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Effects of Lemongrass Essential Oil on Galleria Mellonella Hemocytes

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EFFECTS OF LEMONGRASS ESSENTIAL OIL ON *GALLERIA MELLONELLA* HEMOCYTES

A Master's Thesis

Presented to

The Graduate College of

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In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Agriculture

By

Kyndra Chastain

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ABSTRACT

In modern agriculture, the use of pesticides is unavoidable. However, improper use has resulted in pest resistance and negative impacts on the environment and human health. Changes are needed in the agricultural industry to account for the damage already caused. Researchers have turned to essential oils as key ingredients for potential biopesticides, with some mixtures already on the market. Most studies involving essential oils and a pest's response concentrate on mortality or interruptions in development during and between life stages. My study, however, focused on immune responses; specifically, lemongrass essential oil's impact on *Galleria mellonella*. Such responses include a change in hemocyte numbers and phagocytosis. This research concludes that lemongrass essential oil lowers the numbers of hemocytes in circulation of a triggered immune system, potentially also counteracting phagocytosis efficacy. This revelation could prove practical in integrated pest management strategies that involve biocontrol agents like parasitic nematodes.

KEYWORDS: *Galleria mellonella*, hemocytes, lemongrass, essential oils, biopesticides, insect immune system, Sumi ink, artificial implants

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I dedicate this thesis to my parents: Chris and Regina Chastain. The support they have given me financially and emotionally are the reason I have been able to keep pursuing my dream. I would not be the woman I am today if it were not for them and for the love they constantly show me.

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INTRODUCTION

The global population is expected to reach almost 10 billion people by 2050 (Crist *et al*., 2017). Agriculture production, for human and livestock consumption, will need to increase approximately 60% to adequately support this projected population (Alexandratos & Bruinsma, 2012). McKenzie and Williams (2015) suggest that such an increase in agricultural production is currently possible if processes were done more efficiently, with appropriate changes including: adjusting nutritional imbalances (referring to both under-nutrition and obesity), enhancing current agricultural land usage, expanding irrigated lands, reducing food waste, combatting climate change, encouraging local food production, and improving fertilizer and pesticide usage.

Pesticides are necessary to maximize crop yield by minimizing crop loss caused by insects. Crop losses can reach up to 80% without proper pesticide treatment (Zhang, 2018). Over 3 billion tons of synthetic pesticides are used annually around the globe (Sharma *et al*., 2020). A 2017 EPA report concluded that the United States accounted for nearly 18% of global pesticide expenses (Atwood & Paisley-Jones, 2017). Zhang (2018) reported that from 2010 to 2014, the average total pesticide use worldwide was 2.8 kilograms per hectare (kg/ha). In 2014, the US applied 3.9 kg/ha, while China and Japan used 10.4 and 19.4 kg/ha, respectively (Zhang, 2018).

Pesticides are a double-edged sword as excessive use causes serious environmental and health problems. Despite attempts to lower synthetic pesticide use in some developed countries, the negative effects of past use can still be seen in a decreased biodiversity of native plants, birds, and insects (Geiger *et al*., 2010). Soil bacterial diversity, and thereby soil fertility, is also negatively affected in a significant way by synthetic pesticides (Johnsen *et al*., 2001). Many pesticides also leach into groundwater, contaminating drinking water in rural areas

(Stavnichenko *et al*., 2017). Heavy or improper pesticide use in farming communities could result in physical ailments, from skin irritations and headaches to increased risk of prostate cancer for applicators (Alavanja *et al*., 2003; Berni *et al*., 2021). However, applicators are not the only ones physically affected by exposure to pesticides. Over a nine-year period in China, almost 3,000 children were treated for pesticide poisonings, with nearly half under the age of six years old (Yimaer *et al*., 2017). Another study estimated that approximately 385 million cases of unintentional acute pesticide poisonings and 11,000 deaths occur annually worldwide (Boedeker *et al*., 2020). In much of the developing world, highly toxic pesticides are still used with barely any regulation (Zhang, 2018) and with insufficient education on personal protection equipment and proper application and disposal methods (Berni *et al*., 2021). Some of these countries, like Brazil, are major exporters of agricultural goods. Braga *et al*. (2020) found that every year in Brazil, an alarming number of new pesticides are manufactured with ingredients banned elsewhere. Traces of these ingredients have been found in exported agricultural goods that may cause health issues if consumed in high quantities (Caldas & Jardim, 2012).

The continuous use of synthetic pesticides at high amounts can lead to pesticide resistance amongst pests (Sharma *et al*., 2020). When resistance occurs and yield loss increases, pesticide end-users who have not been given adequate information tend to increase pesticide dosage, only worsening the problem (Jutsum *et al*., 1998).

Considering both the strong need for and negative effects of pesticide use, there has been a strong push for alternative strategies, e.g., biopesticides, to be implemented. Biopesticides are derived from natural means and comprised of microbes, animals, plant-extracts, insect-growth regulators, insect hormones, or plant-based extracts (Kumar *et al*., 2021). However, the EPA simplifies this by dividing them into three groups: microbial pest control agents, biochemical

pesticides, and genetically engineered organisms (Arora *et al*., 2016). Synthetic pesticides can take around three years before receiving approval by the EPA (Handford *et al*., 2015), while biopesticides require only one year due to less human and environmental risk (Kumar, 2012). Biopesticides offer a range of advantages over synthetic pesticides: faster biodegradability, target specificity, efficacy in small amounts, and efficacy comparable to that of synthetic pesticides when used in integrated pest management (IPM) strategies (Kumar, 2012).

Despite the quick registration time and numerous advantages over synthetic pesticides, biopesticides have minimal market significance, accounting for only 5% of pesticides sold (Damalas & Koutroubas, 2018). The US, Canada, and Mexico constitute 44% of the biopesticide market (Bailey *et al*., 2010), and 90% of biopesticides contain *Bacillus thuringiensis* (Kumar & Singh, 2015), an entomopathogenic bacterium more commonly known as Bt, which negatively affects insect digestion (Whalon & Wingerd, 2003). Although biopesticides are slowly gaining popularity, the transition is delayed for a few reasons. The first issue is that natural compounds are sensitive to temperature and humidity, resulting in short shelf lives, need for climate-controlled storage, and varying effectiveness in different parts of the world (Kumar *et al*., 2021). The next issue is that the high target specificity means that multiple different treatments may be needed if dealing with more than one pest (Kumar *et al*., 2021). These issues give motivation to find potential biopesticides in once unlikely places.

Essential oils, although historically used for food preservation, flavor additives, medical treatment, and insect repellent (Koul et al., 2008), have recently become a focus for biopesticide usage(Sharma *et al*., 2020). These aromatic oils have the potential to act as pesticides that attract (Kendra *et al*., 2014), repel (Papachristos & Stamopoulos, 2002), possess antifeedant properties (Hummelbrunner & Isman, 2001; Rajkumar *et al*., 2019), or cause mortality. It has also been

found that some essential oils affect the octopaminergic nervous system, a site specific to insects (Koul *et al*., 2008). Therefore, they are target-specific and safer to use around mammals and humans (Zhang *et al*., 2016). The volatility of these oils means that under field conditions they have limited field persistence. This enables them to target crop pests that are on the treated plants while also allowing biocontrol agents like natural predators/parasitoids to re-enter shortly after treatment (Koul *et al*., 2008).

Lemongrass plants from the genus *Cymbopogon* are native to tropical Asia, where it has been widely used as flavoring for foods and teas. Today, species are cultivated worldwide (Joy *et al*., 2006). Lemongrass has been used individually as an insect repellent for mosquitoes throughout history (Koul *et al*., 2008).

The effect lemongrass essential oil (LGEO) has on the mortality and general health of insects is fairly well researched. One study found that lemongrass (*C. nardus*) could be used as a killing agent while fogging against mosquitoes (Aedes aegypti) that carry yellow fever(Zulfikar & Sitepu, 2019). Plata-Rueda *et al*. (2020) found that lemongrass (*C. citratus*) lowered the respiratory rate, had a repellant effect, and negatively influenced locomotion of granary weevils (*Sitophilus granaries*). Chintalchere *et al*. (2021) found that lemongrass (*C. citratus*) was most effective as a fumigant against common house fly (*Musca domestica*) pupae, but also had promising results on larval and adult development of house flies using different methods of exposure. Rani *et al.* (2019) also tested lemongrass on the same species common house flies, finding similar results with fumigation in the pupal stage. These studies and more have shown LGEO's potential to combat insect pests.

A vertebrate's immune system is both innate and acquired or more simply put, natural and adaptive (Fearon, 1997). This two-fold system works in tandem, with innate immunity

responding immediately and immunity that is acquired active within the next several days to two weeks (Zimmerman *et al*., 2010). Innate immune systems contain receptors that preexist and cannot adapt any further. Whatever threat that is not eliminated by the innate system, triggers the acquired immune system. This system can clone receptors suitable to fight the infection. Once the infection is eradicated, the cloned receptors remain, building immunity against said infection in case of reinfection (Fearon, 1997). Insects historically were found to only have an innate immune system, meaning they were not able to build immunity as with an acquired immune system. However, recent evidence prompts further study into this issue as some Drosophila spp. have displayed transgenerational viral immunity in subsequent generations after parental priming (Mondotte *et al*., 2020).

The innate immune system of an insect operates within its open circulatory system, along with other processes, such as transportation of molecules and nutrients, thermoregulation, and ventilation through the tracheal system (Glenn *et al*., 2010). Here, hemolymph pumps through a single dorsal vessel, alternating between pumping towards the head and towards the abdomen. The dorsal vessel opens on both ends into the open body cavity to allow the hemolymph to flow freely (Glenn *et al*., 2010). In doing so, the immune system can respond faster upon invasion (Dubovskiy *et al*., 2016). Hemolymph contains hemocytes, which are akin to blood cells in vertebrates.

Hemocytes can be categorized into five or six hemocyte types, depending on the source and insect order (Lavine & Strand, 2002; Strand, 2008; Pereira *et al*., 2018). The most common consensus for Lepidopterans includes granulocytes, plasmatocytes, spherulocytes, oenecytoids, and prohemocytes. Granulocytes and plasmatocytes are adhesive cells responsible for immune defenses. Strand *et al*. (2006) found that in some cases with Lepidopteran species, granulocytes

are most efficient at phagocytosis while plasmatocytes are most efficient at encapsulation. Spherulocytes aid in transporting cuticle components (Lavine & Strand 2002; Tungjitwitayakul & Tatun, 2019). Oenecytoids release enzymes that catalyze prophenoloxidase (proPO), which aids the encapsulating hemocytes to start the melanization process (Lavine & Strand, 2002). Lepidopteran species also can have prohemocytes. These cells are considered stem cells as they can differentiate into required cell types if an immune system is triggered (Baghban *et al*., 2018).

When an innate immune system is triggered, the number of hemocytes in the circulatory system increases to perform the immune defenses. For convenience, innate responses are divided into two categories: humoral and cellular defenses. Hemocytes have been found to provide some resources necessary for humoral strategies (Strand, 2008), so the distinction between the two categories is blurred regarding functionality.

The predominant feature of humoral defenses involves molecules, e.g., proteins and antimicrobial peptides, that act as the first line of defense (Sheehan *et al*., 2018), but also incorporates hemolymph clotting and melanization. Hemolymph clots the entry wound via a transglutaminase cascade, which is activated rapidly and simultaneously with the prophenoloxidase (proPO) system (Sheehan *et al*., 2018). The proPO system cascade is catalyzed by the phenoloxidase enzyme produced and secreted by hemocytes (Sheehan *et al*., 2018), specifically oenecytoids in Lepidopteran species (Strand, 2008). The proPO cascade leads to the production of melanin at the injury site (Wojda, 2017). The humoral defense of melanization is not only an important immune function but also is responsible for exoskeleton coloration and sclerotization during ecdysis (Vilmos & Kurucz, 1998). Melanization also occurs at the end of cellular defense encapsulation and nodulation (Wojda, 2017).

Cellular defenses fully utilize hemocytes to perform either phagocytosis, nodulation, or encapsulation. Phagocytosis is the process where individual cells absorb small particles of bacteria and yeasts, or abiotic agents used for experimentation, e.g., chromatography beads and ink particles (Lavine & Strand, 2002; Strand, 2008). Within an hour of engulfment, the hemocyte can digest the target particle (Rosales, 2010). Nodulation and encapsulation refer to groups of hemocytes adhering to and layering around foreign invaders to suffocate them (Strand, 2008). These processes have been observed to begin fifteen minutes after invasion (Dubovskii *et al*., 2010). Nodulation typically forms around small clusters of bacteria, while encapsulation forms around larger pathogens like nematodes and parasitoid eggs (Lavine & Strand, 2002). Plasmatocytes and granulocytes have been noted in literature to be the only hemocytes with adhesion capabilities in Lepidopterans (Strand & Pech, 1995); therefore, they are the only ones used in capsule formation. In these defense actions, granulocytes create the first layer, and plasmatocytes form many layers on top of them (Lavine & Strand, 2002). A final layer of granulocytes forms to prohibit further plasmatocyte adhesion (Strand, 2008). At this point, phenoloxidase is released from oenecytoids to begin the proPO cascade for production of melanin (Dubovskiy *et al*., 2010).

Although the aforementioned studies of LGEO causing mortality and developmental issues have deemed the oil fit for a biopesticide ingredient, there is no research on how LGEO affects insect immune systems. My study objective was to test the effects of LGEO on the immune response of a model insect species. I asked three specific questions: (i) will LGEO lower the cell response in a triggered immune system, (ii) will LGEO impair phagocytosis, and (iii) if LGEO affects insect immune system, what would be the active ingredient responsible for such

effects? The answers to these questions may show potential for LGEOs to be used in or as a biopesticide.

METHODS

Materials

I used *Galleria mellonella* larvae, i.e., greater wax moth, from the insect order Lepidoptera and family Pyralidae. This insect is a pest on honeybee hives, where it feeds on wax combs during its larval stage (Wojda *et al*., 2020). *G. mellonella* has become a model study species in toxicology and immunology (Allegra *et al*., 2018; Pereira *et al*., 2018). This species is found worldwide and is simple to rear and perform experiments on (Wojda, 2017).

I purchased the larvae in their seventh instar stage from Knutson's Live Bait in Brooklyn, Michigan. After being separated into ventilated jars of approximately 60 larvae with prepared diet, I stored the jars in unlit incubators (VWR Scientific Products, Model 2005) set at 30°C and 80% relative humidity. For the diet, I combined 37ml of glycerin USP 99.5% (Humco), 25ml of C&H brand pure granulated cane sugar, and 25ml of purified water. This solution was heated in a low-power microwave for ~20 seconds, stirred thoroughly to dissolve the sugar, and poured into 400 ml of Gerber multigrain cereal. The cereal contains vitamins (A, B1, B2, B3, B6, B12, C, and E) and minerals (calcium, iron, phosphorus, and zinc) necessary for high-quality larval health. I placed this mixture in a one gallon ZipLock bag and kneaded it by hand for two minutes until the cereal was consistently saturated with liquid ingredients. The diet could be stored in a refrigerator at 4°C for up to two months.

I used two chemicals in the injections procedure (below): black Sumi ink and LGEO. Yasutomo black Sumi ink was centrifuged for five minutes to draw large particles down. Using a pipette, I pulled up 40µl of supernatant and added it to a separate microcentrifuge tube with 60µl of distilled water to form a 40% concentration. This concentration was found to be the most

effective as an artificial immune trigger that mimics bacterial infection in prevous work (Haszcz, 2016).

LGEO, i.e., *C. flexuosus*, purchased from NOW Foods, was stored at 4°C in the original glass packaging. To find the most effective doses, LGEO dissolved in cholesterol-free vegetable oil was applied at various concentrations. Control larvae received vegetable oil only. Cholesterol-free vegetable oil was selected because this species does not produce cholesterol and adding it into their system would affect the physiology of the larvae and could skew the results of my experiment.

Anticoagulant buffer was made from 0.157g NaOH, 0.435g NaCl, 0.315g citric acid, and 0.253g Na_2EDTA. I purchased the NaOH and NaCl from Sigma-Aldrich, and the citric acid and Na_2EDTA from Thermo Fisher Scientific. These were weighed using an Ohaus Galaxy 1600 scale and mixed. To obtain the final product at a volume of 40 mL, distilled water was added, vortexed, and acidity tested to ensure a final pH of 4.58. I combined 2mg of acto-neutral red dye (Difco Laboratories, Detroit, MI) and 1mL of anticoagulant to obtain a neutral stain. This stain was used to prevent hemolymph from clotting and to add pigment to hemocytes for easier identification under a microscope.

Injections Procedure

The following injections procedure was the initial step in mortality assays and differential hemocyte count experiments. I randomly selected two groups of 10–15 larvae to anesthetize in tap water for 15 minutes. Individual larvae were removed from the water, blotted dry, and injected with their respective chemicals using a Hamilton microliter syringe (24G/2”/30°) into one proleg of the first or second set. For the control group, each larva was injected with 5µl of

40% Sumi ink / 60% dH_2O (Haszcz, 2016) and 5µl of 100% vegetable oil. The experimental group was injected with 5µl of Sumi ink and 5µl of tested LGEO concentration. After both injections, each larva was gently rolled to ensure that the chemicals spread throughout the body cavity. I stored the injected larvae in a clean ventilated jar and inside the incubator for four hours (as in Haszcz, 2016), after which one of the following two experiments was performed.

Experiment One: Mortality Assays

Because I wanted to observe LGEO's effect on the physiology of the test subject, it was important to keep the oil concentrations below the lethal level.To that end, mortality assays were performed by injecting the larvae with LGEO and ink and calculating the mortality rate after the four hour incubation period following injections. Larvae that reacted when disturbed were counted as alive. Concentrations of lemongrass in cholesterol-free vegetable oil at percentages of 1.25, 2.5, 5, 10, 20, 30, 40, and were applied along with the previously mentioned Sumi ink concentration. Vegetable oil alone was applied to the control larvae. Results from these experiments were plotted on a graph and inspected visually, or subjected to probit analysis. Concentrations of LGEO lower than LD_{10} were used in subsequent experiments.

Experiment Two: Differential Hemocyte Counts and Phagocytosis

In preparation for both differential hemocyte counts and phagocytosis cell counts, I combined 4µl of neutral red stain and 40µl of anticoagulant buffer in 3–5 microcentrifuge tubes for the treated hemolymph to be placed. This mixture sat at room temperature for five minutes. Then a single treated larva was bled by carefully clipping off one proleg with micro iris scissors and pinching the body over a square of parafilm to place a few drops of hemolymph. I

immediately extracted 5µl of hemolymph with a micropipette and added it to one of the prepared microcentrifuge tubes with the stain and anticoagulant and then vortexed the tube. After resting for five minutes to allow staining of the cells, I pipetted 10µl of the mixture into each of the two chambers of an improved Neubauer hemocytometer covered with a Corning cover glass (thickness 1, 22 x 22mm). Each chamber had four quadrants, or grids, eight in total for one sample of hemolymph. The hemocytometer was then inspected under a Galen III microscope fitted with an oblique illumination filter (Peterson, 2019), which improved clarity and depth of field. In both tests, the cells that fell into the boundaries of one of the quadrants on the hemocytometer were counted boustrophedonically (i.e., left to right, down, right to left, down, and so on) through the four rows of the grid.

For differential hemocyte counts, total plasmatocytes, granulocytes, spherulocytes, and oenecytoids were counted individually using a tally counter. In my research, prohemocytes were not counted as they were too low in numbers. Based on probit analysis of larval mortality (see Results), I selected 0.625, 1.25, 2.5, and 5% of LGEO. Control larvae received vegetable oil only. In tandem with differential hemocyte counts, phagocytosis was evaluated.

Each of the cell types were counted in two groups: phagocytizing and not phagocytizing. To be considered phagocytizing, the cell must have one or more particles of black Sumi ink absorbed in its cytosol. The percentage of phagocytized versus not phagocytized was calculated for each lemongras essential oil concentration. All data from Experiment Two were averaged ± SEM for each concetration and subjected to ANOVA followed by Tukey comparison of means.

Delineation of Candidate for Active Ingredient of LGEO

To delineate biologically active substances that could be responsible for LGEO effects on Galleria immune system, I subjected the lemongrass oil to qualitative analysis by high-performance thin-layer chromatography as in Wagner and Bladt (1996). Based on Wagner and Bladt (1996), I selected citral for standards in this analysis. Briefly, a silica gel F_{254} glass plate was washed with 100% methanol, air dried and spotted using a CAMAG Nanomat 4 HPTLC spotter. Five microliters of pure citral diluted in methanol at 0.5% and 0.125%, and five microliters of 1% and 5% of LGEO were applied on the plate.

The plate was developed in a horizontal CAMAG developing chamber for 10 minutes with a mobile phase made of toluene and ethyl acetate (93/7 v.v.). I air dried the plate under a hood for one hour and derivatized it in vanillin reagent. The derivatized plate was then heated at 100°C for 10 minutes on a CAMAG Plate Heater III, cooled and scanned using a Canon CanoScan 5200F flatbed scanner in white light.

Statistical Analysis

The data collected from the mortality assays was subjected to a probit analysis to find the LD_{10} and LD_{50} using Polo Plus, by LeOra Software.

For differential hemocyte counts and phagocytosis percentages, I used ANOVA followed by Tukey multiple comparison of means with the significance value at $p < 0.05$. The data was tested on GraphPad InStat and results were plotted with SigmaPlot.

RESULTS

Experiment One

Mortality of *Galleria mellonella* larvae was positively correlated to tested LGEO concentration (Figure 1). Visual inspection of the data plot (Figure 1) revealed that the control group had an average of 14% mortality. Concentrations ≤ 5% had a mortality rate ranging from approximately 29–40%, while concentrations ≥ 10% ranged between 52–100% mortality. For precise results, I subjected my data to a probit analysis to find the LD_{10} and LD_{50}. The LD_{10} was about 4% with a 95% confidence interval (CI) ranging from approximately 1.6 to 6.6%. The LD_{50} was about 15% with a 95% CI ranging from roughly 10.5 to 20.3% (Table 1). LD10 is widely accepted in experiments studying physiological responses (Beggel *et al*., 2010). For that reason, I selected concentrations 0.625, 1.25, 2.5, and 5% LGEO for subsequent tests.

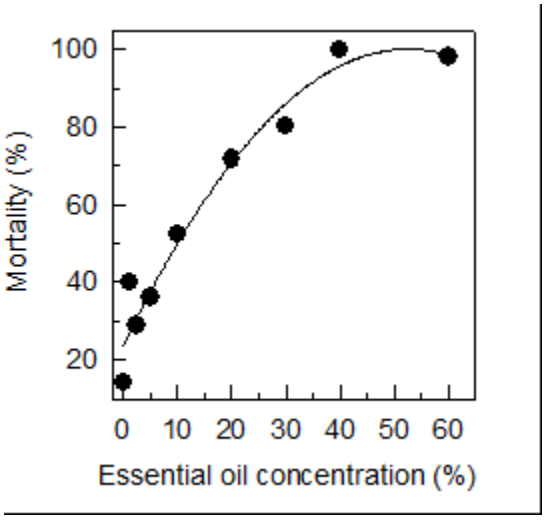

Figure 1. Mortality rate (%) based on dose-dependent injections of 5 µl LGEO in cholesterol-free vegetable oil into larvae challenged with 5 µl 4:6 Sumi ink to dH_2O. The zero-concentration solution was 100% cholesterol-free vegetable oil. N = 15–17 larvae per data point.

Table 1. Probit analysis of LGEO mortality data.

N	Slope	LD_{10} (95% CI) % concentration	LD_{50} (95% CI) % concentration	χ^2	Df
282	2.244	4.072 (1.618 – 6.616)	15.164 (10.469 – 20.270)	7.768	5

Experiment Two

LGEO was effective at significantly lowering both plasmatocytes and granulocytes in triggered *Galleria mellonella* immune systems (Figure 2a–b). In the control group, plasmatocytes (Figure 2a) occurred at a rate of 10.25 x 10^5 cells per ml of hemolymph. LGEO treatments significantly decreased plasmatocyte counts from about 9 x 10^5 cells per ml of hemolymph to about 4 x 10^5 cells per ml of hemolymph in a dose-dependent manner. Granulocytes (Figure 2b) had a similar trend in being negatively correlated with LGEO treatments, starting with the control having 51.04 x 10^5 cells per ml of hemolymph. The treatment groups significantly decreased in granulocyte numbers from approximately 32 x 10^5 cells per ml of hemolymph to nearly 27 x 10^5 cells per ml of hemolymph in a dose-dependent manner. At 1.25, 2.5, and 5% LGEO concentrations, the decrease in individual plasmatocyte and granulocyte counts was statistically significant ($p < 0.05$, ANOVA followed by Tukey multiple comparison of means test).

The trends for spherulocytes and oenecytoids differed from the trends seen in plasmatocytes and granulocytes. The control count for spherulocytes (Figure 2c) was 3.49 x 10^5 cells per ml of hemolymph. Spherulocyte counts in the treatment groups remained at approximately 1.5 x 10^5 cells per ml of hemolymph throughout all tested LGEO concentrations.

All were considered statistically signficiant when compared to the control ($p < 0.05$, ANOVA followed by Tukey multiple comparison of means test). Oenecytoids (Figure 2d) were present at 6.5 x 10^5 cells per ml of hemolymph in the control group. Only at 0.625% LGEO was there a statistically significant depletion in hemocyte numbers observed, with about 1 x 10^5 cell per ml of hemolymph ($p < 0.05$, ANOVA followed by Tukey multiple comparison of means test).

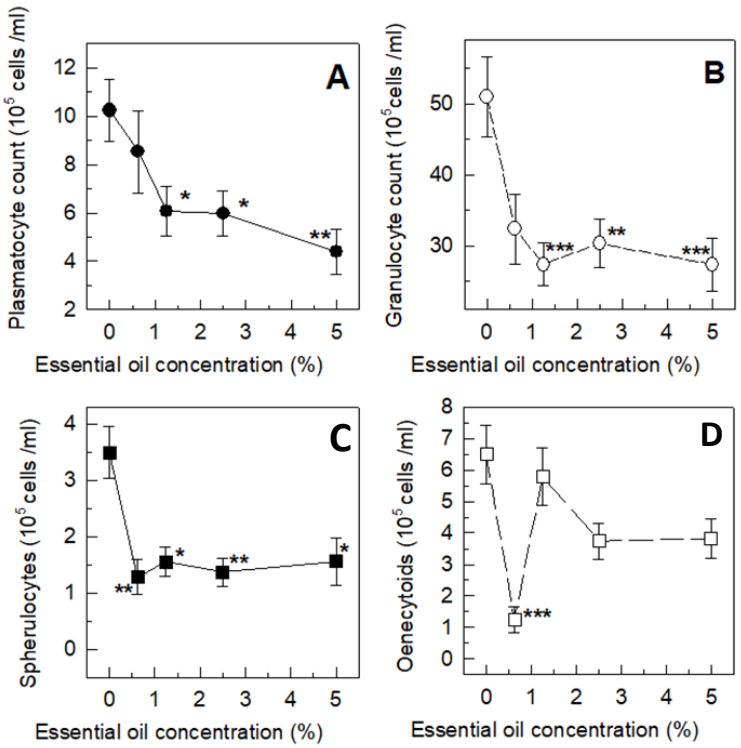

Figure 2. Plasmatocyte (a), granulocyte (b), spherulocyte (c), and oenecytoid (d) counts in response to dose-dependent injections of LGEO in *Galleria mellonella* immune systems challenged with 40% Sumi ink. The zero-concentration solution was cholesterol-free vegetable oil. N = 15-35 larvae per data point. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ in ANOVA followed by Tukey comparison of means.

The percentage of phagocytized cells appeared to not be affected by LGEO (Figure 3). The phagocytized cells in the treatment groups were all above 94%, indicating that phagocytosis still occurred at a high rate in the dose-dependent injections.

Qualitative High Performance Thin Layer Chromatography

Qualitative HTPLC analysis indicated that citral was present in the lemongrass oil used in my study. Derivatized citral produced a band that collocated at Rf 0.55 with a massive band in the lanes containing derivatized LGEO (Figure 4).

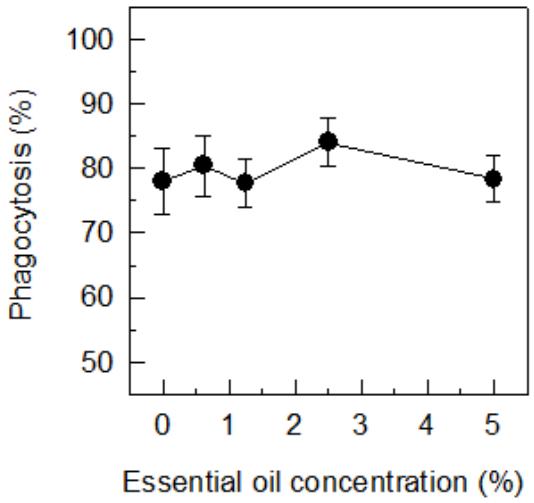

Figure 3. Percent of phagocityzed cells in total hemocyte counts of dose-dependent injections of LGEO and 40% Sumi ink. N = 15 larvae per data point.

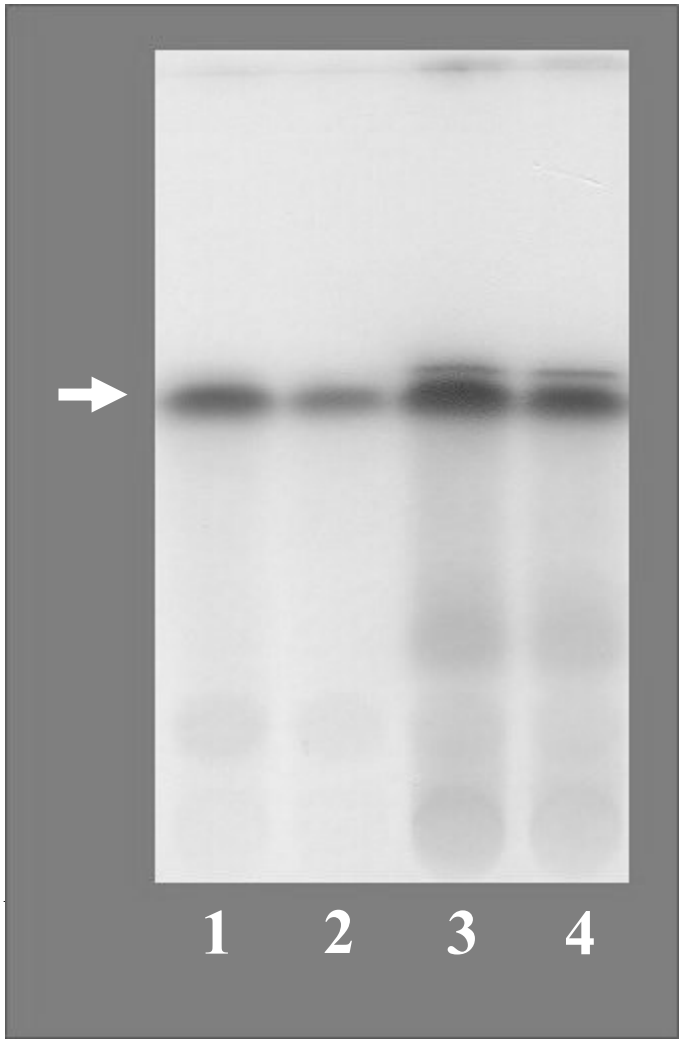

Figure 4. HPTLC plate confirming presence of citral in LGEO. Lanes: 0.5% citral (1), 0.125% citral (2), 1% LGEO (3), and 5% LGEO (4). All concentrations were dilluted in methanol. Plate was developed in 93:7 toluene to ethyl acetate and derivatized in vanillin reagent over heat. Citral standards collocate with the main ingredient of LGEO (arrow).

DISCUSSION

In this study, I addressed whether LGEO had the potential to counteract cellular immune responses of *Galleria mellonella* larvae on two fronts: lowering circulating cells produced in a triggered immune system, and lowering the rate of phagocytosis. LGEO treatments were effective in lowering all four cell types that I examined, with statistically significant results for plasmatocytes, granulocytes, and spherulocytes in a dose-dependent manner. Oenecytoid numbers were lowered with treatments, but only one concentration yielded statistically significant results. However, due to the elevated oenecytoid numbers in the other concentrations, I can surmise that this significance is an outlier.

The rate of phagocytosis was not affected by dose-dependent LGEO treatments. However, the total hemocyte count was lowered, specifically plasmatocyte, granulocyte, and spherulocytes counts. These were the only cell types that I observed to display phagocytosis, correlating to results found in literature (Lavine & Strand, 2002; Peterson, 2019). I speculate that because of lowered hemocyte's numbers the total efficacy of phagocytosis was in fact lowered by the LGEO treatment. If studied further with the proper equipment and tools, the negative correlation would be likely observed with a decrease in phagocytosis rate in a dose-dependent manner.

The HPTLC analysis (Figure 4) shows that citral does make up the majority of the LGEO used in my tests. This is confirmed in literature by Wagner and Bladt (1996). Rice (2021) performed preliminary investigation of citral effects on differential hemocyte copunts in Galleria larvae, using the same methods that I used here with citral instead of LGEO. She observed that citral lowered numbers of circulating plasmatocytes, and was effective at lower concentrations.

This is, in a part, congruent with my findings, confirming that citral is responsible for lowering hemocyte numbers.

Rice (2021) also studied citral application methods and found that citral could influence hemocyte counts in Galleria when applied topically or by alimentary system. This raises the question about citral's potential as biopesticide. Specifically, if citral can lower levels of circulating hemocytes under field conditions, and, consequently, counteract encapsulation. Since shutting off this mechanism increases the chance of survival for the invader, citral could enhance biocontrol treatments used in IPM strategies against parasitic roundworm nematodes. Further studies are needed on lemongrass's and other essential oils' potential against insect immune systems.

Such studies have been recently undertaken in Dr. Pszczolkowski's lab and produced promising results. For example, invasion by a parasitoid can be mimicked by implantation of nylon implant into the hemocoel of the larvae. The larvae respond by encapsulation and melanization of the implant. Later, a video imaging system was used for measuring grey values of light passing through the implants (Rantala *et al*., 2002). In our experiments, citral injections prior to implant insertion inhibited implant melanization at doses as low as 0.3 µg/larva.

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