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Using Pseudo Nomarski Contrast to Study Hemocytes of Galleria Mellonella

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USING PSEUDO NOMARSKI CONTRAST TO STUDY HEMOCYTES OF *GALLERIA MELLONELLA*

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By

Westley David Peterson

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ABSTRACT

Differentiation of insect hemocytes requires microscopes equipped with differential interference contrast (DIC), s.c. Nomarski's contrast. However, the high price of DIC limits speed and efficacy of hemocyte studies. For my thesis, I developed two low cost microscope sets equipped with custom oblique illumination filters. The oblique illumination filters, that I produced, provided high quality images comparable with those produced by DIC, for a fraction of the cost and allowed me to train laboratory personnel quickly. Microscopes with these oblique illumination filters were used in studies of spherulocytes and for rapid screening of plant essential oils to impair insect immune system. Nine plant essential oils were screened for effects on hemocytes in circulation of Sumi ink-challenged larvae. *Galleria mellonella* larvae injected with artificial pathogen, Sumi ink, responded by increasing numbers of circulating spherulocytes in a dose-dependent manner. In addition, phagocytosis of Sumi ink particles by spherulocytes was observed. Two oils (bergamot and frankincense) markedly lowered numbers of plasmatocytes and granulocytes in circulation of *Galleria mellonella* larvae. One (lavender) lowered numbers of oenocytoids.

KEYWORDS: *Galleria mellonella*, hemolymph, Nomarski's differential interference contrast, spherulocytes, sumi ink, oblique illumination filter

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I dedicate this thesis to all my friends and family that supported me throughout this long journey. To my father, Neil Peterson, who passed away so suddenly, I love you and I miss you very much. I especially dedicate this work to my wife, Stephanie M. Peterson, who had to sacrifice so much over the past two years. Her strength gave me the strength, to see this journey through to the end.

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INTRODUCTION

Introductory Remarks

Agricultural crop protection from insect pests relies on synthetic substances used to prevent, kill, repel, or eliminate insects and other organisms (Khater, 2012). Initially, these substances, insecticides, appeared very effective and relatively safe. The carcinogenic, teratogenic, spermatotoxic, and environmentally dangerous properties were unknown at the time of first use (Khater, 2012). However, the long-term overuse of insecticides resulted not only in alarming ecological consequences but also in development of resistance of the pests to insecticides; thereby forcing farmers to increase insecticide application rates to secure effective pest management (Lewis, Van Lenteren, Phatak, & Tumlinson, 1997). Due to global human population growth, crop production will have to increase considerably over the next decades to provide food security. Alternative pest management strategies are needed, and "soft interventions" such as the use of biopesticides instead of broad-spectrum insecticides are advocated (Lewis et al., 1997; Chandler, Grant, & Greaves, 2010).

The United States Environmental Protection Agency (EPA) defines a part of biopesticides as "naturally occurring substances that control pests" (Environmental Protection Agency of the USA, 2012). In my graduate work, I concentrated on plant secondary metabolites and studied the inhibitory effects of these compounds on an insect immune system. In particular, I investigated effects of selected plant essential oils on insect hemocyte counts.

To that end, I utilized a model experimental system previously used for studying insect innate cellular immune responses (Haszcz, 2016; Fenske, 2018). In this system, larvae of *Galleria mellonella* (Lepidoptera, Pyralidae) are challenged with micro particles of oil soot that

mimics infection with pathogens (such as bacteria). This treatment elevates numbers of hemocytes in circulation (Haszcz, 2016; Fenske, 2018), which increases potential of the insect to combat the pathogen. Concurrent treatment of experimental larvae with plant secondary metabolites may reverse the effects of the artificial pathogen, indicating its ability to impair insect immune system. Continuation of such research can lead to identification of chemical substances that impair insect immune response to naturally occurring pathogens, and identify potential adjuvants that can enhance insecticides currently on the market but need to be used in large quantities or for extended periods to exert desirable effects.

Microscopes Used in Studies of Insect Blood Cells

Potentially, any brightfield microscope could be used for the investigation of insect hemocytes, if combined with staining dead cells. The Giemza staining used by Altuntas, Kılıç, Uçkan, and Ergin, (2012) is an example. However, to differentiate among specific classes of living insect hemocytes, one needs a microscope with a reliable contrast system (Gupta, 1985). Although brightfield may be used in some applications (Haszcz, 2016), differentiating among the classes of lepidopteran hemocytes (plasmatocytes, granulocytes, spherulocytes, oenocytoids) using brightfield is inefficient.

Brightfield produces only two-dimensional images of little or no quality. The reason for this is that brightfield does little in manipulating the light waves before passing through the sample. It simply creates a field of light behind the sample for observation. Phase contrast was the next logical step. By using the levers on the phase contract turret, the light passing through the condenser is shifted slightly. This helps outline the cell walls, making it easier to see the cell. Though phase contrast did improve the image quality, it was not improved to a satisfactory

degree. Instead, I used a microscope set up for differential interference contrast (DIC), also known as Nomarski contrast.

Differential interference contrast was developed by Polish born Georges Nomarski using a set of prisms invented by William Wollaston. Nomarski's contrast set-up utilizes two polarizing lenses and two sets of modified Wollaston prisms to manipulate light waves used to view the object being observed (Pluta, 1994). It is important to understand that the difference between Nomarski's prisms and Wollaston's prisms is that the focal point, which is inside Wollaston's prisms, differs from the focal point being outside Nomarski's prisms. Georges Nomarski simply modified Wollaston's prism design in order to make this change in focal points. Nomarski's contrast starts at the light source with a polarizer lens directly between the light source and the condenser, where the first set of prisms are often mounted. Some microscopes have the prisms mounted separate from the condenser. The light passes through the stage and sample and the analyzer that contains the second polarizing lens captures the light, and then the light goes on to meet the eye or camera viewing the sample.

It is important to note that light waves have two directions: horizontal and vertical. Polarizing light means to extinguish, partially or completely, the light waves in one of the aforementioned directions. The first polarizing lens partially or fully eliminates one direction of light waves, as the modified light passes through the first Nomarski prism is then split. Light waves then move through the sample being viewed and into the second set of Nomarski prisms that refocuses the light. Afterwards, it passes through the second polarized lens at the analyzer, which partially or fully extinguishes the remainder of one of the paths of light (Pluta, 1994).

Nomarski's method produces excellent contrast without sacrifice to magnification or resolution of the sample being viewed. However, it is expensive. A microscope with Nomarski

contrast may cost over ten-thousand dollars. Typically, college level and high-grade laboratories have only one microscope capable of DIC. One of my goals was to screen large numbers of plant essential oils quickly for effects on hemocyte numbers. To accomplish this, I needed at least three microscopes allowing reliable differentiation of particular hemocyte classes; however, costs of three DIC attachments were prohibitive. In order to solve this dilemma, I designed oblique illumination filters to use in combination with economy grade compound microscopes.

Based on work by Shaw (2014), I designed and tested more than thirty different filters cut from plastic. Most of the filters did not produce desirable images. One filter though, produced very good results almost matching that of DIC. When I coupled my newly designed oblique illumination filter with a camera, the result was a pseudo Nomarski contrast, obtained at a fraction of the cost of true DIC. Microscope sets based on this prototype were used for studying a class of insect hemocytes called spherulocytes, and for fast screening of ten plant essential oils for the ability to impair the insect immune system.

Immune System of *Galleria melonella*

Galleria mellonella was selected for these experiments because it was established as a model system for insect immune system studies (Wojda, 2017) and the methodology of the studies were developed by my predecessor (Haszcz, 2016).

There are two types of immune systems in *Galleria mellonella*, humoral and cellular. The humoral immune system relies on specific proteins that binds to and destroys foreign substances (Boman & Hultmark, 1987). In contrast, cellular defense refers to hemocyte-mediated immune responses like phagocytosis, nodulation, and encapsulation (Schmidt, Theopold, & Strand 2001; Strand and Pech, 1995).

The humoral response involves the synthesis of defense molecules. These include reactive intermediates of oxygen, nitrogen, and antimicrobial peptides (AMPs) with molecular weights between 10 and 4 kDa possessing antibacterial and/or antifungal properties (Bogdan, Röllinghoff, & Diefenbach, 2000; Casanova-Torres & Goodrich-Blair, 2013; Nappi & Vass, 2001). These molecules do not actively seek foreign invaders, rather these act as mines floating throughout the hemocoel that destroy a foreign invader upon contact. Most often, these molecules destabilize the cell membrane and interfere with translation, transcription, and replication of the cell.

Cellular immune response contains five types of cells within the *Galleria mellonella* hemolymph: granulocytes, plasmatocytes, spherulocytes, oenocytoids, and pro-hemocytes. Granulocytes, the most common, are a part of the white blood cell system that attacks foreign materials introduced into the body. Plasmatocytes are the second most abundant hemocytes and act in a similar manner. It is still undetermined what role oenocytoids or spherulocytes play. Spherule cells have been suggested to transport cuticular components (Sass, Kiss, & Locke, 1994), while oenocytoids contain cytoplasmic phenoloxidase precursors that likely play a role in melanization of hemolymph (Ashida and Dohke, 1980; Iwama and Ashida, 1986; Jiang, Wang, Ma, & Kanost, 1997). Pro-hemocytes, however, act as the stem cells in the *Galleria mellonella* hemolymph and often become granulocytes or plasmatocytes when a foreign substance is introduced.

Once the hemocytes have found foreign matter, the cells either phagocytize, encapsulate, or nodulize the target depending on the size or type of invader. If the foreign matter is large, granulocytes and plasmatocytes surround it forming a capsule; completing a process known as encapsulation. Neither granular cells nor plasmatocytes form capsules alone, but plasmatocytes

readily encapsulate this target following attachment of granular cells (Lavine and Strand, 2002). If the foreign matter is bacteria, then multiple cells bind to the many bacterial cells in a process called nodulation. If the foreign material is small enough for one cell to engulf the particle, then the single cell will engulf it, phagocytizing the particle on its' own. Plasmatocytes and granulocytes are the only cells believed to participate in these processes for the lepidopteran order (Lavine and Strand, 2002; Strand, 2008).

Studies performed by my predecessors (Fenske, 2018; Haszcz, 2016) found that injections of oil soot particles (contained in Sumi ink) could imitate bacterial infections in the *Galleria mellonella* and give rise to the number of circulating plasmatocytes and granulocytes. However, prior to my work, it was unknown if or how spherulocytes responded to ink injections. To delineate what role the spherulocytes play in the cellular immune response for *Galleria mellonella*, I studied the dynamics of spherulocyte counts in Sumi ink–challenged Galleria larvae. In addition, I examined whether or not *Galleria mellonella* spherulocytes were capable of phagocytosis of Sumi ink particles.

Essential Oils

From cosmetics to medicine, essential oils have many uses. The food industry uses essential oils as a preservative against bacteria, molds, and fungi growth on foods for longer shelf life of product (Vergis, Gokulakrishnan, Agarwal, & Kumar, 2015). The cosmetics industry utilizes essential oils in various products (Carvalho, Estevinho, & Santos, 2016). The medical industry uses essential oils mostly for anti-bacterial and anti-microbial properties (Hammer, Carson, & Riley, 1999; Vergis et al., 2015).

The focus of my research was utilizing essential oils for pest control. Whether it be as an antifeedant, a reproductive inhibitor, a growth inhibitor, or a killing agent, essential oils have been used as a pesticide and show real promise in that area (Isman, 2000). An excellent example of this comes from the neem tree (*Azidirachta indica*). For centuries, Indo-Pakistani farmers 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 stored grain (Ahmed & Grainge, 1986).

The necessity to identify essential oils that could serve as biopesticides is becoming more important as the global population inflates and the damage traditional pesticides cause increases. The main problem is that there are many pesticides being used in what is called the pesticide treadmill. The pesticide treadmill refers to a practice of rotating pesticides in and out of use, so that the insects do not develop a resistance to one particular pesticide (Lewis et al., 1997). However, this practice does not resolve the problem of polluting the waterways and soils with these chemical pesticides. Essential oils are natural, eco-friendly and break down returning as beneficial nutrients to the soil over a short period; reducing the impact of harmful pollutants that enter the ecosystems and atmosphere (Tripathi, Upadhyay, Bhuiyan, & Bhattacharya, 2009).

Although the potential of essential oils as insecticides or regulators of insect behavior, reproduction, and growth is largely known (Isman, 2000), their effects on insect immune system are poorly understood. Only recently, it has been found that neem essential oil lowers numbers of circulating plasmatocytes in Galleria larvae (Haszcz, 2016; Fenske, 2018). More extensive screening studies on effects of other essential oils on insect hemocytes are needed.

To that end, three inexpensive microscope sets (a microscope, my custom-made oblique illumination filter, a digital camera, and a micro PC) where assembled. Nine different essential

oils were screened for an ability to impair *Galleria mellonella's* immune system. With an average of eight to fifteen samples per oil accompanied by eight to fifteen controls evaluated alongside the test samples, the results provided valuable information on the oils' potential for affecting insect hemocyte numbers and identified particularly promising formulations for further research.

METHODS

Chemicals

For the artificial pathogen to mimic bacterial infection, particles of tung tree soot contained in KY2 black sumi ink (Yasutomo, San Francisco, CA) was used. In order to separate the soot from bigger particles of nonuniform size, the ink was centrifuged at 2000g for five minutes. The supernatant was placed in an Eppendorf tube, diluted to 40% in double distilled water, and vortexed. The 40% concentration of sumi ink was established previously as most effective (Fenske, 2018; Haszcz, 2016).

An anti-coagulant buffer was necessary to prevent coagulation of sampled hemolymph. In addition, the anti-coagulant served as a mixture component for the cell stain so the cells would absorb the stain and become more visible under the microscope. The anti-coagulant consisted of 0.157g NaOH, 0.435g NaCl, 0.315g citric acid, 0.253g Na_2EDTA, with a final pH of 4.58. NaOH and NaCl was obtained from Sigma-Aldrich® (St. Louis, MO). The citric acid and Na_2EDTA were obtained from Thermo Fisher Scientific (Pittsburg, PA). All the substances were weighed out using an Ohaus Galaxy 160D scale and combined in a test tube. Distilled water was added to bring the final volume to 40mL and the tube was vortexed.

As previously stated, the cell stain utilized the anti-coagulant buffer with bacto-neutral red dye from Difco Laboratories (Detroit, MI). I weighed out 2mg of the bacto-neutral dye in an Eppendorf tube then added 1mL of the anti-coagulant buffer. The substance was then vortexed and checked on a clean hemocytometer under the microscope to ensure no large particles existed. Pertinent information concerning the other chemicals used will be disclosed later in the appropriate sections.

Insects

Greater wax moth (*Galleria mellonella)* larvae were selected as the subject for these studies. A care system for the larvae was established previously (Fenske 2018; Haszcz, 2016). Galleria larvae are inexpensive and easy to acquire. Their relatively large size makes injections easy to perform.

Galleria mellonella of the Lepidopteran order of the family Pyralidae were acquired in their seventh instar stage, from Knutson's Live Baits (Brooklyn, MI). When the package of larvae arrived from Knutson's, the larvae were separated from the wood shavings used in the packing process to protect the larvae during shipping. I prepared eight glass Ball jars (Fishers, IN) that had plastic lids with aeration holes. I would then measure the total weight of the larvae and divide into eight equal portions into respective jars. Along with the larvae, I would place food at a minimum of one and a half times the body weight of the larvae into each jar. In pilot experiments, I found that when this feeding procedure was used, cannibalism amongst the larvae was no longer a concern.

The food for the larvae contained four ingredients: Gerber® multigrain cereal (Gerber, Fremont, MI), glycerin USP 99.5% (Humco, Texarkana, TX), pure granulated cane sugar (Domino Foods Inc., Yonkers, NY), and distilled water. First, I mixed 25mL of sugar, 25mL of the distilled water, and 37mL of glycerin in a small plastic bowl. The bowl was microwaved for ten to twenty seconds to help dissolve the sugar in the mixture. Then 400mL of the multigrain cereal was placed in a one-gallon food storage bag. All of the contents of the small bowl were added and kneaded for no less than two minutes to ensure the contents were mixed evenly. The

food was made either the day before or on the day of arrival of the new larvae shipment, and was refrigerated for no longer than two months.

This insect diet contained many valuable nutrients such as vitamins A, B1, B2, B3, B6, B12, C, and E, as well as minerals such as calcium, iron, phosphorus, and zinc. Along with being easy to produce, this diet was also inexpensive. Once the food was placed into the jars with the larvae, the jars were stored in incubators (VWR Scientific Products, Model 2005) with the atmosphere kept at a constant 30°C and 80% relative humidity with no light source.

Microscopes

Three models of microscope sets were used for comparative and reference observations including an Olympus BX-40 equipped with a UPLFLN40XPH Plan Fluorite 40X objective, U-UCD8-2 Universal condenser, and Nomarski's DIC (Olympus America, Central Valley, PA). For the remaining work (spherulocyte counts, phagocytosis evaluation, and screening of essential oils), microscope sets based on Galen III (Cambridge Instruments, Watertown, MA), or National Ecoline (National Optical and Scientific Instruments, Inc., Schertz, TX) were used. Galen III was equipped with Cambridge Instruments PLAN 40Ph 40X objective, phase contrast turret, and Abbe condenser. National Ecoline microscope was equipped with an EA 40 objective, Abbe condenser, and swing-out filter holder.

Prototyping of the Oblique Illumination Microscope Systems

The Galen III microscope had a turret housing four phase contrast filters and an open port equipped with iris diaphragm for brightfield observations (Figure 1A). The diaphragm assembly was placed in a recessed mount into which a filter was inserted and was immobilized using a

rubber O-ring (Figure 1B). In the process of prototyping, over 20 filters were manufactured by hand based on Von Egmond (2002) and Shaw (2014) and were evaluated using the Galen III microscope. Only one filter gave satisfactory results, a modified oblique illumination filter (Figure 1A). The modified oblique illumination filters were made from a Mead Five Star thin black plastic file folder (ACCO Brands Corp, Bentonville, AR) cut to fit into the condenser with the hole offset of center.

Two types of oblique illumination filters were produced, depending on the type of microscope. For the Galen III microscope, I first had to measure the opening for the brightfield iris diaphragm. The opening measured 27.9 millimeters. The filter outer edge was then cut to 27.8 mm of diameter so that the filter would fit into the brightfield opening. Afterwards, a relief line of roughly three millimeters was drawn just inside the outer edge of the filter, so that the hole to be cut was not covered up by the rubber O-ring needed to retain the filter in the brightfield opening. Consequently, the Galen III filters had the holes on the interior edge of the relief lines (Figure 2 A-C).

The National Ecoline microscopes utilized an LED light source, as opposed to the Galen III that used a halogen light bulb. Since the LED light source was much more intense and directional, the hole for the Ecoline filters had to be cut with the hole edge on the center of the filter (Figure 2 D-F). In addition, the National Ecoline microscope did not have a turret. However, below the condenser, there was a swing-out holder housing a filter made of frosted glass (Figure 3 A). The frosted glass filter was removed from the holder and replaced with an oblique illumination filter (Figure 3 B, C). Then the filter holder was mounted to the microscope and swung into place underneath the condenser (Figure 4 A, B).

Three oblique illuminations filters were initially cut for the Galen III and another three for Ecoline microscopes (Figure 2). For the Galen III microscope the holes measured; 3.1mm, 6.2mm, and 9.3mm. The holes for the Ecoline microscope measured; 3.79mm, 7.59mm, and 11.39mm. Three independent knowledgeable individuals evaluated the efficacy of these filters. To that end, a series of three charts showing pictures of the same insect blood cell taken with microscopes equipped with a given oblique illumination filter was presented to each evaluator, who was asked to grade each picture on a scale from 1 to 4 or 1 to 12. An example of evaluation chart three is given in Appendix A.

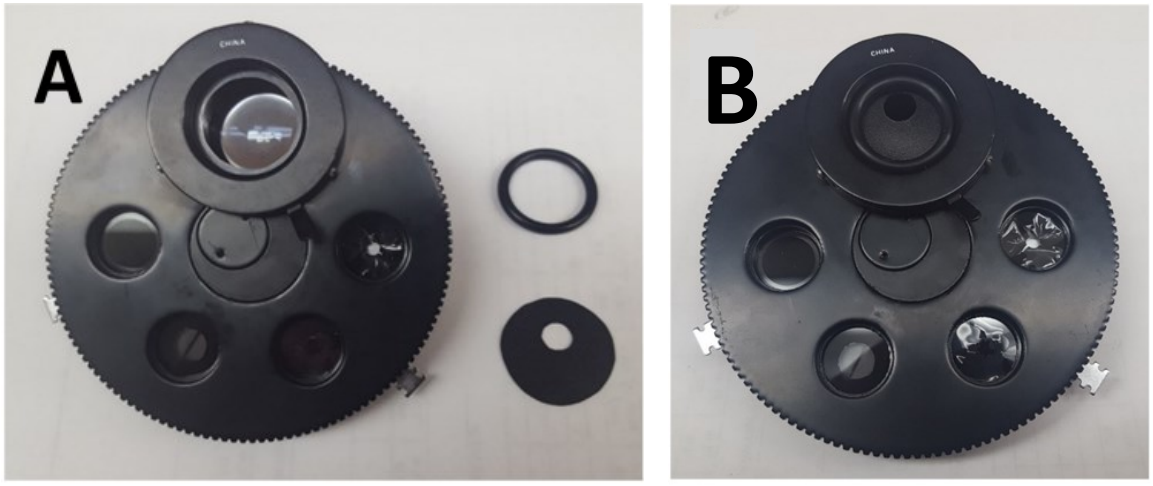

Figure 1. **A**. Galen III phase contrast turret with open port for brightfield observations. The oblique illumination filter and a rubber O-ring are shown on the right. **B**. The oblique illumination filter installed in the brightfield port, and secured with the rubber O-ring.

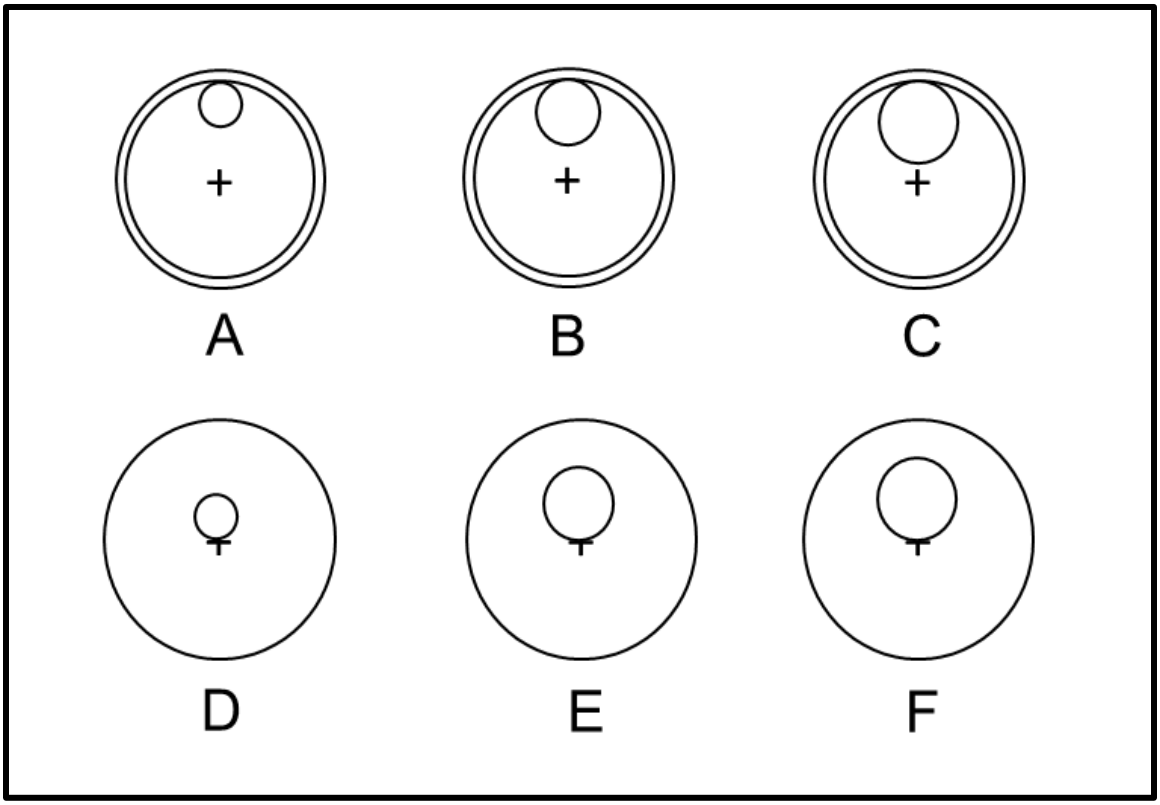

Figure 2. Oblique illumination filters produced by myself for the Galen III microscope (**A, B, C**) and for the National Ecoline microscopes (**D, E, F**). The diameters of the openings are given in text.

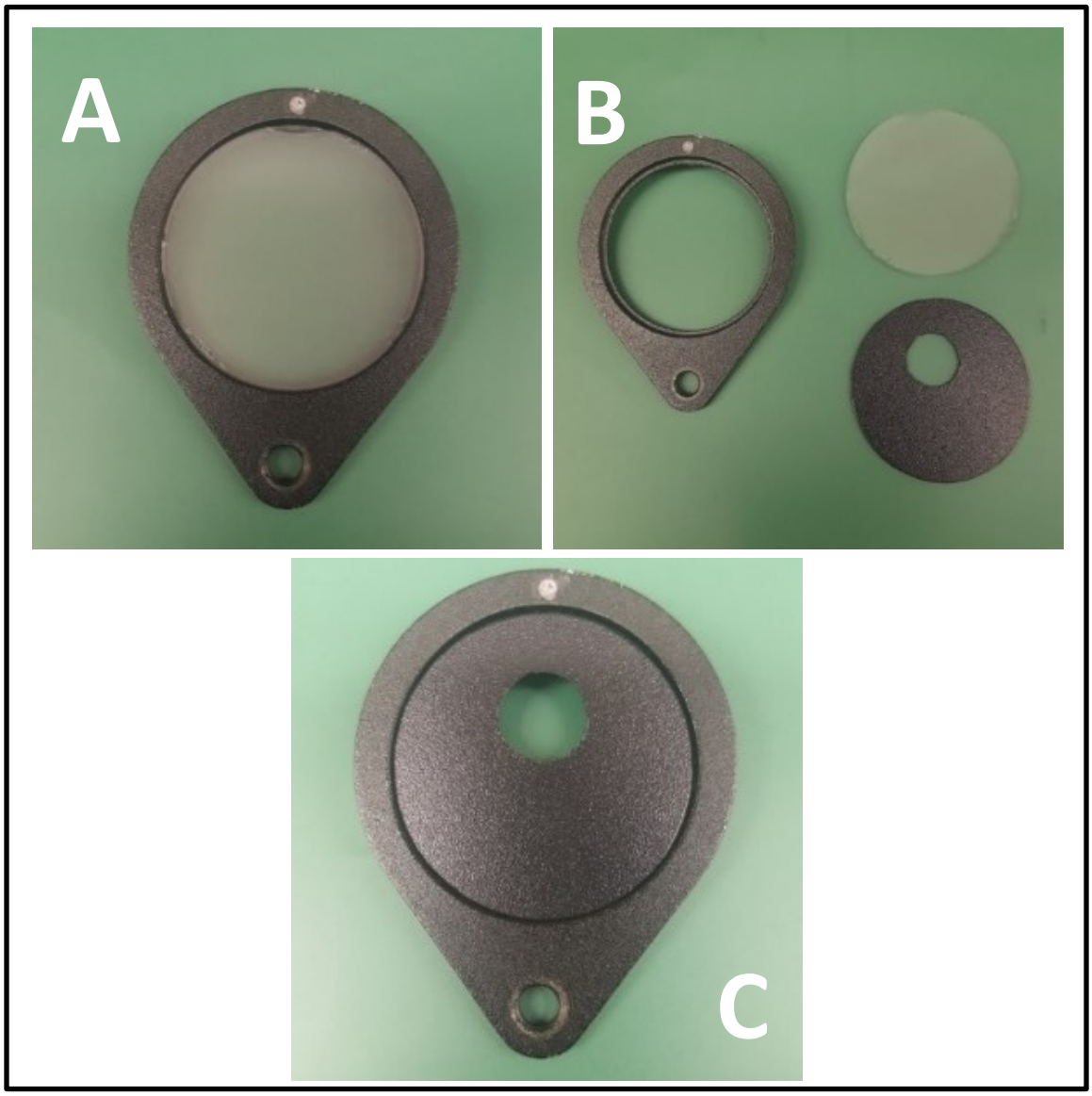

Figure 3. A. Frosted glass filter holder for Ecoline microscope. **B**. Frosted glass filter removed from holder. **C**. Oblique illumination filter inserted into the filter holder.

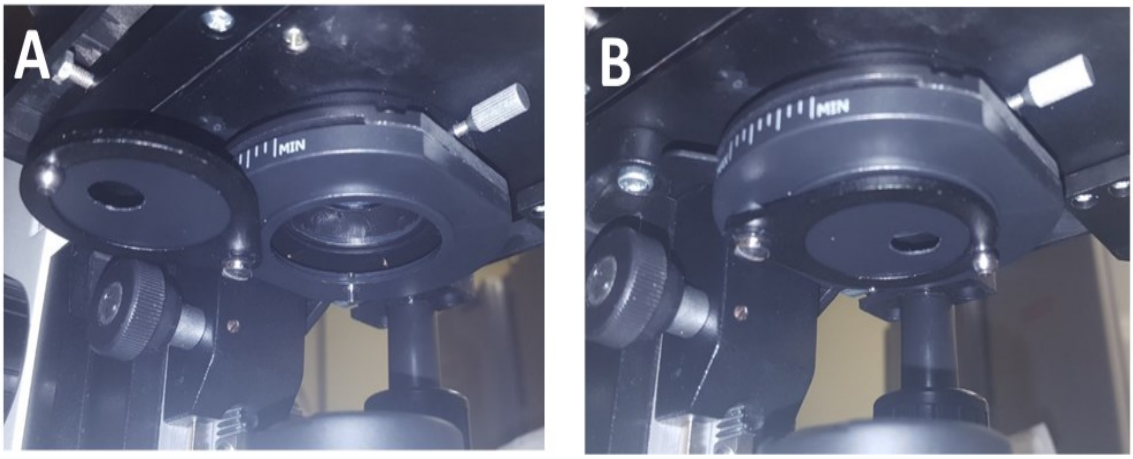

Figure 4. A. The frosted glass holder mounted to the National Ecoline microscope in the swung out position with the oblique illumination filter in place. **B**. Frosted glass holder with the filter swung into place underneath the condenser and ready for use.

Digital Microphotography

Each microscope was additionally equipped with a Dino-Lite digital camera (AnMo Electronics Corporation, New Taipei City, Taiwan) inserted into the eyepiece of the microscope and then connected to a PC. For the Galen III microscope, I used a Dell Optiplex GX620 PC running Microsoft Windows XP. For the Ecoline Microscopes I purchased two new fan-less PC's made by LIVA (Elitegroup Computer Systems, Taipei, Taiwan). Both were LIVA model X's running Microsoft Windows 10. Dino-Lite provided the driver software with each camera. It was necessary to download the drivers off the internet for the fan-less PCs' because those did not have a CD player built in. All images were saved to either to the PC or a flash drive, into folders sorted by cell type designation and whether or not the larvae absorbed Sumi ink.

Experiments with Spherulocytes

The artificial pathogen used in experimenting was particles of soot of tung tree oil contained in Sumi Ink (Yasutomo, San Francisco, CA), prepared as described previously. *Galleria mellonella* larvae, of the last instar, were anesthetized by submerging them in water for fifteen minutes prior to injections. Three different doses of ink per larva, 0.25, 1, and 2 µg, were injected. Each dose was dissolved in double distilled water, and applied in a 5 µL aliquot. Control larvae received 5 µL of double distilled water.

When injecting, the needle was inserted through either the first or second set of prolegs. Next, the larvae were rolled gently to ensure the ink spread through the body. Injected larvae were placed in a glass jar with perforated lid and incubated for 4 hours at 30 °C, 80% humidity, in total darkness. Cell staining solution was prepared in Eppendorf tubes by mixing 4 µl of Bacto-Neutral red dye (DIFCO Laboratories Inc., Detroit, Michigan) and 40 µl of an anti-coagulant buffer to keep the blood from clotting. The larvae were bled by cutting one of their rear prolegs. A drop of the blood was gathered onto a piece of parafilm (American National Can, Chicago, IL). Then 5 µl were taken from the drop and placed in a tube with the dyeing mixture. This tube was set aside for five minutes at room temperature to stain the cells.

Ten microliters of the dyeing mixture and cells were put into each of the two chambers of the hemocytometer slide (Levy Ultra Plane, C. A. Hausser & Son, Philadelphia, PA). The slide was viewed under a microscope at forty times magnification. Different classes of hemocytes were identified with concurrence to Altuntas et al., (2012). Spherulocytes were counted boustrophedonically, in a left to right, right to left pattern down each of the four quadrants in the two chambers. Hemocyte counts were recorded as the averages ± the standard errors. There were two separate variants of this experiment. In the first variant, every spherulocyte was counted

regardless of whether it had ink absorbed or not. In the second variant, the level of phagocytosis was assessed additionally. Here, numbers of the cells containing ink were recorded additionally. Intensity of phagocytosis was expressed as percentage of spherulocytes that had at least one ink particle embedded.

Screening Plant Essential Oils for their Effects on Insect Hemocytes

Effects of neem essential oil from *Azidirachta indica* (Juss) on insect blood cells were studied previously (Fenske, 2018; Haszcz, 2016). The new, economic design of the microscope with oblique illumination filter allowed for rapid and efficient screening of other essential oils for the potential in impairing of insect immune system. Since neem is used in folk medicine in India as an antipyretic (fever-reducing) drug (Subapriya & Nagini, 2005), I selected nine essential oils for which antipyretic properties had been advertised (Table 1). All essential oils were manufactured by NOW Foods, Bloomingdale, IL.

Many plant essential oils are toxic. Thus, in the initial stage of the experiments, toxicity of all plant essential oils was assessed. To that end, the essential oils were dissolved to 30% in cholesterol-free vegetable oil (soybean oil) prior to injection. Ten larvae, challenged with ink, were injected with 5µl of a given plant essential oil, incubated for 4 hours as described above, and number of dead larvae was counted. If no mortality was observed at 30% concentration, the larvae were bled and the blood cells stained and counted. If injections with 30% plant essential oil produced mortality higher than 30%, subsequent mortality assays were done at 20%, 10%, and 5% concentrations of a given essential oil. The results of mortality tests are shown in Appendix B. The highest concentration that caused mortality equal or lower than 29% was used

for subsequent experiments showing effects of essential oils on *Galleria melonella* blood cells (Table 1).

The plant essential oils were injected into *Galleria mellonella* larvae challenged with Sumi ink. The procedures used for ink preparation, plant essential oils injection, larvae incubation and bleeding, and hemocyte dyeing were the same as those described in section "Experiments with Spherulocytes." Numbers of plasmatocytes, granulocytes, spherulocytes, and oenocytoids were counted, and recorded as average numbers ± standard error measure for each plant essential oil separately.

Table 1. Concentrations of plant essential oils screen tested for their effects on insect hemocyte counts.

Trade name	Scientific name of the plant	Percentage concentration of essential oil injected into larvae
bergamot	*Citrus bergamia* Risso	30%
clove	*Eugenia caryophyllata* Thunb.	5%
eucalyptus	*Eucalyptus globulus* Labill.	30%
frankincense	*Boswellia carterii* Engl.	30%
lavender	*Lavandula angustifolia* Mill.	30%
rosemary	*Rosmarinus officinalis* L.	20%
sandalwood	*Santalum album* L.	20%
spearmint	*Mentha spicata* L.	20%
tea tree	*Melaleuca alternifolia* Chee	20%

Statistical Analysis

The data obtained for screening of essential oils were analyzed using a Student's t-test. In all remaining experiments, ANOVA, followed by Tukey multiple comparison of means was used.

The data were tested using GraphPad InStat, (GraphPad Software, San Diego, CA). Statistical significance level was set at $P<0.05$.

RESULTS

Microscopy

It was found that for the Galen III microscope, the 9.3mm filter produced the best quality images. For the Ecoline microscopes the 7.59mm and 11.38mm filters produced acceptable images, a result that prompted experimentation with another filter containing a 9.48mm hole, which produced superior images. Figure 5 shows the Galen III and Ecoline oblique illumination filters.

The images produced using my oblique illumination filters in combination with Galen III or Ecoline economic grade microscopes gave satisfactory results, comparable with Olympus BX-40 equipped with Nomarski's DIC (Figure 6). The resolution and quality of the images warranted further experimentation using those economic oblique illumination microscopes.

Experiments with Spherulocytes; Dose-dependent Response to Sumi Ink

It was found that spherulocytes responded to ink injections by an increase in numbers. Although the counts of circulating spherulocytes were not high, a marked dose-dependent elevation of spherulocyte numbers was observed at the doses of 1 and 2 μg per larva (Figure 7, ANOVA followed by Tukey comparison of means, $P<0.05$, N=11-14).

Experiments with Spherulocytes; Phagocytosis

It was found that spherulocytes of *Galleria mellonella* larvae were capable of phagocytosis (Figure 8). Particles of Sumi ink were found in spherulocytes coming from larvae

injected with 0.25, 1, and 2 μg of ink. This response was also dose-dependent (Table 2, ANOVA followed by Tukey comparison of means, $P<0.05$, N=7-10).

Screening Plant Essential Oils for their Effects on Insect Hemocytes

Six out of nine plant essential oils tested had no effect on hemocyte counts in *Galleria mellonella* larvae. These were clove, eucalyptus, rosemary, sandalwood, spearmint, and tea tree (Tables 3-8, $P>0.05$, Student's *t*-test). However, three oils (bergamot, frankincense, and lavender) influenced the numbers of hemocytes mobilized by ink injections. Bergamot essential oil, at 30% concentration, markedly decreased numbers of plasmatocytes in circulation (Table 9, $P<0.05$, Student's *t*-test). Numbers of granulocytes also dropped after application of 30% bergamot oil (Table 9, $P<0.05$, Student's *t*-test). Original formulation of frankincense essential oil markedly lowered the numbers of plasmatocytes at 30% (Table 10, $P<0.05$, Student's *t*-test).

However, the original formulation of frankincense oil contained only 20% of the active ingredient. Therefore, it should be emphasized that the effective concentration of frankincense administered to the larvae was only 6%. Lavender essential oil, at 30% concentration lowered numbers of oenocytoids (Table 11, $P<0.05$, Student's *t*-test).

Table 2. Effect of Sumi ink concentration on phagocytosis by spherulocytes. N= number of replicates per datum point. *** $P<0.001$ in ANOVA followed by Tukey comparison of means.

Sumi ink concentrations (μg/larva)	Phagocytosis (Percentage ± SEM of cells that phagocytized)	N
0†	0	10
0.5	8.60 ± 4.66	7
1	7.67 ± 2.77	10
2	34.02 ± 4.34 ***	10

† Double distilled water was used

Table 3. Effects of clove essential oil on hemocyte counts in ink-challenged *Galleria mellonella* larvae. Control larvae were challenged with ink injection and subjected to injection of vegetable oil (solvent). Experimental larvae were challenged with ink injection and subjected to injection of 5% clove essential oil dissolved in vegetable oil. Means in rows followed by the same letter are not significantly different (P>0.05, Student's t-test, comparison of means between controls and experimental). N=9-12 per datum point.

Cell type	Control larvae	Experimental larvae	P value
Plasmatocyte	6.75 ± 1.44 a	4.46 ± 1.50 a	0.29
Granulocyte	39.68 ± 6.59 a	27.07 ± 7.71 a	0.23
Spherulocyte	1.83 ± 0.60 a	0.99 ± 0.12 a	0.24
Oenocytoid	7.21 ± 2.97 a	2.72 ± 1.45 a	0.24

Table 4. Effects of eucalyptus essential oil on hemocyte counts in ink-challenged *Galleria mellonella* larvae. Control larvae were challenged with ink injection and subjected to injection of vegetable oil (solvent). Experimental larvae were challenged with ink injection and subjected to injection of 30% eucalyptus essential oil dissolved in vegetable oil. Means in rows followed by the same letter are not significantly different (P>0.05, Student's t-test, comparison of means between controls and experimental). N=9 per datum point.

Cell type	Control larvae	Experimental larvae	P value
Plasmatocyte	10.40 ± 2.29 a	14.51 ± 2.79 a	0.27
Granulocyte	50.39 ± 13.39 a	68.86 ± 12.36 a	0.33
Spherulocyte	4.07 ± 1.45 a	2.69 ± 0.73 a	0.41
Oenocytoid	8.89 ± 1.37 a	11.39 ± 3.75 a	0.54

Table 5. Effects of rosemary essential oil on hemocyte counts in ink-challenged *Galleria mellonella* larvae. Control larvae were challenged with ink injection and subjected to injection of vegetable oil (solvent). Experimental larvae were challenged with ink injection and subjected to injection of 20% rosemary essential oil dissolved in vegetable oil. Means in rows followed by the same letter are not significantly different (P>0.05, Student's t-test, comparison of means between controls and experimental). N=11 per datum point.

Cell type	Control larvae	Experimental larvae	P value
Plasmatocyte	17.68 ± 2.70 a	10.70 ± 2.72 a	0.08
Granulocyte	58.23 ± 10.50 a	44.33 ± 12.39 a	0.40
Spherulocyte	2.51 ± 0.46 a	3.69 ± 1.04 a	0.31
Oenocytoid	7.73 ± 1.85 a	4.72 ± 1.14 a	0.18

Table 6. Effects of sandalwood essential oil on hemocyte counts in ink-challenged *Galleria mellonella* larvae. Control larvae were challenged with ink injection and subjected to injection of vegetable oil (solvent). Experimental larvae were challenged with ink injection and subjected to injection of 20% sandalwood essential oil dissolved in vegetable oil. Means in rows followed by the same letter are not significantly different (P>0.05, Student's t-test, comparison of means between controls and experimental). N=12 per datum point.

Cell type	Control larvae	Experimental larvae	P value
Plasmatocyte	14.80 ± 2.53 a	10.98 ± 1.85 a	0.24
Granulocyte	58.79 ± 9.70 a	41.96 ± 6.46 a	0.16
Spherulocyte	5.19 ± 1.30 a	3.00 ± 0.43 a	0.12
Oenocytoid	7.64 ± 1.94 a	6.67 ± 2.50 a	0.76

Table 7. Effects of spearmint essential oil on hemocyte counts in ink-challenged *Galleria mellonella* larvae. Control larvae were challenged with ink injection and subjected to injection of vegetable oil (solvent). Experimental larvae were challenged with ink injection and subjected to injection of 20% spearmint essential oil dissolved in vegetable oil. Means in rows followed by the same letter are not significantly different (P>0.05, Student's t-test, comparison of means between controls and experimental). N=7-8 per datum point.

Cell type	Control larvae	Experimental larvae	P value
Plasmatocyte	5.54 ± 1.08 a	8.52 ± 1.95 a	0.22
Granulocyte	34.64 ± 5.20 a	54.42 ± 14.75 a	0.25
Spherulocyte	1.73 ± 0.70 a	3.09 ± 0.84 a	0.25
Oenocytoid	5.23 ± 1.76 a	6.67 ± 2.36 a	0.64

Table 8. Effects of tea tree essential oil on hemocyte counts in ink-challenged *Galleria mellonella* larvae. Control larvae were challenged with ink injection and subjected to injection of vegetable oil (solvent). Experimental larvae were challenged with ink injection and subjected to injection of 20% tea tree essential oil dissolved in vegetable oil. Means in rows followed by the same letter are not significantly different (P>0.05, Student's t-test, comparison of means between controls and experimental). N=14 per datum point.

Cell type	Control larvae	Experimental larvae	P value
Plasmatocyte	16.97 ± 3.01 a	13.80 ± 1.80 a	0.38
Granulocyte	55.32 ± 9.55 a	52.82 ± 7.73 a	0.84
Spherulocyte	2.73 ± 0.38 a	3.47 ± 0.49 a	0.24
Oenocytoid	8.90 ± 2.22 a	6.19 ± 1.58 a	0.33

Table 9. Effects of bergamot essential oil on hemocyte counts in ink-challenged *Galleria mellonella* larvae. Control larvae were challenged with ink injection and subjected to injection of vegetable oil (solvent). Experimental larvae were challenged with ink injection and subjected to injection of 30% bergamot essential oil dissolved in vegetable oil. Means in rows followed by the same letter are not significantly different (P>0.05, Student's t-test, comparison of means between controls and experimental). N=8-10 per datum point.

Cell type	Control larvae	Experimental larvae	P value
Plasmatocyte	12.56 ± 2.20 a	5.20 ± 1.04 b	0.01*
Granulocyte	50.31 ± 6.62 a	29.71 ± 4.75 b	0.02*
Spherulocyte	1.45 ± 0.24 a	1.85 ± 0.43 a	0.43
Oenocytoid	1.94 ± 0.55 a	1.79 ± 0.50 a	0.84

*P<0.05, Student's *t*-test

Table 10. Effects of frankincense essential oil on hemocyte counts in ink-challenged *Galleria mellonella* larvae. Control larvae were challenged with ink injection and subjected to injection of vegetable oil (solvent). Experimental larvae were challenged with ink injection and subjected to injection of 30% frankincense essential oil dissolved in vegetable oil. Means in rows followed by the same letter are not significantly different (P>0.05, Student's t-test, comparison of means between controls and experimental). N=12 per datum point.

Cell type	Control larvae	Experimental larvae	P value
Plasmatocyte	13.20 ± 1.45 a	7.56 ± 1.96 b	0.03 *
Granulocyte	47.00 ± 4.8 a	29.48 ± 7.63 a	0.06
Spherulocyte	2.96 ± 0.59 a	2.88 ± 0.70 a	0.93
Oenocytoid	7.17 ± 1.52 a	5.22 ± 1.32 a	0.34

*P<0.05, Student's *t*-test

Table 11. Effects of lavender essential oil on hemocyte counts in ink-challenged *Galleria mellonella* larvae. Control larvae were challenged with ink injection and subjected to injection of vegetable oil (solvent). Experimental larvae were challenged with ink injection and subjected to injection of 30% lavender essential oil dissolved in vegetable oil. Means in rows followed by the same letter are not significantly different (P>0.05, Student's t-test, comparison of means between controls and experimental). N=13-16 per datum point.

Cell type	Control larvae	Experimental larvae	P value
Plasmatocyte	9.96 ± 2.16 a	9.19 ± 2.93 a	0.83
Granulocyte	65.7 ± 8.1 a	33.01 ± 4.95 a	0.71
Spherulocyte	1.70 ± 0.30 a	1.92 ± 0.57 a	0.71
Oenocytoid	9.47 ± 2.87 a	2.81 ± 0.78 b	0.05*

*P<0.05, Student's *t*-test

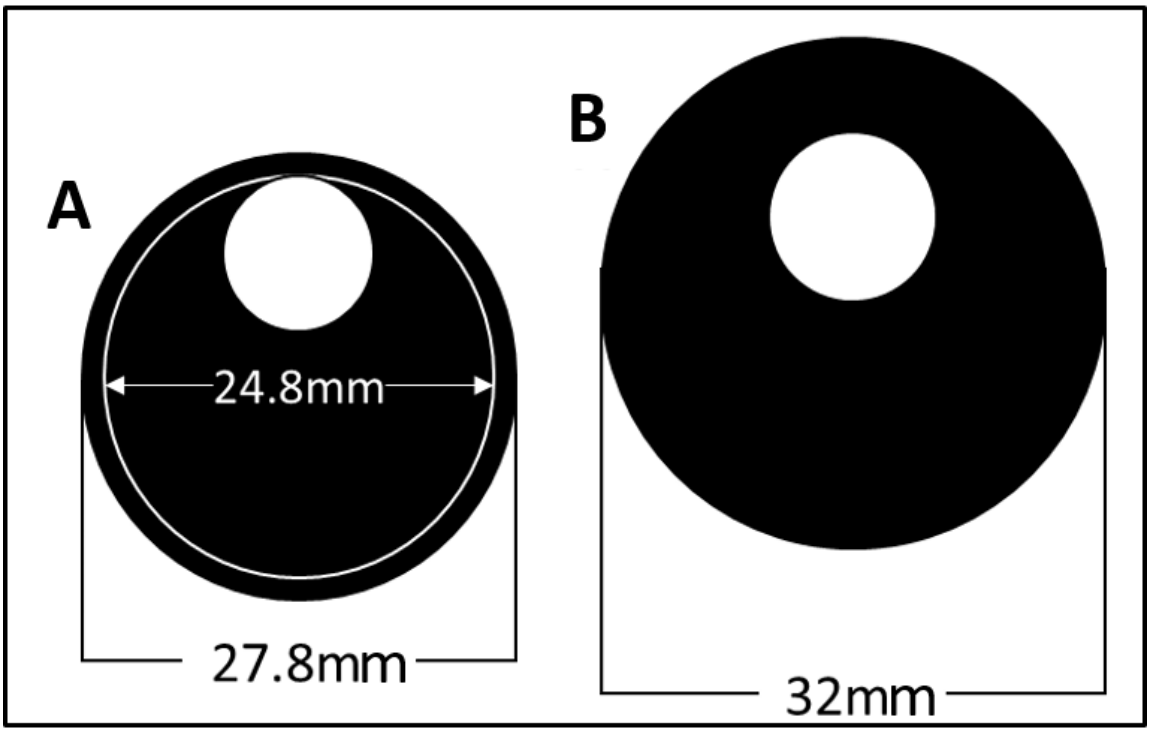

Figure 5. Illustrations of the oblique illumination filters used in the study on *Galleria mellonella* hemocytes. **A.** The filter for the Galen III microscope with 9.3mm hole and relief line. **B**. The filter for the Ecoline microscope the 9.48mm hole.

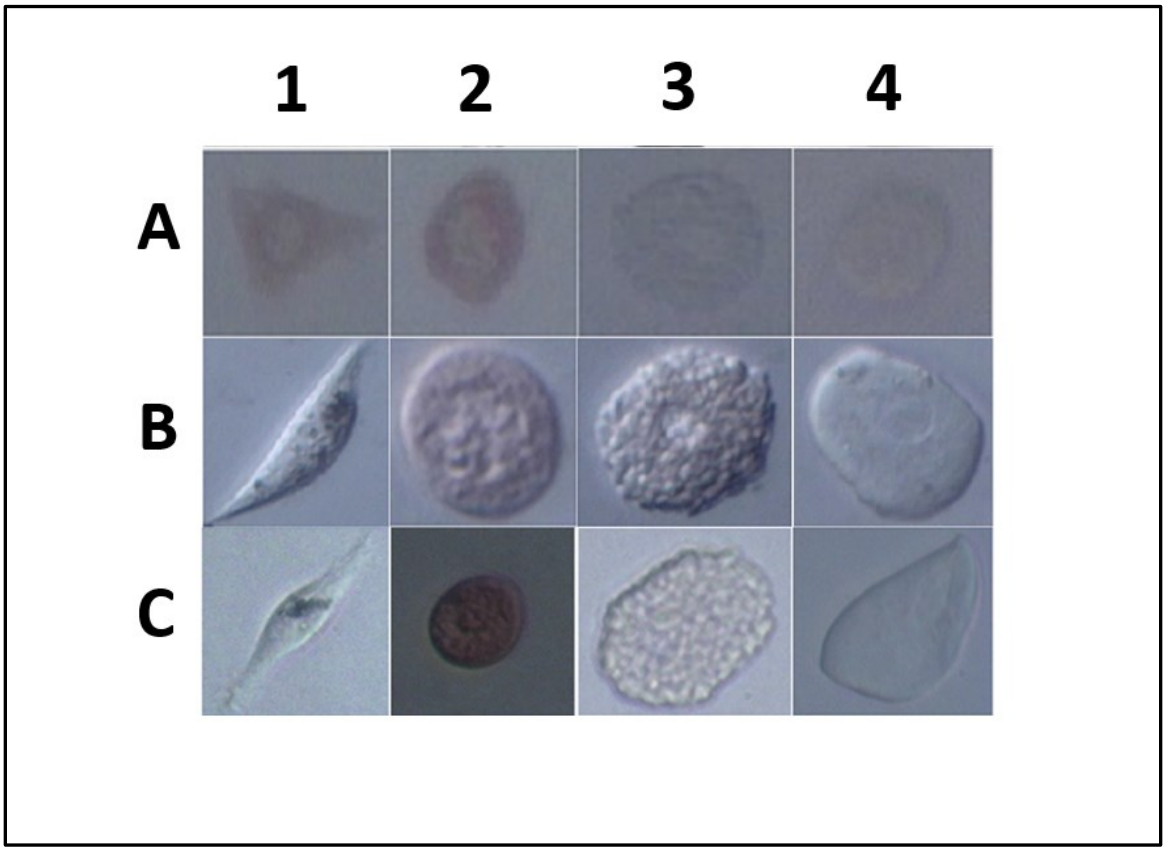

Figure 6. Four classes of *Galleria mellonella* hemocytes (**1.** Plasmatocytes; **2**. Granulocytes; **3**. Spherulocytes; and **4**. Oenocytoids) visualized by (**A**) brightfield microscopy, (**B**) Nomarski's differential interference contrast, and (**C**) oblique illumination.

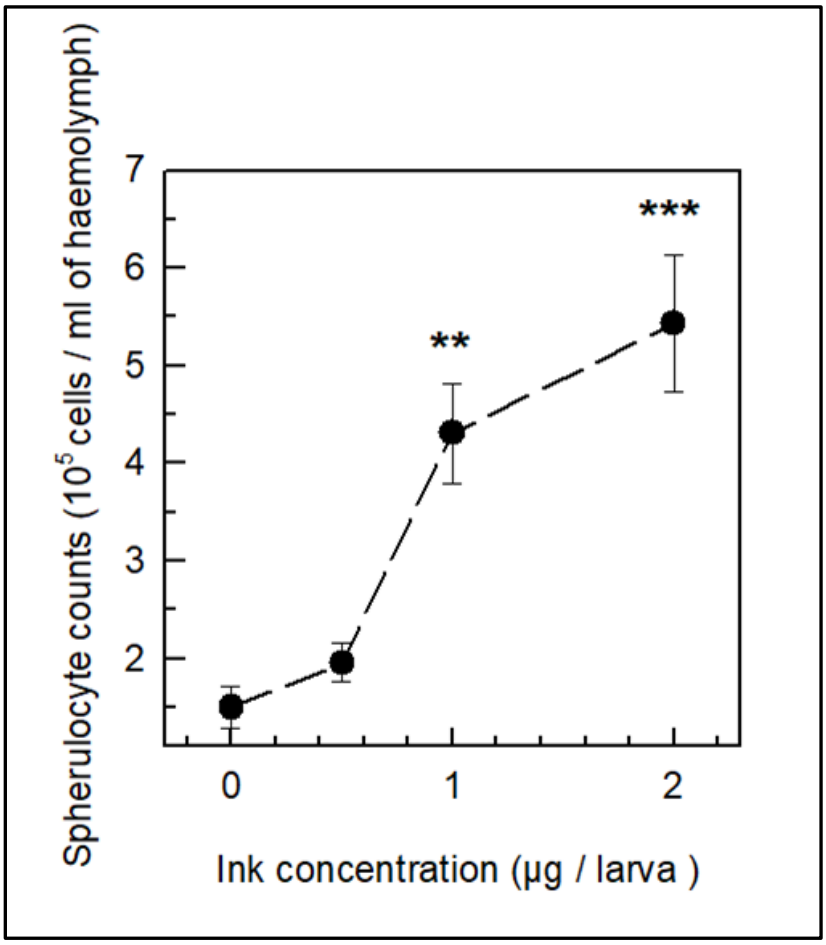

Figure 7. Dose-dependent mobilization of *Galleria mellonella* spherulocytes by Sumi ink.

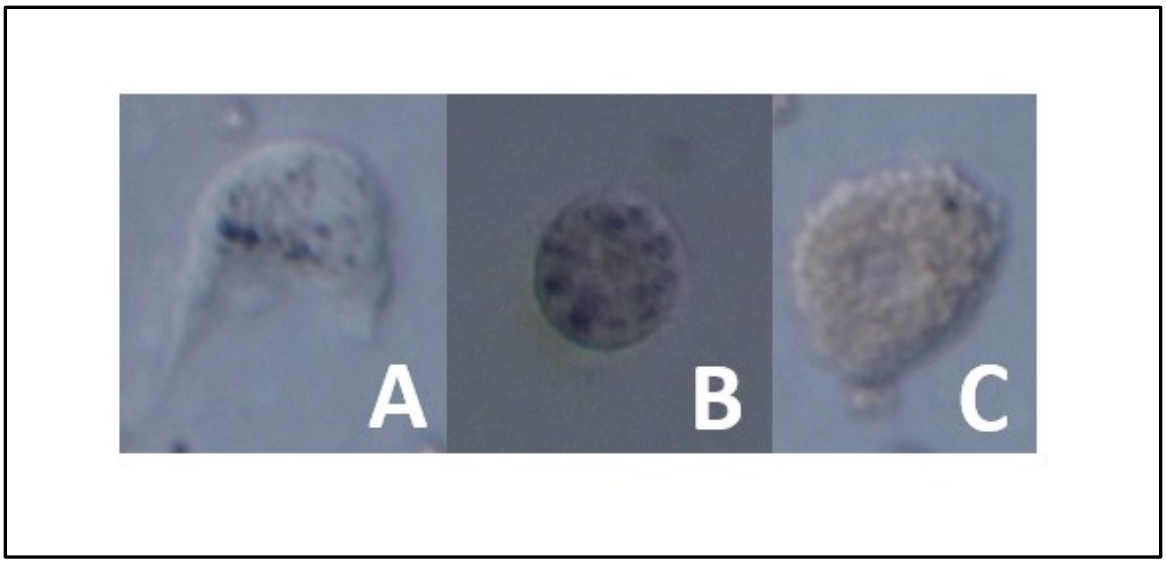

Figure 8. Phagocytosis of Sumi ink particles by *Galleria mellonella* plasmatocyte (**A**), granulocyte (**B**), and spherulocyte (**C**).

DISCUSSION

Microscopy

Employing oblique illumination for improvement of image contrast was postulated as early as the 1950s (O'Neill, 1956). However, applying oblique illumination filters to biological investigations using optical microscopes has never been a popular idea. An excellent paper on investigating protozoans using an oblique illumination filter, similar to my design, by Wessenberg and Reed (1971) attracted only two citations by 2019 and their work was not continued in academia for more than three decades. Citizen scientists later developed oblique illumination filters. Von Egmond (2002) presented this idea for studies and imaging of leaf surfaces and Shaw (2014) published a book about making polarization and oblique illumination filers for amateur and professional microscopy. More recently, a group of researchers from Spanish universities and institutes presented a low-cost oblique illumination microscope system for investigation of diatoms (Ruiz-Santaquiteria, Espinosa-Aranda, Deniz, Sanchez, Borrego-Ramos, Blanco, & Bueno, 2018; Sanchez, Cristóbal, Bueno, Blanco, Borrego-Ramos, Olenici, & Ruiz-Santaquiteria, 2018)

In their study, a low-cost microscope was equipped with a custom-made plastic turret with seven oblique illumination filters. The turret was printed using a 3D printer, and mounted on a pin below the condenser. At the time of designing my own filters (summer 2017) the works of Sanchez et al. (2018) and Ruiz-Santaquiteria et al. (2018) had not been published yet, thus their works were not an inspiration for my accomplishments. Noteworthy, my oblique illumination filters provided much better imaging when used with the Ecoline microscope than used with the Galen III. I attributed that to the fact the Ecoline microscope used LED as an illumination source as opposed to the Galen III that was equipped with a simple incandescent

bulb. A very recent work of Sugimoto, Maruyama, Tamada, Arimoto, and Watanabe (2019), showed the same.

Advantage of my microscope design is evident when reasonable quality of microscope imaging, by using my oblique illumination filters, is combined with the low cost of instrumentation. Assuming that one possesses a microscope, the minimum price of Nomarski's DIC upgrade (two prisms and the analyzer) equals roughly $4,000, and may even reach $7,000 if a new condenser and nosepiece are required. Manufacturing one oblique illumination filter of my design costs less than $15, including costs of materials, tools, and labor. This opens avenues of research and teaching activities to establishments that do not have financial resources enabling studies of plant and animal cells with accuracy hitherto available only to research or academic institution. Schools, clubs, senior or citizen scientist organizations are potential users of my microscope design.

Experiments with Spherulocytes

In my experiments, spherulocytes responded to ink injections by increases in numbers and phagocytosis of ink particles. These results are not easy to discuss because publications about function of spherulocytes are sparse. These cells have been suggested to transport cuticular components and to play a role in cuticle regeneration (Sass et al., 1994). Response of spherulocytes to biotic and abiotic factors was also studied, but no increase in spherulocyte numbers or phagocythosis by spherulocytes were found. Huang, Yang, Shi, Li, Chen, Chen, & Chen (2010) co-incubated hemocytes of *Plutella xylostella* larvae with FITC labelled *Escherichia coli* and observed phagocytosis in plasmatocytes and granulocytes, but not in spherulocytes. Abdel-Al et al. (2011) administered *Bacillus thuringiensis* with food to

Spodoptera littoralis larvae at the dose of LC 50, which increased numbers of plasmatocytes and granulocytes in circulation. However, the numbers of spherulocytes did not change (Abdel-Al, Osman, & Ryad, 2011). Nayar and Evans (2012) exposed larvae of *Oryctes rhinoceros* to *Bacillus thuringiensis* and *Escherichia coli*. In both cases, they observed an increase of granulocyte counts and decrease of spherulocyte numbers. Berger and Jurcova (2012) co-incubated hemocytes of *Spodoptera littoralis* and *Pyrrhocoris apterus* with a copolymer of 2-hydroxyethylmethacrylate (HEMA) and all classes of hemocytes, except spherulocytes, ingested HEMA particles.

In light of sparse literature data, my findings that spherulocytes are capable of increasing numbers and phagocytizing in response to an artificial compound are novel and calls for further experimentation.

Screening Plant Essential Oils for their Effects on Insect Hemocytes

Either total insect hemocyte (Khanikor and Bora, 2012) or oenocytoid counts (Asiri, 2017; Gad and Dakheel, 2009; Sharma, Sharma, & Saxena, 2008; Zahran and Gad, 2013) may increase in response to plant essential oils or plant tissue extracts. However, decreases in either total hemocyte, plasmatocyte, or granulocyte counts is a more frequent response of insect innate immune systems to plant oils and extracts. Lowering of total hemocyte counts was observed in *Helicoverpa armigera*, in response to oils from *Artemisia annua* and *Ageratum conyzoides* (Padmaja and Rao, 2000) and to extracts from *Cledodendron inerme* (Kalyani &Vass Holirosur, 2015).

Spodoptera litura responses to *Acorus calamus* oil experience a decrease in total hemocyte counts (Sharma et al., 2008). The same is observed in *Papilio demoleus* exposed to

Ageratum conyzoides and *Allium sativum* extract (Pandey, Pandey, & Tiwari, 2012), and *Ephestia kuehniella* exposed to essential oil from *Ferula gumosa* (Ghasemi, Yazdib, Tavallaie, & Sendi, 2013).

Decreases in plasmatocytes counts due to plant oils or extracts administered to insects were reported in *Spodoptera litura* exposed to essential oil from *Acorus calamus* (Sharma et al., 2008). Larvae of two mosquito species, *Culex quinquefasciatus* and *Culex pipiens*, exhibited similar responses to oils from *Cinnamomum osmophleum*, *Matricharia chamomella* and extracts from *Solanum nigrum*, *Acokanthera spectabilis* and *Heliotropium aegyptianum* (Gad and El-DaKheel, 2009; Zahran and Gad, 2013).

Several publications reported decreases in insect granulocyte counts after treatment with plant essential oils or extracts. Such response was observed in *Sesamia cretica* exposed to *Festuca ovina* (Sadeghi, Raeisi, & Jamshidnia, 2017), and *Spodoptera littoralis* responding to extract from *Atriplex halimus* (Asiri, 2017).

I am not aware of any report currently showing a decrease in oenocytoid counts caused by plant essential oils or extracts. A recent comprehensive review article on effects of plant – derived substances on immunological parameters in insects (Ghoneim, 2018) also does not refer to such reports.

From among the nine plant essential oils screened in this study, none were previously tested for effects on insect hemocytes. I located only one paper reporting effects of *Eucalyptus globulus* aqueous extract on differential hemocyte counts in larvae of *Papilio demoleus* (Pandey et al., 2012). Here, the extract was administered *per os* with lemon leaves decreased plasmatocyte, granulocyte, and spherulocyte counts after 24 hours of exposure. In my experiments, *Eucalyptus globulus* essential oil, injected to *Galleria mellonella* larvae, did not

affect counts in any class of hemocytes (Table 4). It has been shown, that immune sytems of different insect species may react differently to the same essential oils. For instance, essential oil from *Ageratum conyzoides* increases total hemocyte counts in *Antharaea assama* larvae (Khanikor and Bora, 2012) but decreases the same immunity parameter in *Helicoverpa armigera* larvae (Padmaja and Rao, 2000). My finding that bergamot, frankincense, and lavender oils decreased insect hemocyte counts (Tables 9, 10, and 11) was in concordance with general trend observed in insects responding to essential oils; in most cases a decrease of hemocyte counts was observed (Ghoneim, 2018).

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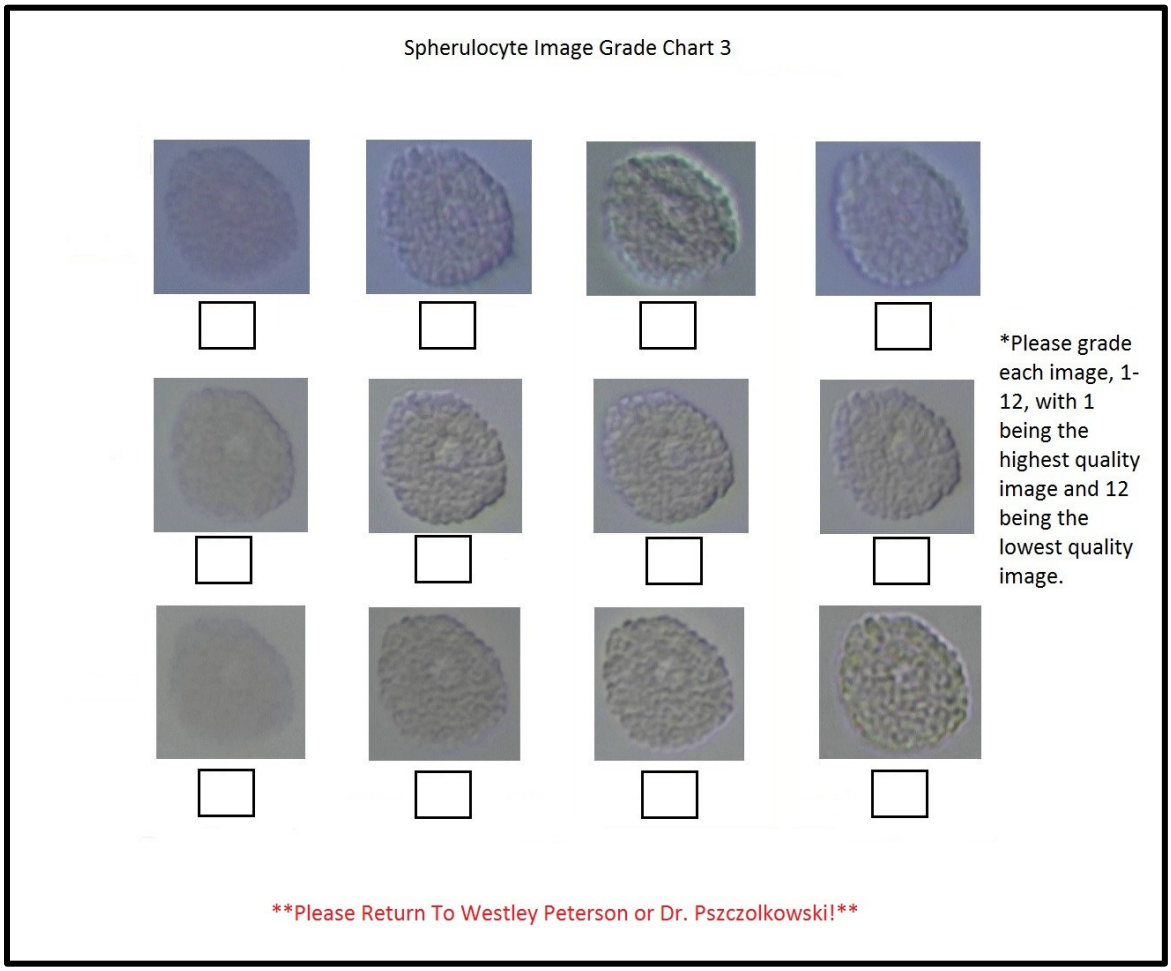
APPENDICES

Appendix A

An example of the chart serving for evaluation of image quality in the microscopes equipped with oblique illumination filters. Grade charts 1 and 2 (not shown) were graded on a scale of 1-4. Chart 3 (shown here) compared all microscopes, and all filters the microscopes used, and was graded on a scale of 1-12. A score of one, throughout all grade charts, remained the highest score.



Appendix B

Mortality percentages in *Galleria mellonella* larvae injected with essential oils screened for effects on insect hemocytes.

Trade name	Scientific name of the plant	% concentration of injected oil	% mortality
bergamot	*Citrus bergamia* Risso	30	0% (10)
clove	*Eugenia caryophyllata* Thunb.	30	100% (10)
		20	87% (15)
		10	87% (15)
		5	27% (15)
eucalyptus	*Eucalyptus globulus* Labill.	30	0% (10)
		20	0% (10)
		10	0% (10)
		5	0% (10)
frankincense	*Boswellia carterii* Engl.	30	0% (10)
		20	0% (10)
		10	0% (10)
		5	0% (10)
lavender	*Lavandula angustifolia* Mill.	30	0% (10)
rosemary	*Rosmarinus officinalis* L.	30	30% (10)
		20	0% (10)
		10	0% (10)
		5	0% (10)
sandalwood	*Santalum album* L.	30	40% (10)
		20	0% (7)
		10	0% (10)
		5	0% (10)
spearmint	*Mentha spicata* L.	30	20% (10)
		20	10% (10)
		10	20% (10)
		5	0% (10)
tea tree	*Melaleuca alternifolia* Chee	30	30% (10)
		20	0% (10)
		10	0% (10)