Fall 2021

Effects of "Lemongrass Factor" on Galleria Mellonella Hemocytes

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EFFECTS OF "LEMONGRASS FACTOR" ON *GALLERIA MELLONELLA* HEMOCYTES

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

Jennifer C. Rice

December 2021

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ABSTRACT

The use of conventional chemical insecticides to control agricultural pest has become problematic as they may have a negative impact towards human health and the environment, resulting in a need to research alternative methods to insect pest control. Plant derived substances like essential oils have been used for generations as toxicants, repellants, and anti-feedants to control agricultural pest. More research is needed to understand how insect immune systems react to essential oils and if cellular immune responses of phagocytosis, encapsulation, and nodulation can be inhibited by such. Subjecting *Galleria mellonella* to various concentrations of "lemongrass factor" lets the researcher/scientist know which concentrations to use during experimentation and which concentrations are lethal to our test subject. By challenging the immune system of *Galleria mellonella* larvae with sumi ink we activate the immune responses within the system, followed by an injection of "lemongrass factor" or topical application of the same essential oil to dorsal side of larva. This allows determination of what hemocytes are present, their numbers, and if cellular processes like phagocytosis is occurring between the hemocyte profiles. In this present study it was determined that "lemongrass factor" may inhibit cellular processes of insects by lowering numbers of hemocytes present and by lowering the intensity of phagocytosis. Our results suggest that "Lemongrass factor" may be suitable as a bio-insecticide to control the greater wax moth, *Galleria mellonella*, a known agricultural pest.

KEYWORDS: *Galleria mellonella*, hemocytes, insect innate immune system, lemongrass, essential oils, bioinsecticides

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ACKNOWLEDGEMENTS

I thank Dr. Maciej Pszczolkowski for the opportunity to continue my education. He offered me a very comfortable position in his lab, with the groundwork done over the experimental design of my project. It has been an eye-opening experience for me. It has been a pleasure learning from him and improving my lab skills. He has been very patient with me through this whole learning experience and has helped me grow as a person and learn how to question the unknown. I had very minimal lab experience before graduate school, and he took a chance on a non-traditional college student and I am forever grateful for his decision. He is a very understanding teacher and advisor, and I wouldn't be where I am today if it wasn't for him. I also thank Kyndra Chastain, my lab Mom, for teaching me our lab procedures and helping me understand our research. I thank Dr. Hwang and Marilyn Odneal for serving on my advisory committee.

I thank Danelle Maxwell with Ozark Technical Community College, for sparking my interest in agricultural and plant science. I thank my Mom, Janet and stepdad, Billy for keeping me grounded and always encouraging me to do and be better. I thank my boyfriend, Matt for being there for me during graduate school. I thank many of my wonderful family and friends for showing me support over the years.

I dedicate this thesis to my Mom, Janet. My mom means everything to me and I'm glad she got to see me achieve yet another goal of mine. Love you forever and always Momma.

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INTRODUCTION

Vertebrates like us mammals, birds, reptiles, amphibians, and fishes have immune systems that consist of innate and adaptive immune responses. These responses help to keep us safe in a world full of threats. Pathogenic threats like viruses, bacterium, and other parasites that have the potential to cause disease and early death, just to name a few.

The innate immune response of vertebrates is the first response to be activated in the presence of a pathogenic invader without prior exposure (Zimmerman *et al*., 2009). This response consists of non-specific molecules, cells, and receptors to stop the spread of further infection and initiates both immune responses (Zimmerman *et al.*, 2009). Once the innate immune response is activated, the adaptive immune response is alerted a few days to a week after exposure (Zimmerman *et al.*, 2009). The adaptive immune responses consist of cellular and humoral responses. These responses work together towards antibody production to fight off the infection from pathogenic invaders and immunological memory is created for future infections (Zimmerman *et al.*, 2009).

Unlike vertebrates, invertebrates, such as insects do not have an adaptive immune response. Insects are dependent on their intricate innate immune response when invaded by a foreign entity or pathogenic organism (Lavine & Strand, 2002). Insect immune responses rely on anatomical and physiological barriers, and humoral and cellular immune responses.

Once the foreign entity penetrates the exoskeleton barrier of an insect it encounters cytotoxic molecules and coagulation of the insect blood, known as hemolymph, to further slow penetration. If foreign entities can make it through the barriers and into the hemocoel, the

primary body cavity of most invertebrates, containing circulatory fluid, they are met with humoral and cellular immune responses (Hillyer, 2016).

Humoral responses include defense molecules such as antimicrobial peptides, radicals, proteins, and the enzymatic cascades that are responsible for clotting and melanization of insect hemolymph (Strand, 2008). Cellular responses include phagocytosis, encapsulation and nodulation which are defense mechanisms performed by the blood cells (hemocytes) of the insect to eradicate the foreign entities (Lavine & Strand, 2002; Strand, 2008; Rosales, 2017). Humoral and cellular immune responses work synonymously together to perform these defense mechanisms and fight off the foreign entity. When insect hemocytes, are challenged in the presence of pathogenic organisms, the cellular immune response is to increase hemocyte production (Haszcz, 2016; Leite *et al.*, 2021).

Phagocytosis is usually the first response and activates the other immune responses, if needed (Hillyer, 2016; Berger & Jurčovà, 2012). This process occurs when hemocytes ingest pathogens directly into the cell itself, degrade with enzymes, and expel from the cell (Rosales, 2017). If phagocytosis is unsuccessful at maintaining the pathogen, the process of nodulation and encapsulation of hemocytes develop around the foreign entities.

If a large number of pathogenic organisms like bacteria are present in the hemolymph the nodulation process is activated and may assist in elimination (Hillyer, 2016; Pereira *et al.*, 2018). Granulocytes adhere first to the foreign organisms, attracting plasmatocytes to form aggregates around them and the foreign organism, eventually forming a nodule, followed by melanization (Hillyer, 2016; Pereira *et al.*, 2018).

Encapsulation is similar to the nodulation response. During encapsulation the same hemocytes enclose larger foreign organisms like parasitic eggs and nematodes in a capsule

consisting of many hemocyte layers and eventually suffocating the foreign organism (Hillyer, 2016; Rosales, 2017; Pereira *et al.*, 2018). Depending on the type and amount of pathogen determines which processes are activated, if not all processes working together to handle the threat of disease or death from the infection.

Hemocytes play in integral role in immune responses of insects. According to multiple studies there are roughly 7 hemocyte classes identified in different insect orders. Not all insects have the same hemocytes. For this thesis project our focus was on *Galleria mellonella* a species found in the insect Order: Lepidoptera. The different types of hemocytes classes found in this species of insects are plasmatocytes, granulocytes, spherulocytes, oenocytoids (Gongqing, 2016). Hemocytes are classified by their morphology and functions they carry out.

Plasmatocytes have adhesion properties and participate in phagocytosis, encapsulation, and nodulation. Granulocytes also have adhesion properties and help aid plasmatocytes in cellular processes. Plasmatocytes build the bulk capsules during the encapsulation and nodules during the nodulation process (Ribeiro & Brehélin, 2006). Plasmatocyes and granulocytes are said to be the most common hemocyte classeses found in *Galleria mellonella* (Pereira *et al.*, 2018).

Spherulocytes are non-adhesive and said to be the source of cuticular components (Strand, 2008; Pereira *et al.*, 2018). Its functions are not well known or studied (Ribeiro & Brehélin, 2006; Pereira *et al.*, 2018). Oenocytoids are non-adhesive and contain phenoloxidase (PO) cascade components, which aide in melanization and hemolymph clotting (Strand, 2008; Pererira *et al.*, 2018).

Hemocytes circulate in the hemolymph (blood) of the insect and are responsible for immune responses. Majority of hemocytes remain in circulation as to where some hemocytes can

attach (sessile) and detach to tissues/organ in the hemolypmh (Hillyer, 2016). Hemocytes originate in two stages. The first set of hemocytes develops during embryogenesis from head or dorsal mesoderm and the second set of hemocytes are developed and released during the larval stage in specialized hematopoietic organs, such as lymph glands, where cellular differentiation occurs (Strand, 2008; Stoepler, 2012). Hemocytes are then maintained in the circulating system of the hemolymph during all life cycles of the insect (Strand, 2008).

Vertebrates have adaptive immune responses which helps them create an immune memory for repeated infection. Even though invertebrates lack an adaptive immune response their innate immune response exhibits certain mechanisms to keep immunity called immune priming (Wojda *et al.*, 2017). Immune priming activates an increased hemocyte production and creates a resistance towards the pathogen as a way to protect itself against a potential re-infection of the same pathogen or similar (Hillyer, 2016).

Cellular and humoral immune responses of an insect immune system have a mutualistic relationship when fighting off pathogenic organisms. Insects are found almost everywhere on this planet, and they are exposed to living and non-living factors that may affect their overall health. The insect immune system recognizes living organisms such as bacteria, fungi, parasites, viruses, and other insects (Hillyer, 2016; Rosales, 2017). The system can also recognize non-living factors such as temperature, humidity, insecticides, and inert objects and particles like synthetic beads, nylon implants, yeast particles, and different types of ink that have been used determine phagocytic potential (Yokoo *et al.*, 1995; Lavine & Strand, 2002; Haszcz, 2016). Indian ink is used to artificially mimic a foreign entity when trying to determine phagocytic potential, which increases the production of hemoctyes within the system. For instance, in a

previous study by Yokoo *et al.* (1995), it was reported that granulocytes were able to phagocytize the particles of Indian ink.

Agricultural operations have become reliant upon conventional chemical control of pest to produce higher yields and profit. Agricultural insect pests are considered organisms that decrease the value of crops and food production, lower crop yields and productivity, and introduce pathogens to crops, affecting food quality. Insect pests can cause crop damage from eating leaves, stems, fruit, and roots causing a loss of revenue.

With an ever-growing human population, it is important to understand insect control to avoid agricultural losses and provide for a growing human population. Many studies have found that conventional insecticide control may have a negative impact towards human health and the environment (Alewu & Nosiri, 2011; Nicolopoulou-Stamati *et al.*, 2016). As most of us know, insecticides cause toxicity to humans and animals, insecticide resistance insects, water and soil pollution, and long persistence in the environment (Alewu & Nosiri, 2011; Köhler & Triebskorn, 2013; Nicolopoulou-Stamati *et al.*, 2016; Richardson *et al.*, 2020). With these problems, comes a need to identify alternative methods to insect control.

Bio-insecticides are natural products that are used to manage agricultural pests. As an alternative to conventional insect control, bio-insecticides create options for pest control, can be used to target certain pest, and have less environmental risk. Bio-insecticides are not a single solution. They are subject to insect resistance as other pesticides but if used correctly and rotated with other forms of pest control, they may help us manage destructive agricultural insect pest with less use of conventional insecticides.

Over time insects have built a resistance to conventional chemical control. Insect resistance occurs when insects become desensitized to the active component in conventional

chemical control and the efficacy of the insecticide decreases. This can lead to an overuse of chemical applications to control insect pests, adding to more resistance and environmental pollution. Agriculturists, scientists, and researchers have been studying alternative management practices to control insects in a more environmentally friendly approach.

Studies on plant-derived oils are a part of such efforts. If we can add a plant-derived essential oil that may inhibit or weaken the cellular responses, then we hope to lower hemocyte numbers of the insect. With a decrease in hemocyte number, we hypothesize that mediated cellular immune response of phagocytosis, encapsulation and nodulation of the pathogen will also decrease.

Moth and butterflies are in the insect order Lepidoptera. Combined there are roughly 180,000 species in this insect order with moths accounting for the majority. Lepidopteran species have a wide distribution range throughout the world (Kwanda, 2017). During the larval stage, is when these insects are the most destructive towards agricultural products. Adults of this insect order are mostly pollinators. Some common larvae/caterpillar species that are well known are: Mexican Rice Borer (*Eoreuma loftini* (Dyar)); Army Cutworm (*Euxoa auxilians*); Common Bagworm (*Thyridopteryx ephemeraeformis* Haworth); Cabbage Looper (*Trichoplusia ni*); Tobacco Hornworm (*Manduca sexta* (L.)); Diamondback Moth (*Plutella xylostella*); Potato Tuber Moth (*Phthorimaea operculella*); Fall Army Worm (*Spodoptera frugiperda*); Cotton Bollworm (*Helicoverpa zea*); and The Greater Wax Moth (*Galleria mellonella*).

Larval Lepidopteran species they have become a special interest to agricultural operations big and small, along with the research world due to their destructive nature. Larval Lepidopteran species feed on almost every agriculture commodity that's used to feed an ever-growing population. Larval lepidopteran species have chewing mouthparts and feed on plant

tissue like foliage, roots, stems, shoots, and fruits. They affect a wide array of agricultural products including rice; sugar; corn; sorghum; honey; a plethora of vegetables and fruits; along with forestry products like conifers and deciduous trees.

Our test subject, *Galleria mellonella*, a Lepidoptera species is not only considered an agricultural pest; it is also considered a novel test subject for immunological research over its innate immune responses (Jiang, 2010). In comparison to mammalian test subject, *Galleria mellonella* is considered a more ethical test subject with fewer requirements and approvals to be used as such (Pereria *et al.*, 2018). *Galleria mellonella* is considered an agricultural pest of honeybees colonies with a wide distribution range that have a major impact towards honey bee colonies and their hives, agricultural products produced from honey bees, and are also considered vectors of disease (Kwadha, 2017; Pereira *et al.*, 2018; Beyene & Woldatsadik, 2019). It is important to understand how to control these pests.

I wanted to investigate an essential oil that could potentially be used to formulate bio-insecticides and how the immune system reacts to the oil. This is an understudied area and has gained popularity from home to farm use. Based on the work of my predecessor (Peterson, 2019; Peterson, personal communication; Pszczolkowski personal communication), I have selected a chemical present in lemongrass oil as a subject of my experimentation. Although chemical name and structure are known to me, for the purpose of confidentiality, I will be referring to that chemical as “lemongrass factor”.

METHODS

Insects and Diet

Galleria mellonella larvae were purchased from Knutson's Live Bait in Brooklyn, MI. The larvae's diet consisted of a handmade mixture of 400ml of Gerber multigrain cereal, 37ml glycerin, 25ml purified water, and 25g cane sugar.

The glycerin, purified water, and cane sugar were mixed in a bowl and placed into the microwave for up to 20 seconds. This allowed the mixture to be stirred and to make sure the ingredients were dissolved. Next, that liquid was added to 400 ml Gerber multigrain cereal and the mixture was placed in a plastic zip-lock bag. To make sure the liquid was mixed with the cereal it was rolled together and the large aggregates pinched into the dry cereal in the plastic bag until there was even saturation throughout. The left-over diet was stored in a refrigerator with a temperature of 4°C. The larvae were reared in glass jars with perforated lids in a dark incubator that was set at 35°C and 80% relative humidity. Last instar larvae were used for all experiments.

Chemicals

Anticoagulant buffer solution and neutral red stain was used in every experiment during this project and stock solutions of each were formulated prior to experimentation. The anticoagulant buffer solution consisted of 0.157g NaOH, 0.435g NaCl, 0.315g citric acid, 0.253g Na2EDTA, resulting in a pH of 4.58. Neutral red stain consisted of 2mg of bacto-neutral red stain to 1ml of anticoagulant buffer. NaCl and NaOH was purchased from Sigma-Aldrich® (St.

Louis, MO). Na2EDTA and citric acid was purchased from Thermo-Fisher Scientific (Pittsburg, PA). Bacto-neutral red stain was purchased from Difco Laboratories (Detroit, MI).

"Lemongrass factor" was purchased from Santa Cruz Biotechnology, Dallas, TX. It was stored in a refrigerator at 4°C. Black Sumi ink was purchased from Yasutomo (San Francisco, CA).

Materials

Pipettes, pipette tips and box for pipette tips, Hamilton 10 microliter syringe (Reno, Nevada) , cleaners for syringes between each injection (Equate distilled water, Equate 91% isopropyl alcohol, acetone ordered from Sigma-Aldrich® (St. Louis, MO). I also used 2 ml centrifuge tubes, plastic disposable cups, timers, benchtop counters to keep track of the hemocytes present, soft tip forceps for handling of the larvae, scissors to cut proleg of the larvae for counting purposes, and parafilm (Bemis, Neenah, WI).

Experiment One: Mortality assays of "lemongrass factor" on *Galleria mellonella* larvae

To measure the toxicity of "lemongrass factor" in vegetable oil injected on the larvae, they were submerged in water for 15 minutes to anesthetize them for easier injection. Next the larvae were dried off by rolling them lightly on a paper towel. Then the larvae were injected with 5 microliters of 40% concentration of sumi ink in distilled water. That mixture was injected in the 1st or second proleg of larvae. Followed by an injection in the same proleg with selected "lemongrass factor" concentration in cholesterol free vegetable oil. The larvae were rolled lightly again on the paper towel to evenly distribute the mixture throughout the hemocoel. Then placed into a small glass mason jar ventral side up.

The selected “lemongrass factor” concentrations that we tested during this experiment were 0.001, 0.01, 0.1, 1, 3.25, 6.5 % in vegetable oil and applied to 25-68 larvae per concentration. The larvae were then placed into an incubator with the settings of 35°C and 80% relative humidity for 4 hours in the dark.

After four hours of incubation the larvae were observed and poked by soft tip tweezers. Larvae that did not move were considered dead. Mortality percentage was calculated for each “lemongrass factor” concentration. Mortality percentage was calculated for each “lemongrass factor” concentration.

To measure the toxicity of “lemongrass factor” applied topically, the larvae were submerged in water for 15 minutes to anesthetize them for easier injection. Next the larvae were dried off by rolling them lightly on a paper towel. Then the larvae were injected with 5 microliters of 40% concentration of sumi ink in distilled water. Followed by an injection in the same proleg with selected “lemongrass factor” concentration in cholesterol free vegetable oil. The larvae were rolled lightly again on the paper towel to evenly distribute the mixture throughout the hemocoel. Then placed into a small glass mason jar ventral side up.

The selected “lemongrass factor” concentrations that we tested during this experiment were 0, 2.5, 5, 10, 20, 40, 60, 80 % mixed in methanol or acetone and applied to 30 larvae per concentration. The larvae were then placed into an incubator with the settings of 35°C and 80% relative humidity for 4 hours in the dark.

After the incubation period the larvae were poked by soft tip tweezers. Larvae that did not move were considered dead. Mortality percentage was calculated for each “lemongrass factor” concentration.

Experiment Two: Effects of "lemongrass factor" injections on hemocyte counts and phagocytosis

The larvae were water anesthetized and dried off by rolling them lightly on a paper towel, then injected with 5 microliters of 40% Sumi ink in distilled water. That mixture was injected in the 1st or second proleg of larvae. Followed by an injection in the same proleg with selected "lemongrass factor" concentration in cholesterol free vegetable oil. The larvae were then rolled lightly again on the paper towel to evenly distribute the mixture throughout the hemocoel. Then placed into a small glass mason jar ventral side up and into the incubator.

Based on the results of mortality assays we decided to use "lemongrass factor" concentrations of 0.2, 0.8, and 3.2 % dissolved in vegetable oil. Control larvae were injected with vegetable oil alone. Fifteen larvae per concentration were used. Larvae were then placed into an incubator with the settings of 35°C and 80% relative humidity for 4 hours in the dark.

After incubation one prolegs of the larvae was cut and the larva was bled on to a piece of parafilm. Five microliters of the hemolymph were added to 4 microliters of red stain dye and 40 microliters of anticoagulant buffer, the mixture was vortexed and incubated for 5 minutes at room temperature. This would stain the hemocytes for easier counts and keep the hemocytes from clumping together.

Ten microliters of that mixture were placed on each side of the Improved Neubauer Hemocytometer and hemocytes were counted boustrophendonically, using a brightfield microscope equipped with a custom oblique illumination filter (Peterson, 2019) was used during the counting of hemocytes. Additionally, classes of hemocytes (plasmatocytes, granulocytes, spherulocytes, and oenocytoids) were identified, and percentage of hemocytes that had ingested the sumi ink was calculated for each hemocyte class.

Experiment Three: Effects of "lemongrass factor" topical application on hemocyte counts and phagocytosis

Water anesthetized larvae were dried and injected with 40% Sumi ink as described before. I addition, each experimental larva received 5 microliters of 2% "lemongrass factor" dissolved in acetone and applied topically onto cuticle on dorsal side of the body. Control larvae received 5 microliters of acetone only. There were 15 larvae per each data point.

After 4 hours of incubation the larvae were bled on to a piece of parafilm. Five microliters of hemolymph were immediately added to 4 microliters of red stain dye and 40 microliters of anticoagulant buffer, vortexed and for 5 minutes at room temperature. Hemocytes were counted and classified, and percentage of phagocytosis was assessed as in Experiment Two.

Experiment Four: Effects of "lemongrass factor" on hemocyte counts via topical application to larval diet

For this experiment we treated pieces of the diet we feed the larvae with 5 microliters of the selected "lemongrass factor" concentration in solvent of methanol or acetone and placed the treated food and larvae in the incubator for 24 hours. Concentrations of 0.05% "lemongrass factor" in methanol, 2% "lemongrass factor" in methanol, and 2% "lemongrass factor" in acetone were used to treat the diet of the larvae. 12-13 larvae per concentration were used in this experiment.

The procedures for staining, counting and classification of the hemocytes were the same as injections and topical applications. Since we did not inject the larvae with sumi ink in this experiment we did not record the intensity of phagocytosis.

Statistics

Sigma Plot was used to determine the mean and standard error of the datasets. The data were subjected to analysis of variance (ANOVA) followed by Tukey comparison of means or Student's t-test using Graph Pad.

RESULTS

Experiment One

The objective of this experiment was to determine the relative toxicity that "lemongrass factor" has towards the mortality rate of *Galleria mellonella* larvae. Injections of "lemongrass factor" in vegetable oil at concentrations ranging from 0.001 to 1% resulted in low mortality of 12 – 16 %. At concentrations of 3.25 and 6.5 % "lemongrass factor" caused over 30% mortality (Table 1). Topical applications of "lemongrass factor" in methanol produced a typical dose-dependent response. Mortality ratios increased together with dose of "lemongrass factor" increasing from 3% and reaching 90% mortality at the highest dose (Table 2). Topical applications of "lemongrass factor" in acetone (Table 3) resulted in a pattern similar to that produced by the experiment involving topical application of "lemongrass factor" in methanol. Based on Experiment One, I decided to use "lemongrass factor" at concentrations not higher than 5% in following experiments.

Table 1. Mortality caused by "lemongrass factor" in vegetable oil injected to last instar *Galleria mellonella* larvae challenged with Sumi ink. Mortality was checked after 4 hours of incubation.

"Lemongrass factor" concentration (%)	Number of dead larvae	Total larvae tested	Mortality (%)
0.001	3	25	12
0.01	3	25	12
0.1	3	25	12
1	4	25	16
3.25	22	68	32.4
6.5	15	40	37.5

Table 2. Mortality caused by "lemongrass factor" dissolved in methanol topically applied to last instar *Galleria mellonella* larvae challenged with Sumi ink. Mortality was checked after 4 hours of incubation.

"Lemongrass factor" concentration (%)	Number of dead larvae	Total larvae tested	Mortality (%)
0	1	30	3.3
5	1	30	3.3
10	2	30	6.6
20	8	30	26.6
40	20	30	66.6
60	25	30	83.3
80	27	30	90

Table 3. Mortality caused by "lemongrass factor" dissolved in acetone topically applied to last instar *Galleria mellonella* larvae challenged with Sumi ink. Mortality was checked after 4 hours of incubation.

"Lemongrass factor" concentration (%)	Number of dead larvae	Total larvae tested	Mortality (%)
0	0	40	0
5	5	40	12.5
10	7	40	17.5
20	22	121	18.18
40	48	120	40
60	45	60	75
80	35	60	58.3

Experiment Two

The objective of this experiment was to evaluate effects of "lemongrass factor" dissolved in vegetable oil on differential hemocyte count (DHC) and phagocytosis in the hemocytes of *Galleria mellonella* larvae challenged with injection of 40% Sumi ink. Injections of "lemongrass factor" in solvent mentioned significantly reduced numbers of plasmatocytes in circulation (Table 4). "Lemongrass factor" did not have effects on numbers of granulocytes, spherulocytes or oenocytoids (Table 4). Phagocytosis was not affected in plasmatocytes, and barely affected in granulocytes (Table 5), but spherulocytes and oenocytoids reacted to "lemongrass factor" by lowering the intensity of phagocytosis (Table 5).

Table 4. Dose- dependent effects of "lemongrass factor" in vegetable oil on differential hemocyte count in last instar *Galleria mellonella* larvae challenged with Sumi ink. N=7 larvae per data point.

"Lemongrass factor" Concentration (%)	Differential Hemocyte Count			
	Plasmatocytes	Granulocytes	Spherulocytes	Oenocytoids
0	20.36 ± 5.84 a	43.52 ± 2.83 a	1.70 ± 0.29 a	2.51 ± 1.15 a
0.2	7.72 ± 1.67 b	30.04 ± 4.74 a	0.82 ± 0.17 a	0.64 ± 0.16 a
0.8	5.30 ± 0.62 b	32.80 ± 4.00 a	1.27 ± 0.29 a	0.45 ± 0.13 a
3.2	6 ± 0.84 b	31.36 ± 5.27 a	1.30 ± 0.44 a	0.68 ± 0.20 a

Mean ± SE values followed by the same letter are not significantly different within a column (ANOVA followed by Tukey comparison, $P > 0.05$)

Experiment Three

The objective of this experiment was to evaluate effects of topical application of "lemongrass factor" had on differential hemocyte count and phagocytosis in the hemocytes of

Galleria mellonella larvae challenged with injection of 40% Sumi ink. Topical applications of "lemongrass factor" significantly lowered numbers of plasmatocytes, granulocytes and oenocytoids, but no effects on numbers of spherulocytes (Table 6). Topical applications of "lemongrass factor" had no effects on phagocytosis by hemocytes of *Galleria mellonella* larvae (Table 7).

Table 5. Dose- dependent effects of "lemongrass factor" in vegetable oil on phagocytosis by hemocytes in last instar *Galleria mellonella* larvae challenged with Sumi ink. N=4-15 larvae per data point.

"Lemongrass factor" Concentration (%)	Percentage of Phagocytosis			
	Plasmatocytes	Granulocytes	Spherulocytes	Oenocytoids
0	74.80 ± 3.70 a	61.30 ± 0.21 a	48.68 ± 13.63 a	28.78 ± 8.54 a
0.2	81.04 ± 6.27 a	82.18 ± 4.31 b	80.31 ± 9.38 a	9.17 ± 4.15 b
0.8	77.40 ± 4.53 a	73.52 ± 2.94 a	58.32 ± 9.87 a	0.00 ± 0.00 c
3.2	70.33 ± 2.05 a	66.05 ± 1.80 a	20.50 ± 8.79 b	5.59 ± 2.54 d

Mean ± SE values followed by the same letter are not significantly different within a column (ANOVA followed by Tukey comparison, $P > 0.05$)

Experiment Four

The objective of this experiment was to evaluate effects of applying "lemongrass factor" in the larval diet on differential hemocyte counts in *Galleria mellonella* larvae that were not challenged with Sumi ink injection. If methanol was used as solvent, 0.5% "lemongrass factor" had no effects on differential hemocyte counts (Table 8), but at 2% concentration lowered counts

of plasmatocytes, granulocytes and oenocytoids were observed (Table 9). “Lemongrass factor” dissolved in acetone at 2% also lowered counts of plasmatocytes and granulocytes (Table 10).

Table 6. Effects of topically applied 2% “lemongrass factor” dissolved in acetone on differential hemocyte counts in *Galleria mellonella* larvae challenged with Sumi ink. Acetone served as solvent. N=7-9 per data point.

Class of hemocytes	Control (Acetone only)	Experimental (2 % “Lemongrass factor”)	P-Value
Plasmatocytes	8.77 ± 1.46 a	5.10 ± 0.69 b	P=0.0372
Granulocytes	20.87 ± 2.36 a	13.39 ± 2.43 b	P=0.0446
Spherulocytes	2.72 ± 0.70 a	1.29 ± 0.23 a	P=0.0749
Oenocytoids	19.07 ± 4.42 a	7.06 ± 1.66 b	P=0.0258

Mean ± SE values followed by the same letter are not significantly different within a row (Student’s *t* test, P > 0.05).

Table 7. Effects of topically applied 2% “lemongrass factor” dissolved in acetone on phagocytosis by hemocytes of *Galleria mellonella* larvae challenged with Sumi ink. Acetone served as solvent. N=7-9 per data point.

Class of hemocytes	Control (Acetone only)	Experimental (2 % “Lemongrass factor”)	P-Value
Plasmatocytes	96.01 ± 0.98 a	96.60 ± 1.25 a	P=0.7123
Granulocytes	97.29 ± 1.17 a	96.81 ± 0.81 a	P=0.7356
Spherulocytes	99.70 ± 0.30 a	99.01 ± 0.68 a	P=0.3583
Oenocytoids	28.39 ± 5.93 a	28.03 ± 5.48 a	P=0.9647

Mean ± SE values followed by the same letter are not significantly different within a row (Student’s *t* test, P > 0.05).

Table 8. Effects of 0.5% "lemongrass factor" dissolved in methanol and applied *per os* on *Galleria mellonella* differential hemocyte counts. N=12 per data point.

Class of hemocytes	Control (Methanol only)	Experimental (0.5% "Lemongrass factor")	P-Value
Plasmatocytes	7.01 ± 2.16 a	5.07 ± 1.66 a	P=0.4847
Granulocytes	21.86 ± 8.04 a	12.94 ± 3.65 a	P=0.3222
Spherulocytes	2.09 ± 0.80 a	2.18 ± 0.50 a	P=0.9306
Oenocytoids	9.30 ± 1.39 a	9.72 ± 2.62 a	P=0.8895

Mean ± SE values followed by the same letter are not significantly different within a row (Student's *t* test, P > 0.05).

Table 9. Effects of 2% "lemongrass factor" dissolved in methanol and applied *per os* on *Galleria mellonella* differential hemocyte counts. N=12-13 per data point.

Class of hemocytes	Control (Methanol only)	Experimental (2% "Lemongrass factor")	P-Value
Plasmatocytes	11.11 ± 1.99 a	6.51 ± 0.89 b	P=0.0459
Granulocytes	36.00 ± 6.43 a	19.92 ± 3.01 b	P=0.0328
Spherulocytes	3.00 ± 0.59 a	2.73 ± 0.58 a	P=0.7464
Oenocytoids	19.52 ± 3.97 a	18.17 ± 4.23 a	P=0.8177

Mean ± SE values followed by the same letter are not significantly different within a row (Student's *t* test, P > 0.05).

Table 10. Effects of 2% "lemongrass factor" dissolved in acetone and applied *per os* on *Galleria mellonella* differential hemocyte counts. N=12-13 per data point.

Class of hemocytes	Control (Acetone alone)	Experimental (2% "Lemongrass factor")	P-Value
Plasmatocytes	8.67 ± 0.93 a	4.51 ± 0.78 b	P=0.0023
Granulocytes	26.67 ± 3.79 a	16.41 ± 3.17 b	P=0.0488
Spherulocytes	2.38 ± 0.63 a	3.24 ± 0.58 a	P=0.3274
Oenocytoids	4.04 ± 0.51 a	2.55 ± 0.60 a	P=0.0690

Mean ± SE values followed by the same letter are not significantly different within a row (Student's *t* test, $P > 0.05$).

DISCUSSION

Background

Previous graduate students, working along with multiple lab assistants, screened 12 plant-derived essential oils and determined that several of them were capable of lowering counts of circulating hemocytes (Haszcz, 2016; Fenske, 2018; Peterson, 2019). These essential oils were effective only at relatively high concentrations, sometimes as high as 30%. However, in subsequent experiments, essential oil of lemongrass lowered numbers of plasmatocytes at concentrations as low as 1.25% (Chastain, personal communication). Chromatographic fingerprinting of lemongrass essential oil suggested chemical nature of the active ingredient of lemongrass essential oil responsible for lowering hemocyte counts. I named this ingredient "lemongrass factor" and acquired it in a form of pure synthesized chemical.

Experiment One

The objective of this experiment was to determine which concentrations of "lemongrass factor" were lethal to *Galleria mellonella* larvae. As expected, mortality increased in a dose-dependent manner together with increased doses of "lemongrass factor". This finding is consistent with mortality trends observed by other researchers in insects exposed to plant essential oils. For instance, Khanikor & Bora (2012) observed mortality in a silkworm *Antheraea assama*, treated with essential oils of *Ocimum sanctum, Ocimum gratissimum* and *Ageratum conyzoides.* Mortality increased together with dose and time. Tawfeek *et al.* (2017) studied the effects of six plant essential oils on *Sitophilus oryzae* (rice weevil)*, Rhizopertha dominica* (lesser grain borer) and *Tribolium castaneum* (red flour beetle). In their experiments, essential oils from

Foeniculum vulgare, Carum carvi, Cinnamomum verum, and *Cymbopogon winterianus* caused increase of mortality percentage together with increasing oil concentration and exposure times.

The same trends in mortality assays are observed in *Galleria mellonella* larvae exposed to plant essential oils. Haszcz (2016) observed dose-dependent mortality in response to *Artemisia annua, Eucaliptus radiata* and *Azadirachta indica* injected in vegetable oil. Comparison of mortality curves obtained by Haszcz (2016) and presented in my current study suggests that "lemongrass factor" is more toxic than *Artemisia annua, Eucaliptus radiata* and *Azadirachta indica* oils; reaching 30-40% mortality from any of these oils required injection of 10, 20, and 80% oil concentrations, respectively (Haszcz, 2019). In my study 30% mortality was obtained at "lemongrass factor" concentration as low as 3.25% (Table 1), and at much shorter time of incubation (4h in our study versus 48 h in Haszcz's study).

Beyenne & Woldatsadik (2019) observed the same dose-dependent mortality trends for essential oils obtained from *Azadirachta indica, Ocimum basilicum, Vernonia amygdalina, Calpurnia aurea, and Vebascum sinatticum benth* and applied to *Galleria mellonella* topically, by spraying the oils to run off onto the larvae. Here, their way of application was probably more efficient than in my study, mostly due to the higher doses applied by Beyenne & Woldatsadik (2019). Here, in 3h long toxicity tests, mortality from 15% oil ranged from ca. 60% for *Azadirachta indica* to ca. 18% for *Vebascum sinatticum benth*. In my experiments, 20% "lemongrass factor" applied topically in 5 µl aliquot caused 19-27% mortality. Given the way of application and the dose, however, it may be surmised that topically applied "lemongrass factor" is not less effective in mortality assays then the oils tested by Beyenne & Woldatsadik (2019).

Plata-Rueda *et al*. (2020) evaluated the toxic properties of lemongrass essential oil applied topically against *Sitophilus granarius*. Here, the mortality increased with dose, but comparison of their results with mine would be difficult; mortality assays were made using adult weevils, mortality is expressed as probits and time of incubation was 24h.

Experiment Two, Three, and Four

The objective of these experiments was to determine if "lemongrass factor" was able to influence differential hemocyte counts and the process of phagocytosis in *Galleria mellonella* larvae. Our results suggest that "lemongrass factor" injected to ink challenged *Galleria mellonella* larvae, significantly lowered the number of circulating plasmatocytes (Table 4). Even though the other hemocytes classes did not exhibit statistically significant declines in density, numeral declining trend between the control and the varying concentration of "lemongrass factor" was observed (Table 4). These findings are similar to trends observed by other researchers in insects injected with plant essential oils. For instance, Sadeghi (2019), injected 4th instar larvae of *Sesamia cretica* Ledere (pink stem borer) with 1 microliter of 4 different ecotypes of *Cuminum cyminum* L. (cumin) essential oil that was dissolved in acetone; they observed significant decreases in circulating hemocyte numbers after 3 hours.

Additionally, lemongrass factor" dissolved in acetone was applied to the dorsal side of *Galleria mellonella* larvae. Significant decreases were observed in plamatocytes, granulocytes, and oenocytoids compared to the control (Table 6). Er *et al.* (2017) studied the effect of a commercial formulation of Azadirachtin (extract from the seed of neem tree) in distilled water, on life history traits and cellular immune reactions of *Galleria mellonella*. In their experiment, they topically applied Azadirachtin to larvae at various concentrations, with an incubation period

of 24 and 48 hours. At the varying concentrations of Azadirachtin they observed significant declines in total hemocyte counts and determined that at 100 ppm concentration, reductions in the differential hemocyte counts of plasmatocytes and granulocytes.

Effects of "lemongrass factor" applied topically to the artificial larval diet of *Galleria mellonella* was investigated as well. At 0.5% "lemongrass factor" dissolved in methanol, no statistically significant declines of circulating hemocytes were observed (Table 8) but at 2 % concentration of "lemongrass factor" dissolved in methanol, significant decreases in numbers of circulating plasmatocytes and granulocytes were observed. The same 2% concentration of "lemongrass factor" in acetone and applied in diet reduced numbers of plasmatocytes in circulation. (Table 10). Pandey *et al*. (2012) observed similar significant reductions of circulating hemocytes for essential oils obtained from the dried leaves of *Eucalyptus globulus* (sefeda), *Ageratum conyzoides* (bottle brush), and cloves extract of *Allium sativum* (garlic). Fresh lemon leaves were dipped in various concentrations of the extracts mentioned in distilled water for 1-2 hours and allowed to air dry before being fed to 5th instar larvae of *Papilio demoleus*. Total hemocyte count and differential hemocyte counts were conducted after 24 and 48 hours and reduction trends were observed in all hemocytes classes. Here, their significant trends were observed at 50% concentration of essential oils tested. In the current study we saw results at concentrations as low as 2% concentration of "lemongrass factor". Comparison of the concentrations may indicate "lemongrass factor" may suppress hemocyte mobilization at a much lower concentration and application rate. Many other studies report the similar findings of essential oils/plant derived substances affecting the numbers of circulating hemocytes (Er & Keskin, 2016; Ghoneim, 2018; Rahimi *et al*., 2018; Sokuti & Ghasemi, 2018; Afraze *et al*., 2021).

To investigate phagocytic potential, we challenged the larval immune system with 40% sumi ink before treatment of varying concentrations of "lemongrass factor" injected or applied topically. Yokoo *et al*. (1995) concluded that hemocytes of *Galleria mellonella* were able to ingest particles of Indian ink. Haszcz (2016), Fenske (2017) and Peterson (2019) observed the same for Sumi ink. My results confirm their findings.

In my experiments, injections of "lemongrass factor" in vegetable oil reduced phagocytosis by oenocytoids at all tested concentrations, and in spherulocytes at 3.2% concentration. Topical applications of 2% "lemongrass factor" did not have effect on phagocytosis by either class of hemocytes (Table 7).

According to multiple sources plasmatocytes and granulocytes are said to be the only hemocytes capable of phagocytosis (Lavine & Strand, 2002; Strand, 2008; Jiang *et al*., 2010; Rosales, 2011; Gongqing *et al*., 2016). In my experiments all tested classes of hemocytes exhibited phagocytic activity, with plasmatocytes and granulocytes being the most effective (Table 5). But spherulocytes and oenocytoids also contributed to phagocytic response, although at a lower level in comparison to plasmatocytes and granulocytes (Table 5). This is a novel finding, and it calls for further experimentation.

Other researchers studying the effects of essential oils, bio-insecticides, or plant derived substances have reported similar findings. Koodalingam *et al*. (2013) examined the effects of aqueous extract of *Sapindus emarginatus* (soapnut) on 4th instar larvae and pupae of *Aedes aegypti*. In their experiment they observed lowering of hemocyte phagocytic potential against yeast cells. The same was observed by Koodalingam *et al*. (2014) for bioinsecticide, NeemAzal, applied to 4th instar larvae of *Aedes aegypti*. In both of these experiments the hemocyte monolayers were washed in a sterile insect saline solution to remove any unattached yeast cells

before counts. Maybe their results are more accurate than ours because we did not wash our hemocytes before cell counts but they did not distinguish which hemocytes were able to phagocytize the yeast cell targets.

Figueiredo *et al*. (2006) observed a reduction in phagocytosis after treating 5th instar larvae of *Rhodnius prolixus* with orally administered azadirachtin (neem) and/or ecdysone (steroid hormone responsible for molting). Larvae were fed either azadirachtin or azadirachtin and ecdysone mixed in blood meal, and then inoculated by an injection of yeast cells. They reported that larvae treated with just the azadirachtin treatment exhibited reduction of phagocytic potential.

Zibaee & Bandani (2010) observed similar trends of a reduction in phagocytic potential in *Eurygaster integriceps* exposed to *Artemisia annua* (sweet wormwood). *E. intergriceps* adults were starved for 12 hours, then fed foliage treated with *Artemisia annua* essential oil, and finally inoculated by injection of *Beauveria bassiana*, to determine phagocytosis potential of hemocytes. Comparison of their results with mine would be difficult because larvae were used during my experimentation, whereas Zibaee & Bandani (2010) performed their experiments on adult pest at a much longer exposure time.

My predecessor, Katherine Haszcz (2016) injected ink challenged *Galleria mellonella* larvae with pure neem oil. Differential hemocyte counts were recorded, along with phagocytosis percentages. Haszcz (2016) detected a significant reduction in the plasmatocyte counts when compared to the control but did not observe a reduction in phagocytic potential of the hemocytes. Haszcz (2016) also performed experiments with nylon implants inserted into *Galleria mellonella* larvae to determine the encapsulation potential of hemocytes and recorded that neem at a concentration of 1.5 mg/larvae showed significantly decreases of encapsulation compared to the

control. Even though her experiments with neem oil did not reduce phagocytic potential, it did have an influence on circulating hemocytes and encapsulation. Which are important cellular processes that insect hemocytes perform when challenged in the presence of a foreign invaders.

In conclusion, in my experiments, injections of "lemongrass factor" lowered numbers of circulating plasmatocytes and percentage phagocytosis in oenocytoids and spherulocytes. Topical applications of "lemongrass factor" lowered numbers of plasmatocytes, granulocytes, and oenocytoids in circulation, but has no effects on intensity of phagocytosis. Application *per os* lowered numbers of plasmatocytes and granulocytes in circulation.

I think that "lemongrass factor" has a potential for impairing insect immune system. Insect hemocytes play an integral role in insect cellular immunity. When insects are infested by a foreign invader, they increase production of hemocytes to eliminate the threat and to slow the infection (Haszcz, 2016; Leite *et al*., 2021). If a chemical decreases hemocytes numbers then less circulating hemocytes are capable of cellular processes such as phagocytosis, encapsulation, and nodulation in reaction to bacteria, fungi, or parasitoid eggs. "Lemongrass factor" could potentially be a more environmentally friendly alternative to insect pest control, compared to conventional insect control that has been shown to have a negative impact towards humans and the environment. To address that issue more studies are needed.

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