpenoids, pentanortriterpenoids, hexanortriterpenoids, and nontriterpenoidal constituents" (Koul et al., 1990, p. 2). The nontriterpenoidal compounds include "hydrocarbons, fatty acids, diterpenoids, sterols, phenols, flavonoids, and glycosides" (Koul et al., 1990, p. 2).

The most studied are the triterpenoids, which include azadirachtin, salanin, and meliantriol. These triterpenoids control more than 100 species of insects, mites, and nematodes (Ahmed & Grainge, 1986). Studies of azadirachtin have shown that it is an effective antifeedant and growth disruptor in insects (Sinha et al., 1999). Recent research with biopesticides has targeted the larval stage, due to the chance of resistance and adaptation by adults (Nicoletti et al., 2012).

Another aspect of pest control is to target the immune response. Multicellular entities have a two-system response to infectious organisms, innate and acquired (Lavine & Strand, 2002). An innate immune system is an evolutionary type of nonspecific

defense, whereas an acquired defense is a learned way of attacking a specific antigen that develops an immunological memory (Lavine & Strand, 2002). Insects only have an innate immune system, but it is well developed. Initial defenses include the integument and gut, clotting reaction, and cytotoxic molecules produced at the wound site (Lavine & Strand, 2002). Researchers discovered numerous hemolymph proteins whose function is innate immune response first in Lepidopteran insects (Jiang, Vilcinskas, & Kanost, 2010).

Insects also have humoral and cellular defense responses. Humoral defenses deal with antimicrobial peptide production, responsive intermediates of either oxygen or nitrogen, and multifaceted enzymatic cascades that control coagulation or melanization of the insect's hemolymph (Lavine & Strand, 2002). Hemolymph contains the insect's blood cells, or hemocytes (Jones, 1962). Cellular defenses represent a hemocyte-facilitated immune response, such as phagocytosis or encapsulation.

Phagocytosis is a primary response of hemocytes to small particles, such as bacteria, and it is a form of receptor-mediated endocytosis (Gillespie, Kanost, & Trenczek, 1997). It is a defense response, in that the target attaches to the receptor and stimulates the immune cell to create a phagosome (Strand, 2008). Hemocytes phagocytose many biotic targets, including bacteria and yeast, or abiotic elements like India ink (Lavine & Strand, 2002). Active polymerization-dependent processes engulf the target, and the phagosome matures into a phagolysosome, through a succession of fission and fusion actions with endosomes and lysosomes (Strand, 2008).

Unlike phagocytosis, which involves a single cell, encapsulation is the result of multiple hemocytes attaching to larger particles, involving multiple cells (Strand, 2008).

Multilayer sheaths surround the material that undergoes encapsulation, which is why

scholars refer to them as capsules. Encapsulation occurs within 1 to 3 days (Ottaviani,

2005). After formation, the capsule may melanize, and it can undergo digestion or

degradation. Sometimes when describing the attachment of multiple hemocytes to

masses of bacteria, it is referred to as nodulation (Strand, 2008).

Researchers have categorized hemocytes by morphological characteristics,

determined by light or electron microscopy (Gillespie et al., 1997). Hemocytes increase

in number and segregate into distinct types due to stress, infection, and injury responses

(Strand, 2008). The most common types of hemocytes are granulocytes, plasmatocytes,

spherulocytes, and oenocytoids. Scholars have identified these cell types in species from

diverse orders such as "Lepidoptera, Diptera other than *Drosophila*, Orthoptera, Blattaria,

Coleoptera, Hymenoptera, Hemiptera, and Collembola" (Strand, 2008, p. 2).

Granulocytes occur in the hemolymph as rounded cells, which firmly adhere to foreign

substances and spread symmetrically. Plasmatocytes also occur as rounded cells, but

they spread asymmetrically after attachment to a foreign substance. In the larval stage of

Lepidoptera, both granulocytes and plasmatocytes make up over 50% of circulating

hemocytes. Oenocytoids are non-adhesive hemocytes that contain phenoloxidase cascade

elements like prophenoloxidase 1, which leads to melanin production. Spherule cells or

spherulocytes are possible sources of cuticle constituents (Strand, 2008). In Lepidoptera,

there are also small numbers of prohemocytes, which are progenitor or stem cells. These

prohemocytes may differentiate into other hemocytes, especially plasmatocytes and

granulocytes, during times of stress or injury (Strand, 2008). Plasmatocytes are the main

capsule-forming hemocytes in Lepidoptera, but granulocytes are also present. In some

species like *Pseudoplusia includens*, granulocytes first attach to the foreign object, then plasmatocytes adhere in large numbers to the target (Strand, 2008). Granulocytes break down to sticky granular cells after attachment to a foreign substance, and they may release a chemoattractant that activates the plasmatocytes to form the capsule (Ottaviani, 2005).

A previous Missouri State University graduate student, Katherine Haszcz, showed that Sumi ink injections increased numbers of circulating plasmatocytes in *Galleria mellonella* larvae, and that crude neem essential oil reversed this process (Haszcz, 2016). However, the bright light microscopy Haszcz (2016) used made classification of hemocytes problematic. Therefore, further work was necessary to validate Haszcz's results.

Also, Haszcz (2016) performed experiments aiming to delineate which compounds of neem could be responsible for the reversing effects of Sumi ink on circulating plasmatocytes. Haszcz tested azadirachtin, one of the most studied triterpenoids of neem oil. Among many effects, it disrupts insect growth by suppressing the process of molting (Sinha et al., 1999). However, Haszcz's research showed that azadirachtin (at doses that inhibited molting) had no effect on numbers of circulating plasmatocytes in *Galleria mellonella* larvae. While crude neem essential oil effectively impaired the immune response in insects by lowering the numbers of circulating plasmatocytes, it was still unclear what neem constituent was responsible for these outcomes.

The aim of this work was to validate the findings of Haszcz (2016) using a microscope with differential interference contrast (also known as Nomarski contrast), and

a new type of microscopy, which is currently under development by my lab partner Westley Peterson, called pseudo-Nomarski contrast.

In addition, I have extended the work of Haszcz (2016) by isolation and identification of the chemical responsible for reversal of Sumi ink effects on *Galleria mellonella* plasmatocytes (Haszcz, 2016). To that end, I extracted the crude neem essential oil with methanol, and tested the extract in bioassays for its ability to reverse increases of plasmatocyte numbers in response to Sumi ink injections. Moreover, using high-performance thin layer chromatography (HPTLC) and bioassays, I identified a specific neem oil component, stigmasterol, as the neem-derived substance that reverses the process of plasmatocyte mobilization by Sumi ink in *Galleria mellonella* larvae.

METHODS

Insects

The experiments used greater wax moth (*Galleria mellonella*) larvae. This moth belongs to the order Lepidoptera and the family Pyralidae. *Galleria mellonella* live in beehives and nests, consuming wax, honey, and pollen (Wojda, 2017). *Galleria mellonella* has a life cycle of 7-8 weeks, and it undergoes seven larval instars. This takes approximately 5-6 weeks at 25-28°C. Two more weeks are necessary to form adult moths from the pupae (Wojda, 2017). The advantages of this insect are simple procedures for injections, and it is an ethically suitable model for experimentation (Mukherjee et al., 2010). Rearing of *Galleria mellonella* is also cost effective (Wojda, 2017). Researchers have used *Galleria mellonella* extensively for studies in physiology, toxicology, biochemistry, and pathology, among other disciplines. For experimentation involving the rearing of parasitic and predatory insects, *Galleria mellonella* larvae are also useful hosts or prey (Mohamed & Coppel, 1983).

I obtained the larvae from Knutson's Live Bait (Brooklyn, MI) in their 7th instar stage. I separated them into one-pint glass mason jars with vented lids at ~ 60 larvae per jar. I kept them in a light-free incubator (VWR Scientific Products, Model 2005) at 30°C and 80% relative humidity to maintain their normal life cycle. I fed them a diet formulated by combining 37ml of glycerin USP 99.5% (Humco), 25ml of pure granulated cane sugar (C&H), and 25ml of purified water in a plastic bowl. I then heated the mixture ~ 10 to 20 seconds in a low-power microwave to dissolve the sugar, and stirred it thoroughly. I placed this mixture and 400 ml of Gerber® multigrain cereal (Fremont, MI) in a gallon Ziploc bag, and I hand mixed it thoroughly, until a spongy texture formed.

The Gerber® multigrain cereal contains essential vitamins and minerals to maintain the health of the larvae. Excess diet mixture may be stored for up to 2 months at 4°C.

Chemicals

I formulated a stock solution of anticoagulant buffer (0.157g NaOH, 0.435g NaCl, 0.315g citric acid, 0.253g Na₂EDTA, pH 4.58) and neutral red stain (2mg of bacto-neutral red to 1ml of anticoagulant buffer). I obtained NaCl and NaOH from Sigma-Aldrich® (St. Louis, MO); I bought Na₂EDTA and citric acid from Thermo Fisher Scientific (Pittsburg, PA); I acquired bacto-neutral red from Difco Laboratories (Detroit, MI). I specify the other chemicals and reagents and their respective vendors in the following part of the Methods.

Validation of the Results from Previous Studies; Response to Ink

In the hemolymph of *Galleria mellonella*, the plasmatocytes remain in a rounded shape, but they spread asymmetrically when they come into contact with plastic or glass. Therefore, it is easy to distinguish them from other classes of hemocytes.

I used black Sumi ink (Yasutomo, San Francisco, CA) as an artificial pathogen. I pipetted an aliquot of 200μl of the ink into a 2ml microcentrifuge tube, and centrifuged it at 2,000g for 10 minutes to eliminate large particles. I pipetted the supernatant into another microcentrifuge tube and diluted it to the desired concentration with distilled water.

I anaesthetized the 7th instar larvae of *Galleria mellonella* by immersion in water for 10 minutes, blotted them, and injected them with 5μl of ink solution through the proleg with a Hamilton® syringe equipped with 24G needle (Reno, NV). Control larvae

received 5µl of distilled water. I then placed the larvae in glass jars, provided them with food, and stored them in an incubator at 30°C.

I prepared microcentrifuge tubes for each larva with 40µl of anticoagulant buffer and 4µl of neutral red stain. After incubation, I collected hemolymph by cautiously cutting off a proleg with micro iris scissors and bleeding the larva onto a piece of parafilm. I immediately pipetted 5µl of the hemolymph into the prepared tube with the buffer and stain, vortexed, and allowed it to set for 5 minutes at room temperature to stain the cells.

When staining was complete, I then vortexed the prepared tube, pipetted 10µl of the solution with cells to the upper and lower chamber of an improved Neubauer hemocytometer and covered it with a Corning cover glass (thickness 1, 22 x 22mm). The chamber of the hemocytometer filled thanks to capillary action. I then inspected the hemocytometer using a Galen III microscope (Cambridge Instruments, Cambridge), equipped with phase contrast or a custom-made oblique illumination filter (W. Peterson, personal communication, September 26, 2017). I counted the plasmatocytes boustrophedonically, using the outside four corner quadrants.

I used this procedure for two different series of experiments. First, I tested several different concentrations of Sumi ink: 0, 10, 20, 40, and 80%. In this experiment, the incubation time equaled 4 hours, based on the work of Haszcz (2016). In the second experiment, I injected the ink at only one concentration of 40%, but I bled the larvae and counted plasmatocytes after various times of larvae incubation: 30 minutes, 2, 3, 4, 6, and 8 hours. I obtained each data point using 6-14 larvae.

Validation of the Results of Previous Studies; Effects of Crude Neem Essential Oil on Plasmatocyte Mobilization by Ink Injections

Haszcz (2016) used neem essential oil from NOW Foods, Bloomingdale, IL for previous experiments in 2015. For this work, I purchased a new batch of neem essential oil from the same vendor in 2017. I sealed the newly acquired oil with parafilm (American National Can, Chicago, IL) to prevent degradation, and stored it in a refrigerator at 4°C. I measured it precisely in a 2ml microcentrifuge tube by weight, and then I diluted it to a 30% concentration of neem oil using cholesterol-free soybean oil (as solvent), purchased from Cal Western Packaging Corporation, Memphis, TN.

I used water-anaesthetized, 7th instar larvae of *Galleria mellonella*. I injected 10 experimental larvae with 5μl of 40% Sumi ink as described before, and additionally, they received 5μl of neem essential oil dissolved to 0.188, 0.375, and 1.125 mg/larva, in cholesterol-free soybean oil. I injected 10 control larvae with 5μl of 40% Sumi ink and with 5μl of cholesterol-free soybean oil. I provided the injected larvae with food, and I placed them in an incubator at 30°C and bled them after 4 hours. I obtained each data point using 5-9 larvae.

Extraction of Crude Neem Essential Oil

I placed 200μg of neem essential oil in a 2ml microcentrifuge tube, and then I added 1ml of methanol, vortexed the tube, and left it for 10 minutes at room temperature. Next, I centrifuged the tube at 2,000g for 10 minutes. I removed the supernatant to a precisely weighed 2ml microcentrifuge tube. I then rotary evaporated the supernatant (Savant Instruments Inc, Holbrook, NY) for approximately 35 minutes to remove any methanol. I reweighted the tube to determine the final mass of the residue, which I

subsequently resuspended with appropriate solvent to the desired concentration. (For thin layer chromatography, I used methanol, whereas for bioassaying, I dissolved the residue in cholesterol-free soybean oil). I used this procedure for two more extractions, one using ethanol, and another using distilled water instead of methanol.

I visualized the extracted compounds using HPTLC. Briefly, I applied methanolic, ethanolic, and aqueous extracts onto Merck 10 x 10 cm silica gel F_{254} glass plates using a Camag Nanomat 4 HPTLC spotter. I then developed the plates for 6 minutes in a horizontal Camag developing chamber. The mobile phase comprised chloroform and ethyl acetate at an 8:2 volume to volume ratio. I added acetic acid to this mixture at 2% concentration. I air dried the developed plates and viewed and photographed them in ultraviolet light at 254nm. I calculated retention factors (Rfs) for each band. I only used methanolic extract for further experiments.

Effects of Methanolic Extract from Neem Essential Oil on Counts of Plasmatocyte Mobilization by Ink Injections

I prepared the methanolic extract as described above, rotary evaporated it, and suspended it in cholesterol-free soybean oil at 0.188, 0.375, and 0.563 mg/larva concentrations. I water anaesthetized *Galleria mellonella* larvae. I injected 10 larvae with 5μl of 10% methanol-extracted neem and 5μl of 40% Sumi ink. I repeated this procedure with 20% methanol-extracted neem (10 larvae) and 30% methanol-extracted neem (another 10 larvae). Each of 10 control larvae received 5μl of pure cholesterol-free soybean oil. I placed the injected larvae in an incubator at 30°C and bled them after 4 hours. I obtained each data point using 9-27 larvae.

Effects of Aqueous Extract from Neem Bark on Plasmatocyte Mobilization by Ink Injections

I tested aqueous neem bark extract (Sigma, St. Louis, MO) at 100% concentration using the aforementioned procedures. Briefly, I anaesthetized *Galleria mellonella* larvae in water for 10 minutes. Ten experimental larvae received 5μl injections of 40% Sumi ink, combined with 5μl injections of 100% aqueous neem bark extract. Ten control larvae received a combination of ink injections and distilled water injections. I then placed the injected larvae in an incubator at 30°C, and I bled them after 4 hours. I obtained the data points with 10 larvae.

Comparative Chromatography of Neem Essential Oil Extract and Neem Bark Extract

To delineate the biologically active substances in methanolic neem essential oil extract, I used comparative HPTLC. To that end, I applied methanolic extracts at four concentrations (3.75%, 7.5%, 15%, and 30%) onto silica gel F_{254} glass plate using a Camag Nanomat 4 HPTLC spotter. I then developed the plate for 6 minutes in a horizontal Camag developing chamber. The mobile phase comprised chloroform and ethyl acetate at an 8:2 volume to volume ratio. I added acetic acid to this mixture at 2% concentration. I air dried the developed plate. I repeated this procedure with another plate to which I had applied aqueous extract from neem bark at four concentrations (3.75%, 7.5%, 15%, and 30%). I then assembled the plates (one with methanolic extract of neem essential oil and one with aqueous extract of neem bark) side by side and photographed them in ultraviolet light at 254 nm. I calculated Rfs for each band.

Confirmation of Stigmasterol Presence in Methanolic Extract of Neem Essential Oil

I extracted neem essential oil with methanol as previously described, rotary evaporated it, and diluted it to 15% concentration using pure methanol. I diluted stigmasterol acetate (Chem Cruz, Dallas, TX) with pure methanol to 10%, 20%, and 30% concentrations. I then applied the chemicals to a silica gel F_{254} glass plate in the following pattern: 10%, 20%, and 30% stigmasterol, 15% methanolic extract of neem essential oil, and again 10%, 20%, and 30% stigmasterol. I then developed the plate as described above, air dried it, and photographed it in ultraviolet light at 254 nm. I calculated Rfs for each band.

Effects of Stigmasterol Acetate on Plasmatocyte Mobilization by Ink Injections

I diluted stigmasterol acetate, which I purchased from Chem Cruz, Dallas, TX, to 0.071, 0.141, and 0.282 mg/larva concentrations in cholesterol-free soybean oil. I then kept all concentrations on ice for the injections. I water anaesthetized the larvae for 10 minutes. I then subjected the experimental larvae to a 5μl injection of 40% Sumi ink concurrently with various concentrations of stigmasterol acetate in 5μl of cholesterol-free soybean oil. The control larvae received 5μl of 40% ink and 5μl of cholesterol-free soybean oil. I placed the injected larvae in an incubator at 30°C, and I bled them after 4 hours. I obtained the data points with 10-16 larvae.

Statistical Analysis

I analyzed the data I obtained for the effects of aqueous extract from neem bark on plasmatocyte mobilization by ink injections using Student's *t* test. In all remaining experiments, I used ANOVA, followed by Tukey multiple comparison of means. I tested

the data using GraphPad InStat, (GraphPad Software, San Diego, CA). I set the statistical

significance level at $p < 0.05$.

RESULTS

Validation of the Results from Previous Studies; Response to Ink

Haszcz (2016) showed that numbers of plasmatocytes circulating in the hemolymph of *Galleria mellonella* larvae increased together with increasing doses of injected Sumi ink, and together with post injection time of insect incubation.

This work confirms the findings of Haszcz (2016). Sumi ink injections increased plasmatocyte counts from about 16×10^5 cells per ml of hemolymph to about 33×10^5 cell per ml of hemolymph, in a dose-dependent manner (Table 1). At 40% and 80% ink concentrations, the increase of plasmatocyte numbers was statistically significant ($p < 0.05$, ANOVA followed by Tukey Multiple Comparison of Means Test). Also, I showed that *Galleria mellonella* larvae respond to Sumi ink injections in a time-dependent manner (Table 2). Plasmatocyte numbers increased significantly at 3 hours after ink injection, and they stayed significantly elevated for the next 5 hours ($p < 0.05$, ANOVA followed by Tukey multiple comparison of means).

Table 1. Effects of various Sumi ink concentrations on plasmatocyte counts in *Galleria mellonella* larvae. Larva bleeding took place 4 hours after injection of the ink. Means followed by the same letter are not significantly different ($p > 0.05$, ANOVA followed by Tukey multiple comparison of means)

Sumi ink concentration (%)	Number of plasmatocytes (10^5 cells/ml)	*N*
0 †	10.21 ± 1.10 a	6
10	16.34 ± 1.91 a	14
20	15.55 ± 2.86 a	13
40	24.73 ± 3.28 b	11
80	32.91 ± 3.99 b	10

† The zero-concentration solution was distilled water.

Table 2. Time dependent effects of Sumi ink on plasmatocyte counts in *Galleria mellonella* larvae. Larvae received injections of 40% Sumi ink and bleeding took place at various times after injection. Means followed by the same letter are not significantly different ($p > 0.05$, ANOVA followed by Tukey multiple comparison of means)

Time after injection (hours)	Number of plasmatocytes (10^5 cells/ml)	*N*
0.5	8.74 ± 1.3 a	13
2	11.50 ± 1.49 a	11
3	17.92 ± 1.81 b	12
4	24.73 ± 3.28 b	11
6	25.41 ± 2.76 b	11
8	28.10 ± 4.27 b	6

Validation of the Results from Previous Studies; Effects of Crude Neem Essential Oil on Plasmatocyte Mobilization by Ink Injections

Haszcz (2016) showed that numbers of circulating plasmatocytes mobilized by Sumi ink decreased after the injection of crude neem essential oil at various doses. The present work confirms the findings of Haszcz.

Injections of Sumi ink combined with cholesterol-free soybean oil resulted in plasmatocyte counts of approximately 11 x 10^5 cells per ml of hemolymph. Injections of neem essential oil, at doses of 0.188, 0.375, and 1.125 mg/larva, to insects challenged with Sumi ink significantly lowered plasmatocyte counts (Table 3, $p < 0.05$, ANOVA followed by Tukey multiple comparison of means).

Extraction of Crude Neem Essential Oil

A high-performance thin layer chromatogram of methanol-extracted neem essential oil revealed four distinct bands (Table 4, Figure 1), while in the case of the ethanolic extract only two distinct bands were visible. The remaining two bands (3 and

Table 3. Dose-dependent effects of neem essential oil on plasmatocyte counts in *Galleria mellonella* larvae challenged with Sumi ink. Larvae received injections of 40% Sumi ink and various concentration of neem essential oil, and bleeding took place 4 hours after injection. Means followed by the same letter are not significantly different ($p > 0.05$, ANOVA followed by Tukey multiple comparison of means)

Dose of neem essential oil (mg/larva)	Number of plasmatocytes (10^5 cells/ml)	*N*
0†	10.92 ± 0.54 a	9
0.188	5.68 ± 0.18 b	6
0.375	4.50 ± 0.27 b	5
1.125	5.91 ± 0.11 b	9

† The zero-concentration solution was cholesterol-free soybean oil.

4) did not separate distinctively (Table 4, Figure 1). The aqueous extract did not produce any bands. Therefore, methanolic extracted neem was suitable for further testing.

Table 4. Summary of the high-performance thin layer chromatogram of three extracts from neem essential oil.

Lane	Extract	Rf Values	Remarks
A	Methanolic	0.289, 0.515, 0.764, 0.838	Well-defined bands
B	Ethanolic	0.277, 0.513	Bands 3 and 4 poorly defined
C	Aqueous	NA	No bands visible

Effects of Methanolic Extract from Neem Essential Oil on Plasmatocyte Mobilization by Ink Injections

Methanolic extracts of neem essential oil reversed the mobilization of plasmatocytes by Sumi ink. Sumi ink injections combined with cholesterol-free soybean oil resulted in plasmatocyte counts of 9.3×10^5 cells per ml of hemolymph. Methanolic extracted neem injected to ink-challenged larvae at doses of 0.188, 0.375, and 0.563 mg/larva, effectively lowered plasmatocyte counts (Table 5, $p < 0.05$, ANOVA followed by Tukey multiple
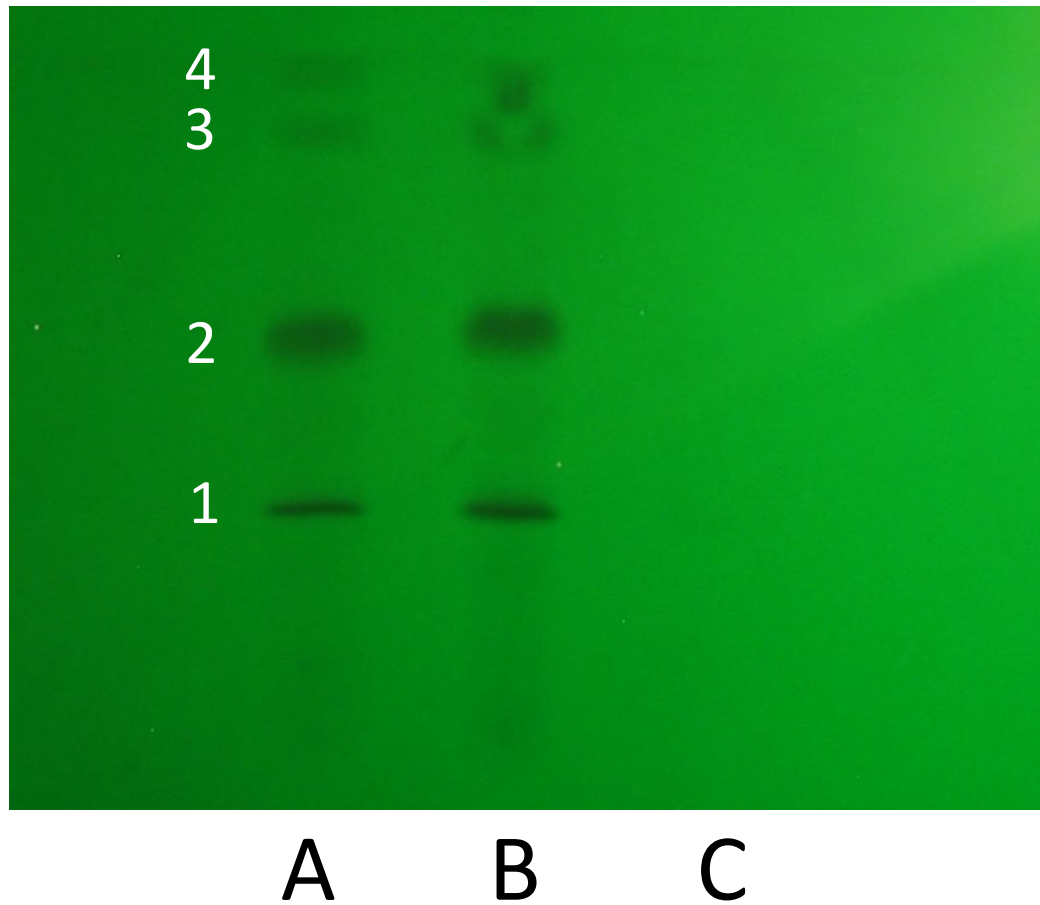
Figure 1. High-performance thin layer chromatogram of three extracts from neem essential oil. (A) methanolic extract, (B) ethanolic extract, (C) aqueous extract. All the extracts had a 30% weight/volume concentration.

comparison of means).

Effects of Aqueous Extract from Neem Bark on Plasmatocyte Mobilization by Ink Injections

Neem bark aqueous extract had no effect on the numbers of plasmatocytes Sumi ink mobilized. Sumi ink injections combined with injections of distilled water resulted in plasmatocyte counts similar to those in previous tests, with approximately 9.3×10^5 cells

Table 5. Dose-dependent effects of methanolic extract from neem essential oil on plasmatocyte counts in *Galleria mellonella* larvae challenged with Sumi ink. Larvae received injections of 40% Sumi ink and various concentrations of methanolic extract from neem essential oil, and bleeding took place 4 hours after injection. Means followed by the same letter are not significantly different ($p > 0.05$, ANOVA followed by Tukey multiple comparison of means).

Dose of the extract (mg/larva)	Number of plasmatocytes (10^5 cells/ml)	*N*
0†	9.30 ± 0.77 a	27
0.188	4.96 ± 0.33 b	10
0.375	4.26 ± 0.17 b	9
0.563	4.90 ± 0.41 b	18

† The zero-concentration solution was cholesterol-free soybean oil.

per ml of hemolymph. Injections of 100% aqueous extract of neem bark to ink-challenged larvae resulted in a slight decrease in plasmatocyte counts numerically, but this difference was not statistically significant. (Table 6, $p > 0.05$, unpaired Student's *t* test).

Table 6. Effect of neem bark aqueous extract on plasmatocyte counts in ink challenged *Galleria mellonella* larvae. Larvae received injections of 40% Sumi ink and one concentration of neem bark aqueous extract, and bleeding took place 4 hours after injection. Means followed by the same letter are not significantly different ($p > 0.05$, unpaired Student's *t* test).

Concentration of neem bark aqueous extract (%)	Number of plasmatocytes (10^5 cells/ml)	*N*
0†	9.26 ± 1.40 a	10
100%	7.83 ± 1.80 a	10

† The zero-concentration solution was distilled water.

Comparative Chromatography of Neem Essential Oil Extract and Neem Bark Extract
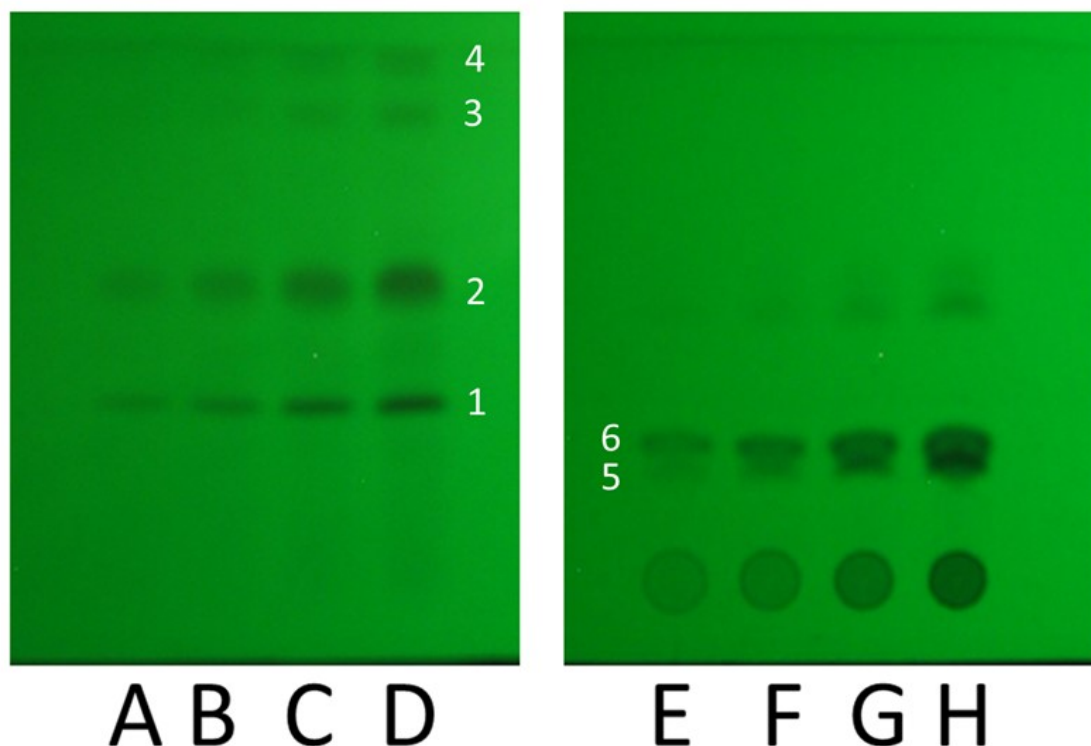
The methanolic extract from neem essential oils showed four well-defined chromatographic bands (Figure 2, Bands 1-4) that had Rfs corresponding with four Rfs from the previous experiment with chromatography of neem methanolic extract (Table 4, Table 7). Chromatography of the aqueous extract from the neem bark extract revealed only two chemical constituents (Figure 2, Bands 5 and 6). The Rfs of Bands 5 and 6 were lower than those of any band in the chromatogram of neem methanolic extract (Figure 2, Lanes A-D). Consequently, the bands produced by aqueous neem bark extract did not collocate with the bands produced by neem methanolic extract, showing that these two extracts do not share their chemical constituents.

Table 7. Summary of comparative high-performance thin layer chromatograms of methanolic extract from neem essential oil and aqueous extract from neem bark

Lanes	Extract	Rf Values	Remarks
A-D	Methanolic extract from neem essential oil	0.295, 0.509, 0.769, 0.850	Well-defined bands
E-H	Aqueous extract from neem bark	0.220, 0.257	Poor separation of bands.

Confirmation of Stigmasterol Presence in Methanolic Extract from Neem Essential Oil

A comparison of chromatographic fingerprints for methanolic extract from neem essential oil with those obtained for stigmasterol acetate showed that the band with Rf 0.296 (Figure 3, Band 1), present in the methanolic extract, collocated with the bands produced by each stigmasterol solution (Figure 3, Table 8). This result proves that stigmasterol is indeed present in methanolic extract of neem essential oil.

Figure 2. Comparative high-performance thin layer chromatogram of methanolic extracts from neem essential oil (Lanes A-D) and aqueous extract of neem bark (Lanes E-H). Both extracts had 3.75, 7.5, 15, and 30% weight/volume concentration.

Table 8. Summary of comparative high-performance thin layer chromatograms of methanolic extract from neem essential oil and methanolic solutions of stigmasterol acetate

Lane	Chemical substance	Rf Values for Band 1	Remarks
A-C	Stigmasterol	0.296, 0.294, 0.293	Collocation with neem
D	Methanolic extract from neem essential oil	0.296	Collocation with stigmasterol
E-G	Stigmasterol	0.296, 0.294, 0.293	Collocation with neem
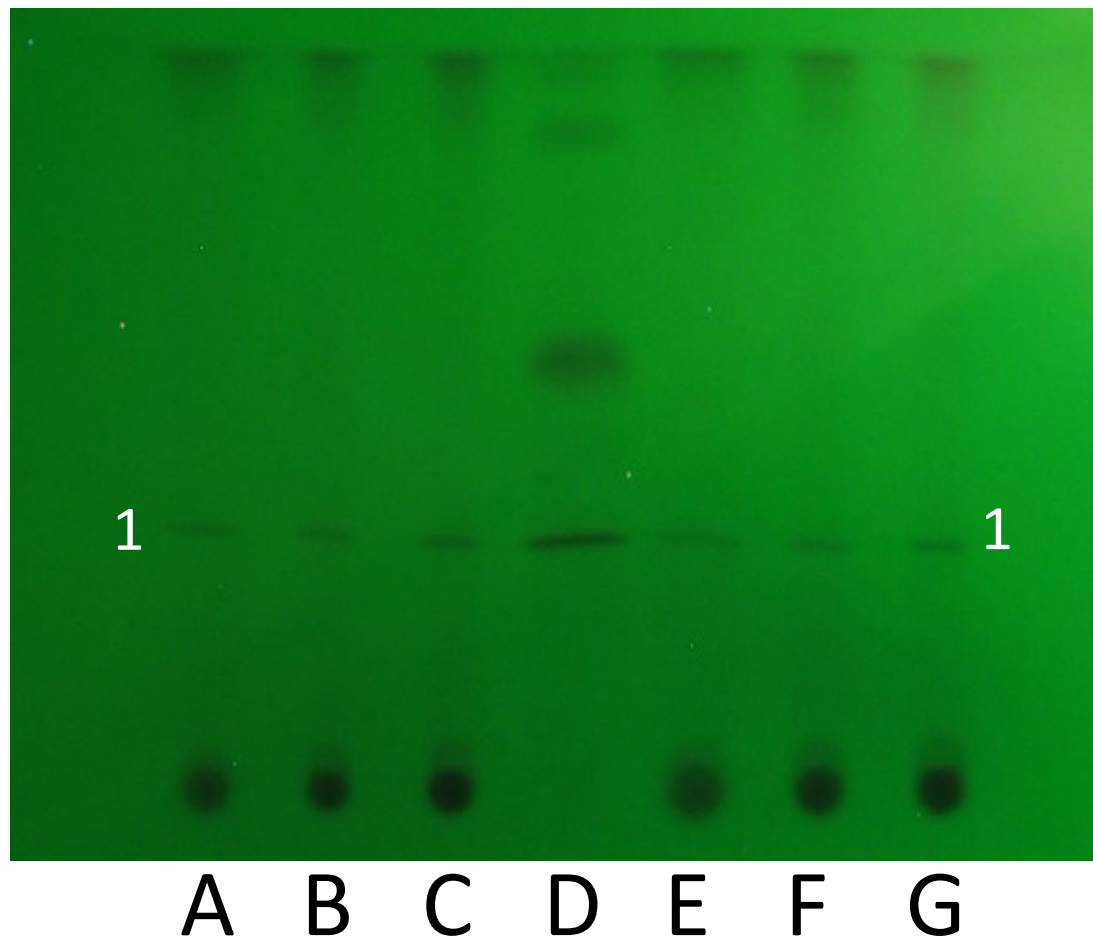
Figure 3. Comparative high-performance thin layer chromatogram of stigmasterol acetate (Lanes 1-3), methanolic extract from neem essential oil (Lane 4), and stigmasterol acetate again (Lanes 5-7). The stigmasterol had 10, 20, and 30% weight/volume concentration. The methanolic extract from neem essential oil had 15% weight/volume concentration.

Effects of Stigmasterol Acetate on Plasmatocyte Mobilization by Ink Injections

Stigmasterol acetate lowered plasmatocyte counts in *Galleria mellonella* larvae challenged with Sumi ink. Together with increasing doses of stigmasterol, plasmatocyte counts dropped from about 17×10^5 to less than 6×10^5 cells per ml of hemolymph in a dose-dependent manner (Table 9). Plasmatocyte counts dramatically lowered,

confirming the effectiveness of stigmasterol acetate ($p < 0.05$, ANOVA followed by

Tukey multiple comparison of means).

Table 9. Dose-dependent effects of stigmasterol acetate on plasmatocyte counts in *Galleria mellonella* larvae challenged with Sumi ink. Larvae received injections of 40% Sumi ink and various concentration of stigmasterol acetate, and bleeding took place 4 hours after injection. Means followed by the same letter are not significantly different ($p > 0.05$, ANOVA followed by Tukey multiple comparison of means)

Dose of stigmasterol acetate (mg/larva)	Number of plasmatocytes (10^5 cells/ml)	*N*
0†	17.09 ± 2.91 a	16
0.071	6.73 ± 0.72 b	10
0.141	4.80 ± 0.69 b	12
0.282	5.08 ± 0.45 b	10

† The zero-concentration solution was cholesterol-free soybean oil.

DISCUSSION

This research examined neem and its constituents for their potential of impairing insect immune systems. Former Missouri State graduate student Katherine Haszcz found that Sumi ink increased the number of circulating plasmatocytes in the 7th instar larvae of *Galleria mellonella*. Plasmatocytes are primary hemocytes involved in encapsulation in the lepidopteran order of insects. Haszcz showed that a 40% concentration of Sumi ink at 4 hours post injection was optimal for the increase in plasmatocytes and their mobilization (Haszcz, 2016). However, she used bright light microscopy in her research, which did not allow for accurate differentiation of particular classes of hemocytes. The results of microscopy based on differential interference contrast and oblique illumination filters, and shown in the present dissertation, confirmed the previous findings of Haszcz (2016).

Sumi ink indeed elevates numbers of circulating plasmatocytes in a dose-dependent manner (Table 1), and in a time-dependent manner (Table 2). Moreover, optimal concentration of the ink and optimal time of incubation in my experiments were the same as in the work of Haszcz (2016). Neem essential oil reversed the effects of Sumi ink, as in Haszcz (2016).

The experimentation presented in the current thesis expanded the findings of Haszcz (2016) by extracting neem biologically active substances and identifying stigmasterol as the chemical that reverses plasmatocyte mobilization by Sumi ink.

For chromatography with neem, I tried three extraction solvents: methanol, ethanol, and water. I subjected all three extracts to comparative HPTLC (Figure 1). Of the three solvents, methanol worked the best: all the bands were visible and well defined.

Ethanol had the first two bands well defined, with the other bands obscure and not distinguishable. Water failed to work in this experiment, as there were no visible bands.

These findings are in accordance with previous work by Sharma, Dua, and Srivastva (2014), who investigated the antibacterial activity of extracts made from neem seed, leaf, bark, and root. In their experiments, methanolic extracts had the highest antibacterial activity against both *Escherichia coli* and *Bacillus amyloliquefaciens*, whereas ethanolic extracts were not as effective. Interestingly, aqueous extracts of neem seed and root also had some antibacterial activity in the experiments performed by Sharma et al. (2014). The fact that water was ineffective in the extraction experiments reported in the present thesis might have been the result of different methods of extraction. Sharma et al. made their aqueous extract by boiling plant tissues in water for 30 minutes at 100°C, whereas in the experiments reported herein, extraction took place at room temperature for 10 minutes.

There are other reports showing that methanol is a good solvent for extraction of biologically active substances from neem. For instance, Stark and Walter (1995) used methanolic extracts of neem to enhance the activity of several insecticides against pea aphids.

De and Ifeoma (2002) conducted their study with extracts of *Azadirachta indica* to compare its antimicrobial spectrum with that of conventional antibiotics used against pathogenic fungi and bacteria. They extracted their samples of powdered neem bark and leaves separately with water, acetone, or methanol. The aqueous extracts failed to show antimicrobial activity at any concentration on the test organisms, whereas the methanolic

extracts of had comparable results to antibiotics generally used to treat infections caused by the test organisms (De & Ifeoma, 2002).

This research shows that aqueous neem bark extract had no effect on plasmatocyte counts in *G. mellonella* larvae, whereas methanol-extracted neem lowered plasmatocyte counts, which is in accordance with the works of Sharma et al. (2014), De and Ifeoma (2002), and Stark and Walter (1995).

I also used HPTLC to delineate substances of interest in neem essential oil. Nicoletti et al. (2012) used HPTLC to analyze several commercially available neem products. In their research, HPTLC displayed significant variations in chemical composition of particular neem products; however, methanol consistently extracted the principal active metabolites of neem: nimbin, salanin, azadirachtin A, and azadirachtin B. It is difficult to compare my comparative HPTLC of neem (Figure 2) with the chromatograms of Nicoletti et al., because these authors used a mobile phase for chromatography, which differed from that in my experiments.

It is clear, however, that methanolic extract of neem essential oil (which lowers plasmatocyte counts in ink-challenged *Galleria mellonella* larvae) contains chemicals that are not present in the aqueous extract of neem bark (which does not affect plasmatocyte counts). In particular, it is the band of Rf equaling 0.295 (Figure 2, Band 1) that collocates with the stigmasterol standard (Figure 3).

Bioassays confirmed that stigmasterol (Figure 4) lowers plasmatocyte counts in ink-challenged larvae of *Galleria mellonella* (Table 9). This finding is novel, and therefore difficult to assess.

However, there is a body of research showing that this plant secondary metabolite exhibits biological activity in animal systems. For instance, stigmasterol has been successful in docking studies against Human Epidermal Growth Factor Receptor-2 (Sugappriya, Sudarsanam, Bhaskaran, Joseph, & Suresh, 2017). Stigmasterol is also the most likely inhibitory resource against the envelope protein VP28, which is a major factor in white spot syndrome virus, a disastrous disease of shrimp (Sahu, Kathiresan, Singh, & Senthilraja, 2012). In another study, a mixture of *β*-sitosterol and stigmasterol extracted from the roots of *P. indica* proved to be effective as an antidote to snake venom (Gomes Saha, Chatterjee, & Chakravarty, 2007). Behmer and Grebenok (1998) tested various sterols in the diet of a lepidopteran, *Plutella xylostella*, and they found that stigmasterol was the only one to lower the survival rate of the moths.

More important, stigmasterol showed its potential as an anti-inflammatory agent in studies of asthma (Antwi, Obiri, & Osafo, 2017), and it has antipyretic effects on mice (Antwi, Obiri, Osafo, Forkuo, & Essel, 2017). Known compounds that have anti-inflammatory and antipyretic effects have also been successful in insect immune response. Büyükgüzel, Tunaz, Stanley, and Büyükgüzel (2007) researched cellular immune responses to viruses in *Galleria mellonella* larvae, and they found that indomethacin, an anti-inflammatory drug, inhibited that response.

This dissertation lays the groundwork for further testing of stigmasterol. This plant-derived compound impaired the immune defense systems in *G. mellonella* larvae. In previous studies with neem oil, it appeared that azadirachtin was the essential compound of interest. Azadirachtin has proven its effectiveness as an antifeedant in

insects, but it failed to show promising results when tested for impairment in the insect immune system (Haszcz, 2016).

Stigmasterol lowered circulating plasmatocyte counts to < 50% in ink-challenged larvae. This significant finding allows for larvicidal treatment, where the immune system of insects is most vulnerable, and allows less chance for adaptation. Future bioassays with the lepidopteran order of insects, as well as others, are necessary to show the potential of stigmasterol as a possible plant-based insecticide.

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