5-2016

The Essential Oil of Lippia Alba Affects Drosophila Behavior and Physiology

Jibin Manimala
University at Albany, State University of New York

The Essential Oil of Lippia Alba Affects *Drosophila* Behavior and Physiology

An honors thesis presented to the Department of Biology, University at Albany, and State University of New York in partial fulfillment of the requirements for graduation with Honors in Biology and graduation from The Honors College.

Jibin Manimala

May 2016

Research Mentor: Dr. Lnenicka

Committee Member: Dr. Rosellini

Abstract

Lippia alba (LA) is a flowering shrub native to Central and South America. Its essential oil has been used in herbal medicine as an anti-anxiety drug and in aquaculture; it is used to sedate fish during transport (Daniel 2014. Essential oils are derived from plants usually through steam distillation. They are labeled as "essential" because it contains the odor of the plant. Its physiological action is unknown. We use the fruitfly Drosophila melanogaster to further define its behavioral actions and determine its underlying physiological effects. We first tested whether *Lippia alba* produced had an anesthetic effect on the fruitfly. We then attempted to determine which components were responsible for the anesthesia. Based upon mass spectroscopy, the major components of LA are Citral (58.54%), Carvone (7.41%), and Limonene (7.32%) (Lopez et al. 2011). In the experiments, a 50ml centrifuge tube was used and each tube contained 10 flies. The cap of each tube contained a piece of paper with 10µL of essential oil. After 30 minutes the tube would be tapped and the flies that failed to crawl upwards were recorded. This was done for 150 minutes. After this test was conducted we would conduct a recovery test. In the recovery tests, a small thin piece of filter paper would be soaked in a 1% Sucrose solution. The purpose of this experiment was to examine how many flies would be able to move upwards and it would show that the oil had a sedative effect on the flies. The recovery test results would be recorded after 1 hour, 3 hours, and 6 hours. *Lippia alba*, Citral, and Carvone are shown to have an anesthetic effect. Limonene is shown to have an irreversible effect and Beta-caryophyllene is shown to have no effect. Propylene Glycol was mixed with the components to equalize the vapor pressure. We then tested the physiological effects of LA essential oil and its components. Since many drugs affect the nervous system by targeting synapses, we examine their effect on the neuromuscular junction. The nerve was stimulated and EPSPs were record from the muscle before, during and after applying the essential oil and its components. We found that LA produced a large reduction in transmitter release. This effect of Citral on synapses was very similar to that seen with LA. It is likely that LA produces its anesthetic effect by blocking synapses and Citral is largely responsible for its action.

Acknowledgements

I would like to thank my research mentor Dr. Gregory Lnenicka for giving me the opportunity to work in his lab opportunity and for giving me a firsthand experience into science research, as well as providing guidance. Everything I have accomplished in research wouldn't have been done without his help, for that I'm truly grateful.

I would like to thank Dr. Lenise Silva. If she from UFOPA for allowing me to work with her on her project. If she hadn't come to Albany this project wouldn't have come to fruition. Next I would like to thank Andrew Powers from the Lnenicka lab, his guidance over the last several years has been invaluable.

Next I would like to thank Dr. Robert Rosellini for being the second reader of my paper. His guidance both in academia as my professor and as my honors thesis committee member has truly been remarkable.

Lastly I would like to thank my family and my friends. Without your constant support during my undergraduate career, this thesis wouldn't have been completed.

Table of Contents

Introduction:

Essential oils are very popular currently and have a wide variety of effects. They have been used in wide variety of aspects ranging from pharmaceuticals to home remedies. The amazon contains many essential oils. The amazon contains a relatively large percentage of the earth's biodiversity including many plants with known economic importance. A number of plant extracts have been used in agriculture and medicine for their behavioral and physiological effects on animals. Although many of these extracts have been used with good success for many years their mode of action is usually unknown. Plants will often release these volatile chemicals in the air, some volatiles are the green leaf volatiles (aldehydes) and terpenes (Unsicker et al. 2009). The essential oil is extracted through steam distillation and called "essential" because it contains the essence (odor) of the plant. Essential oils are mainly composed of terpenes (monoterpenes, sesquiterpenes, and diterpenes) and oxygenated compounds (esters, aldehydes, ketones, alcohols, phenols, and oxides.) Volatiles can disrupt the cellular membranes of organisms, inhibit intake of nutrients, inhibit ion transport, inhibit signal transduction, and inhibit many other physiological processes. The volatiles will deter herbivores (often insects) from attacking the plant or it will attract herbivore predators. The volatiles in the essential oils stem from the active compounds. Essential oils can affect humans as well. For example, Lavender oil derived from the plant *Lavandula angustifolia* can be used to treat anxiety. Using essential oils could be a novel way of trying to lower anxiety levels, rather than using pharmaceuticals. Aromatherapy is claimed to be beneficial to the mental, psychologic, spiritual, and social aspects, although they are less quantitatively measurable (Lee et al. 2011). There is no clear understanding of how essential oils work. Essential Oils have been used commercially as a way to anesthetize fish (Cândida, et al.

2014). The purpose of this project is to examine the behavioral effects of the essential oil of *Lippia alba* (LA) and its components on *Drosophila*. It is the ideal organism to use in this experiment because it reproduces very quickly and the study of its nervous system can be used for human application. The ultimate goal is to identify the active components LA and determine how they affect the nervous system to produce their behavioral actions.

The major active compounds of LA are Citral (58.54%), Carvone (7.41%), and Limonene (7.32%) (Lopez et al. 2011). It would be beneficial for agriculture and medicine to identify the active components and their mode of action (Cunha et al. 2015). The effects of essential oils may have evolved from plants as a way to defend themselves from herbivores.

Materials and Methods:

The essential oils were obtained from Federal University of Western Pará and the components were ordered online. The *Drosophila* used in this experiment were the Canton S flies. We chose to test the effects of Citral, Carvone, and Limonene on *Drosophila* because these 3 have the highest % area in *Lippia* alba. Beta-caryophyllene was also used because we wanted to see if a minor component would have an anesthetic affect. The % area was found using mass spectrometry and the results of the mass spectrometry can be seen on table 1.

Jazz food preparation:

The food for the flies were prepared using Jazz media, which is a powdery substance. 454g of Jazz media was mixed with water and boiled in a crockpot with approximately 2.5L of water. After the media was thoroughly boiled and cooled, it was poured into plastic bottles. Each bottle contained approximately 33mL of the Jazz media. The Jazz media was then cooled in the bottles for three hours before being placed in the freezer. 22 bottles were used every week to

replace the stock bottle from the previous week. These stock bottles in the freezer are needed to flip the flies.

Transferring *Drosophila*:

Flipping the flies is when the flies from the stock bottle were transferred to a new bottle, which were then become the stock bottle. The old stock bottle become the experimental bottle. The old stock bottle was placed on top of the new stock bottle and gently tapped to release the flies into the new stock bottle. The target age for the flies is 5 days. The media loses its integrity over the course of the week due to the flies and the larvae constantly eating the media. So when the flies are being transferred the media may drip down the side. If that occurs then only a few flies can be obtained from that particular bottle. As a result, there are many old stock bottles in the lab that were used to increase the amount of stock flies in each stock bottle. All the flies used in the experiment were obtained from the experimental bottle. Before the flies were flipped the bottles had to be dried using a kim wipe. If the water that aggregated due to condensation wasn't dried it would be extremely detrimental to the flies. It would be detrimental because the flies would either stick to the water or drown. An adequate number of flies in a stock bottle would be when the flies essentially formed a carpet on top of the media. It was important to have plenty of stock flies because this would be the easiest way to produce the greatest number of experimental flies. After the flies were flipped they would be placed in an incubator, which is set for 25.8ºC and 31% humidity. When the flies are flipped, the old stock bottle should not have any living flies because this will cause some of the flies to be substantially older than other flies. The experimental bottle will have larvae, pupae, and eggs inside of it. When all these flies are formed they will be anywhere from 1-7 days old and they will be used in the experiments

Behavioral Tests:

When the flies were at least 5 days old they were taken out and placed in various 50mL centrifuge tubes. CO_2 was used to momentarily anesthetize the *Drosophila* and transfer them into the centrifuge tubes. Each tube contained 10 flies. Each centrifuge tube had 10 µL of a certain essential oil. The essential oils used in the experiment were Citral, Beta-caryophyllene, Carvone, and Limonene. A small piece of filter paper was placed in the cap and it was saturated with a specific chemical. A fumigation test is done when the flies are exposed to the chemical for up to 2.5hours. Recordings were done every 30 minutes. The tube would be tapped and the number of flies that failed to move back up the tube were recorded. WE would normally wait around 30 seconds to see if the flies would be able to crawl upwards. After 2.5 hours the flies were transported into the recovery tube. The recovery tube contained a flat piece of filter paper immersed with 1% sucrose solution. The water was used to mimic humidity. High Humidity is the natural environment for the flies. The sucrose serves as a food source for the flies. The recovery test would last for 6 hours and recordings would be made after 1 hour, 3 hours, and 6 hours. The amount of flies that failed to move upwards on the tube were recorded.

Electrophysiology:

Electrophysiology is the branch of physiology that deals with the electrical phenomena associated with nervous and other bodily activity. In this experiment Saline (HL3) was used as a control and LA and its components were then added the Saline. The Saline was left at room temperature and poured into a 10mL beaker. Then another 10mL beaker was filled with Saline. Ethanol was also poured into a separate 10mL beaker, which contained 6.3µL of ethanol and 0.7µL of the essential oil. This solution was then vortexed and then pipetted into the 10mL

beaker containing the Saline. This is the experimental syringe and the control syringe contained just saline both of these solutions were then transferred to a 10mL beaker which is was used in the experiment. The larvae will be prepped for electrophysiology by this time and there are very precise needles inserted by the nerve endings of the larvae for stimulation. A different larvae will be used for each experimental condition. The experimental conditions are Lippia Alba, Citral, Ethanol, Carvone, Limonene, and Caryophyllene. Each larvae used in this experiment is a third instar. The first three minutes of the experiment there was only electrical stimulation and this was used to establish the baseline. Then from minute 3 to minute 8 the drug was administered. During this 5 minute interval we examined the effects of the experimental syringe on the larvae. After the eight minutes the larvae was washed with Saline to remove any trace of the essential oil. The wash out was used to see if the essential oil had a reversible effect. The reversible effect can only be observed if the larvae can go back to baseline. The electrophysiology results are recorded in pClamp.

Clampfit:

The results from the electrophysiology experiment were recorded as a trace on pClamp. That file was then saved and then uploaded on Clampfit. Clampfit was then used to examine the effects of the essential oils on the peak, half width, decay, and baseline. To obtain values for peak, half width, decay, and baseline the cursors must be situated a certain way. Cursor 1 and 2 must be placed right before the artifact, cursor 3 must be placed directly before the EPSP, and Cursor 4 must be placed at the end of the trace. Then click the statistics option and the results will appear in a new window.

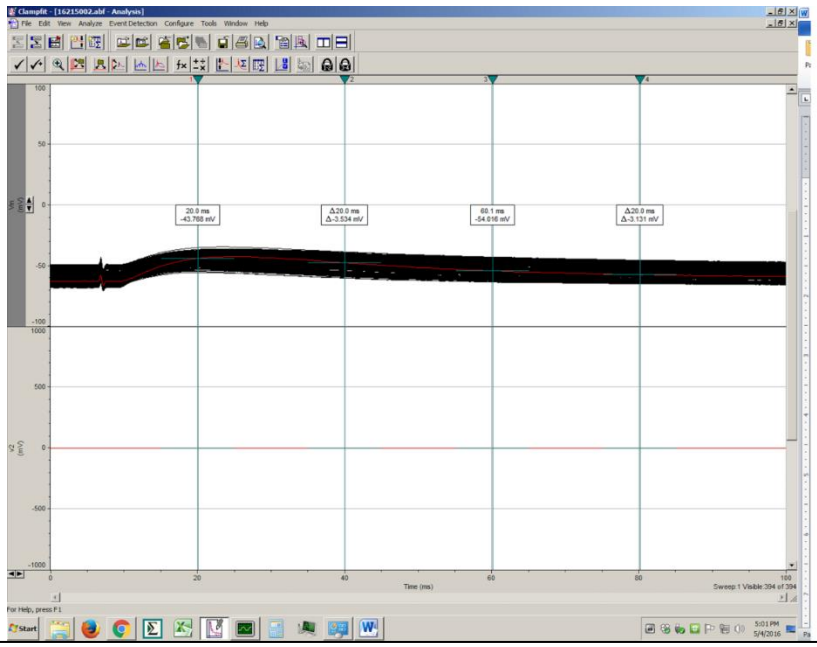

Figure 1: Displays the trace of the electrophysiology experiment from pClamp.

SigmaPlot

After the values for peak, half width, decay, and baseline were obtained they were saved on an Excel file before being transported to Excel. The values were transposed pasted on a SigmaPlot file. The average was taken for each 15 second interval. This was considered a bin. A bin contains a cluster of information over a course of time. The average value for each bin was then recorded on a template file and this was repeated for all experimental conditions.

Results:

The first part of the experiment consisted at observing the effects of the fumigation test. This was accomplished by placing the flies from the experimental bottles in the incubator into

various centrifuge tubes containing various concentrations (0.5µL to 20µL) of either *Lippia alba* or its various components. Each test tube contained a small filter paper that was saturated with *Lippia alba* or its components. The flies were exposed to the chemical for up to 2.5 hours and recordings were done every 30 minutes. The results were recorded using a tap down test. After the tap down test, the number of flies that failed to move up the test tube was recorded. The biggest factors that had to be observed was the relationship concentration of the oil and the number of flies that were immobilized. It was expected to be a direct relationship. The results of this experiment can be seen in Figure 2.

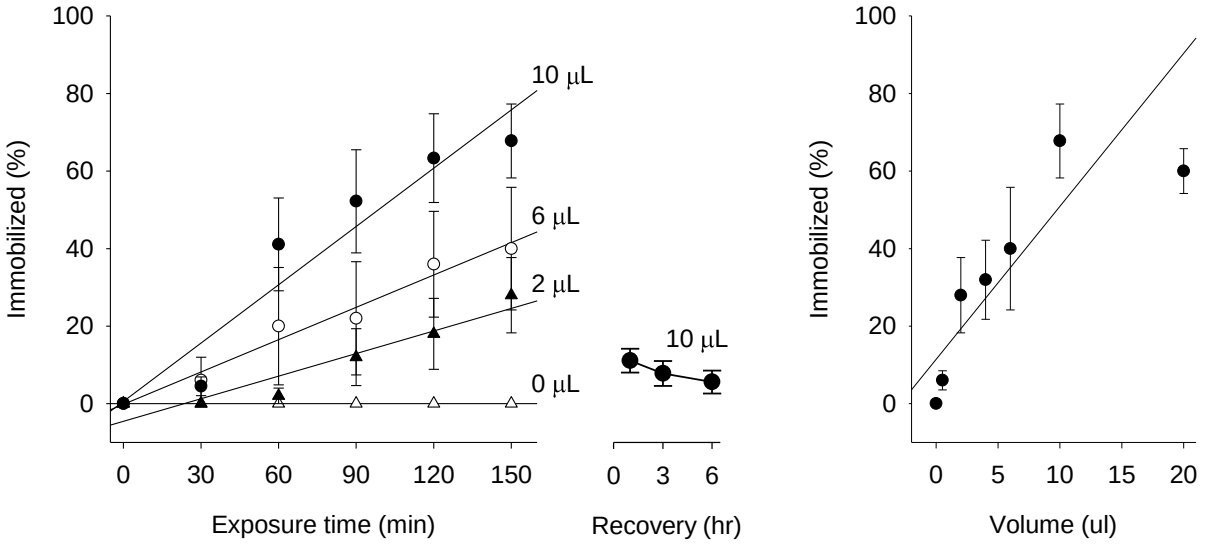

Figure 2: Illustrates the various effects of different concentrations of *Lippia alba* over the course of 2.5 hours on the *Drosophila.* This figure also illustrates the relationship between volume and immobilization.

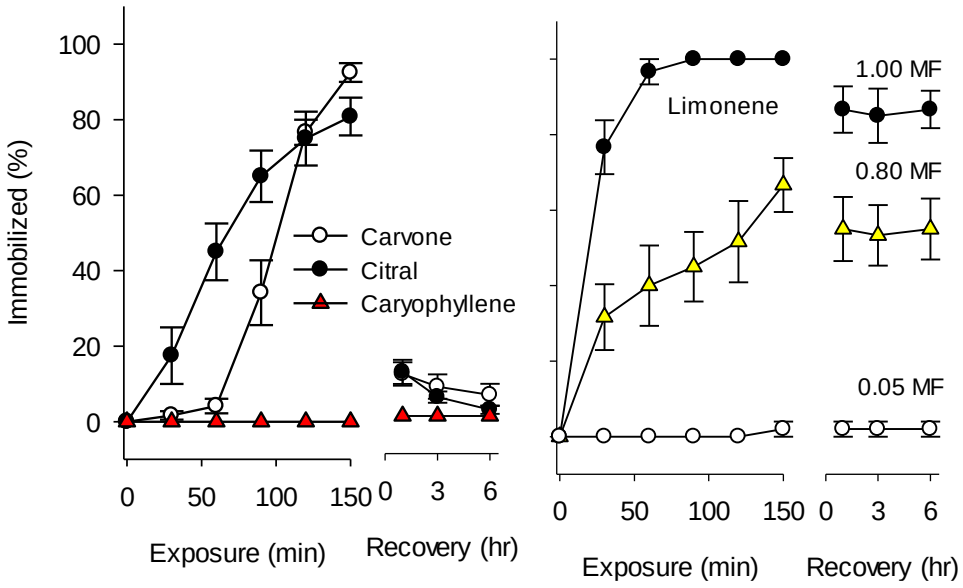

Figure 11: On the left hand side examines the immobilization rate between all 3 components. The right side examines the immobilization rate of Limonene at various mole fractions (MF).

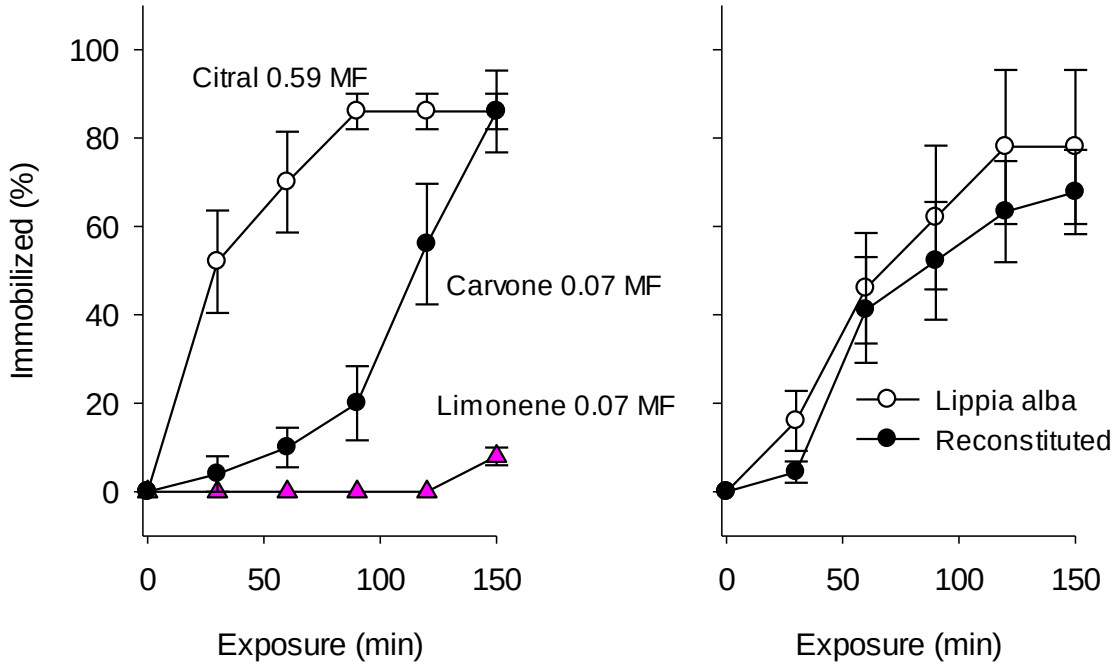

Figure 12: On the left hand side examines the immobilization rate between all 3 components with their respective MF in LA. The figure on the right examines the immobilization % between natural LA and the reconstituted LA.

The experiment shown in Figure 11 was conducted because we wanted to examine the effects of vapor pressure on immobilization. We wanted to know which components in particular are responsible for the anesthetic affect found in *Lippia alba*. As shown in Figure 7, Limonene has the highest immobilization rate, when at 1 mole fraction. It has 100% immobilization and we believe that Limonene will react with the *Drosophila* in an irreversible manner. We used Propylene glycol to dissolve the Limonene and to lower its vapor pressure. The reason we used Propylene glycol was that Limonene's vapor pressure is approximately 20 times larger than that of Carvone and Citral. The Propylene glycol was used essentially to equalize all the partial pressures. We needed equal partial pressures because Limonene's vapor pressure was much larger than the other components we used in the study. Raoult's law** was used to determine the partial pressure of each component. Since we already knew the vapor pressure and the mole fraction solving for partial pressure was pretty simple. 0.05 MF causes Limonene's partial pressure to be equal to that of Carvone and Citral. When all partial pressures were equalized Limonene is shown to have next to no effect on the *Drosophila*. As shown in Figure 11 it can be concluded that only Carvone and Citral have an anesthetic effect on the *Drosophila*.

Next in Figure 12, we lowered the MF for each component. We lowered the MF of each component my mixing Propylene glycol with each component. The vortex machine was used to create a through mixture between the two. Each MF on Figure 12 represents the MF each component would have in LA. This was done so we could further examine, which component gives LA its anesthetic property. We used the MF to examine how each component is naturally found in LA. On Figure 12 it can be seen that Citral and Carvone will cause immobilization to occur, whereas Limonene causes a minimal affect. After creating the MFs found in Figure 12, we combined them to form a reconstituted form of LA. We then examined the effect of natural LA

to reconstituted LA. The results between the natural LA and the reconstituted La are very similar.

****Raoult's Law: Partial Pressure= Vapor Pressure* Mole Fraction**

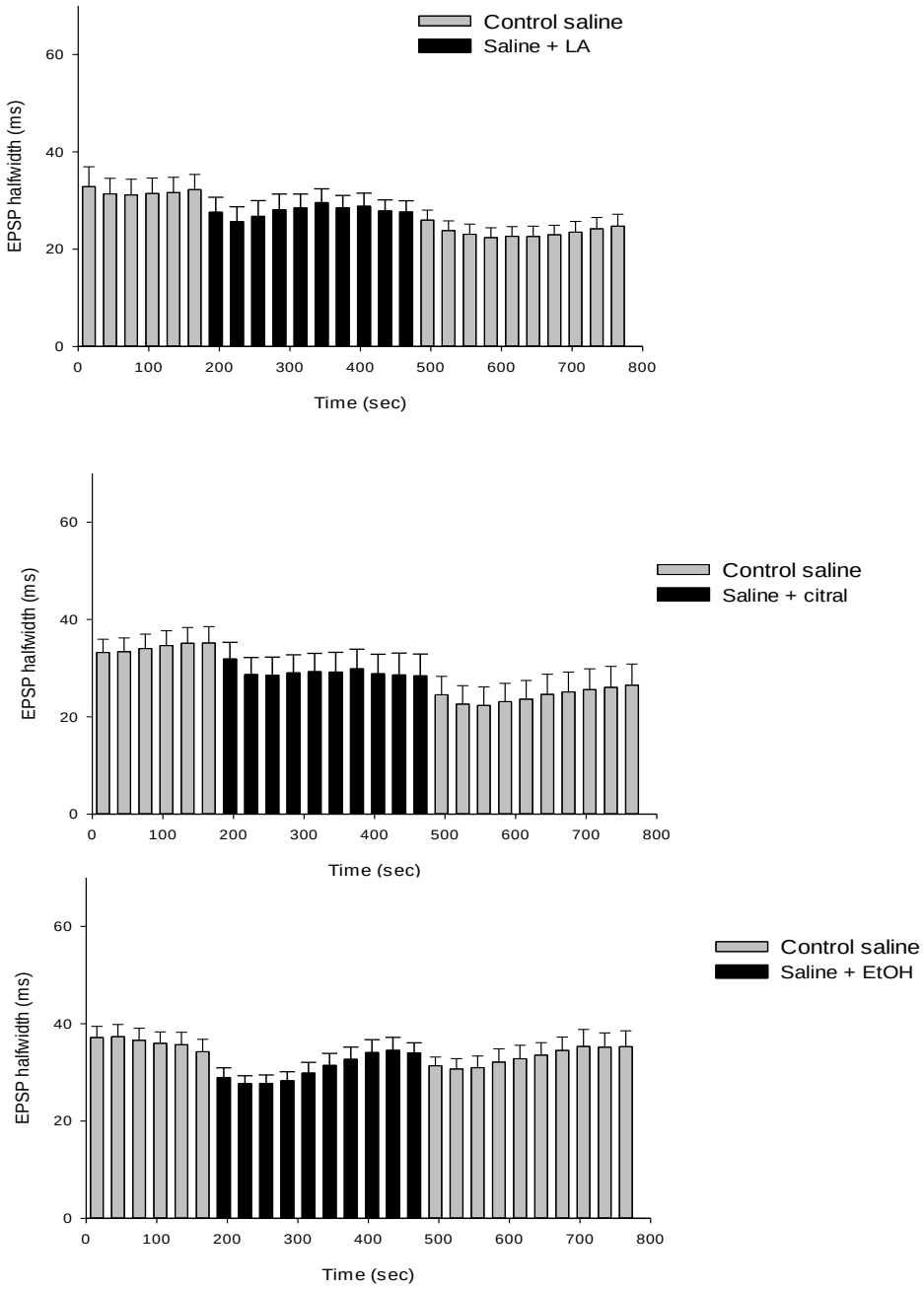

Figure 3: Illustrates the effects the essential oil and its components on the EPSP half width.

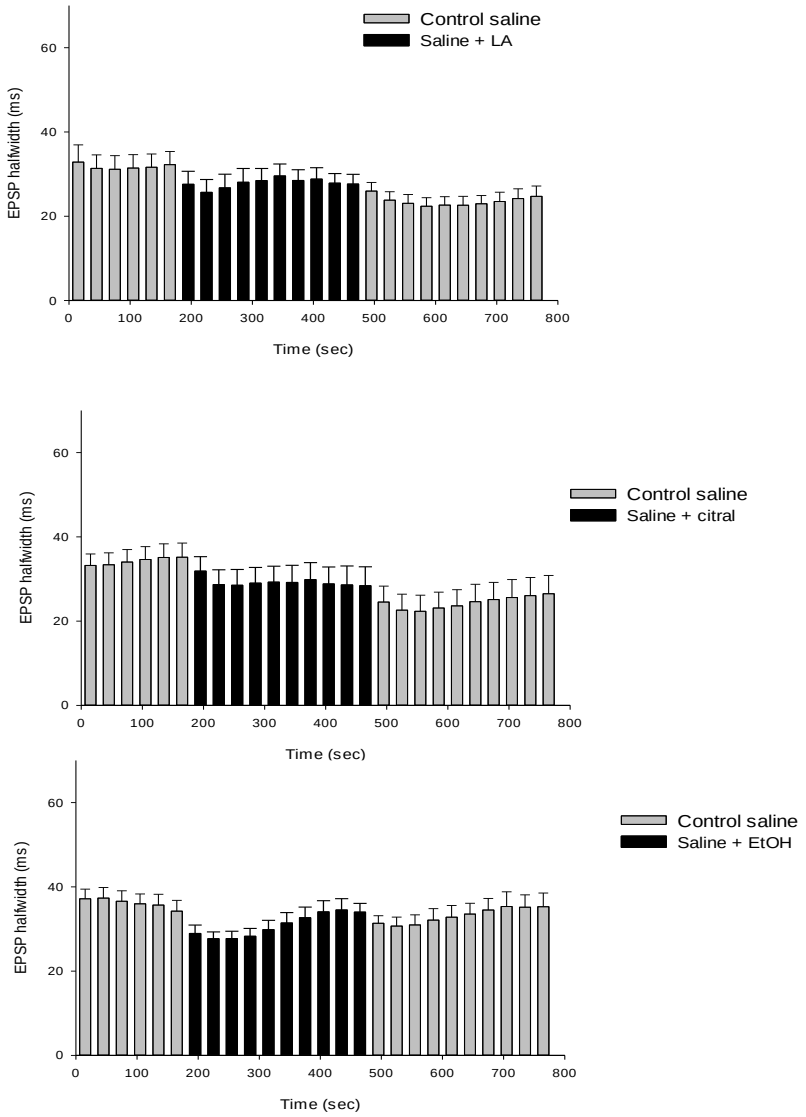

Figure 4: Illustrates the effects the essential oil and its components on the EPSP half width.

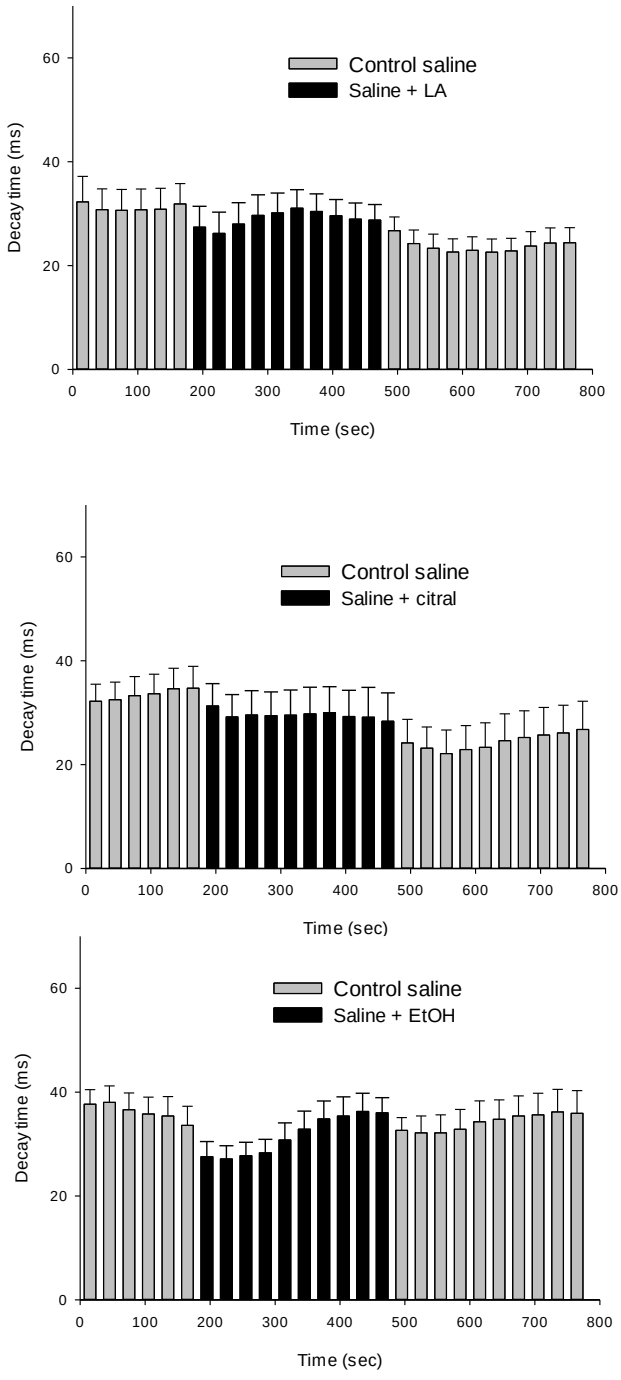

Figure 5: Illustrates the effects the essential oil and it's components on the EPSP decay time.

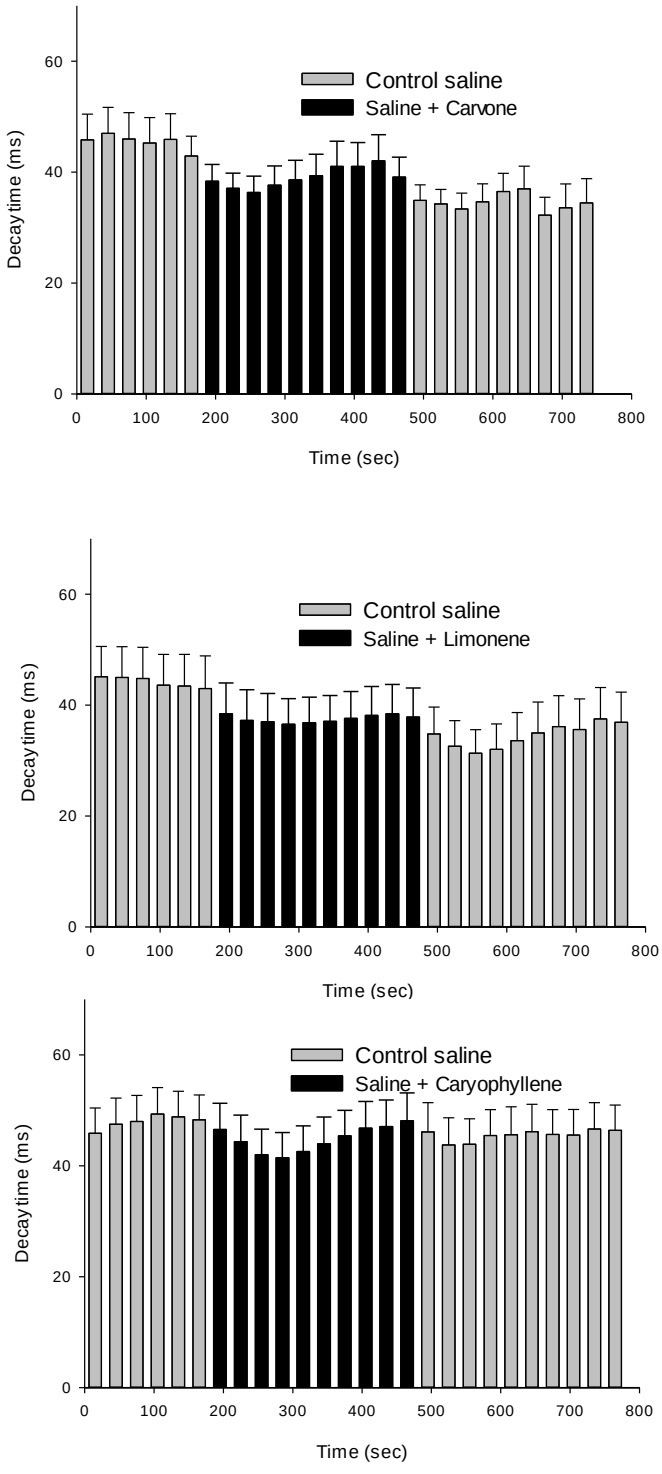

Figure 6: Illustrates the effects the essential oil and it's components on the EPSP decay time.

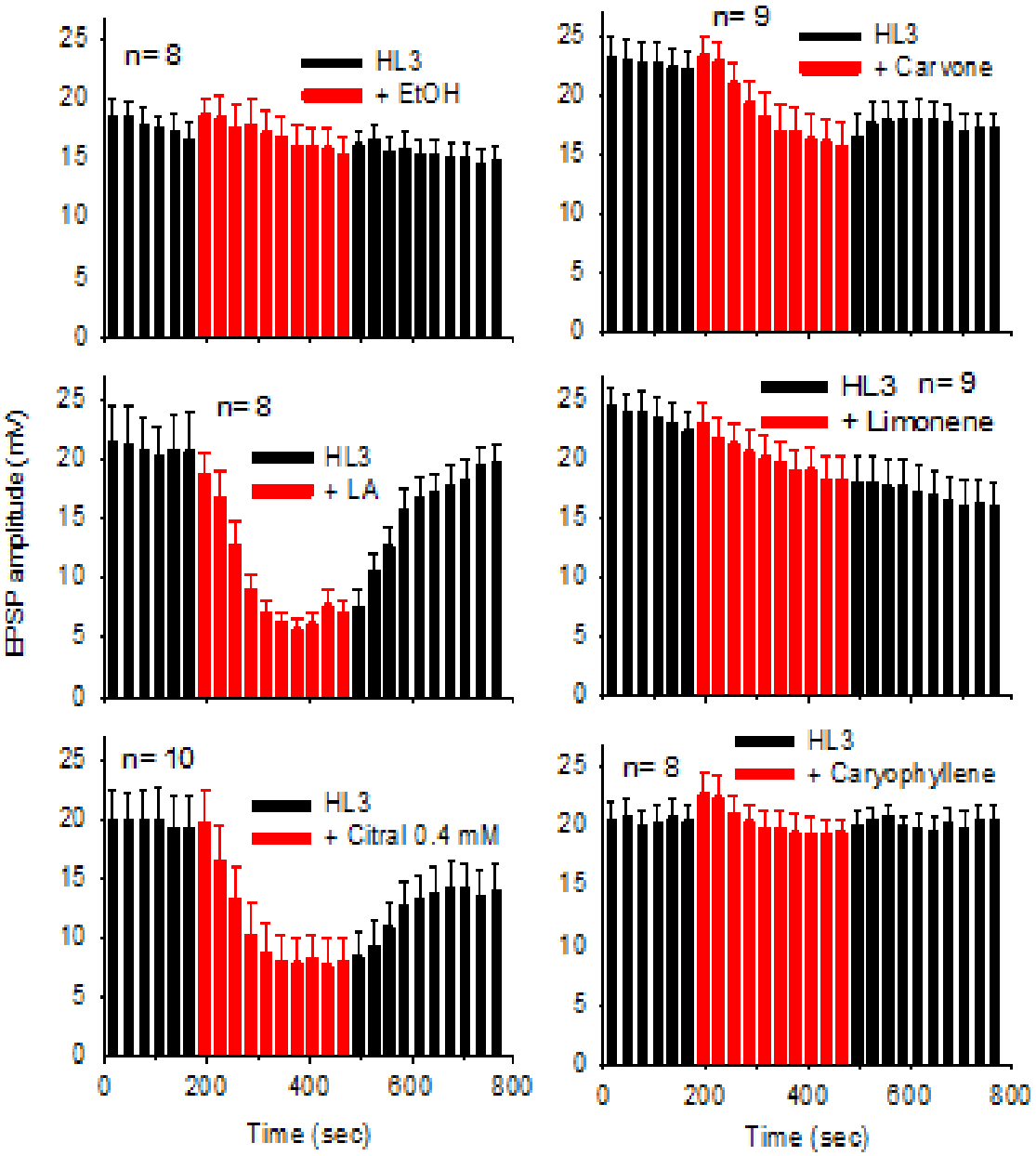

Figure 7: Examines the change in EPSP amplitude between LA and the various components. The *Drosophila* larvae were used in this experiment.

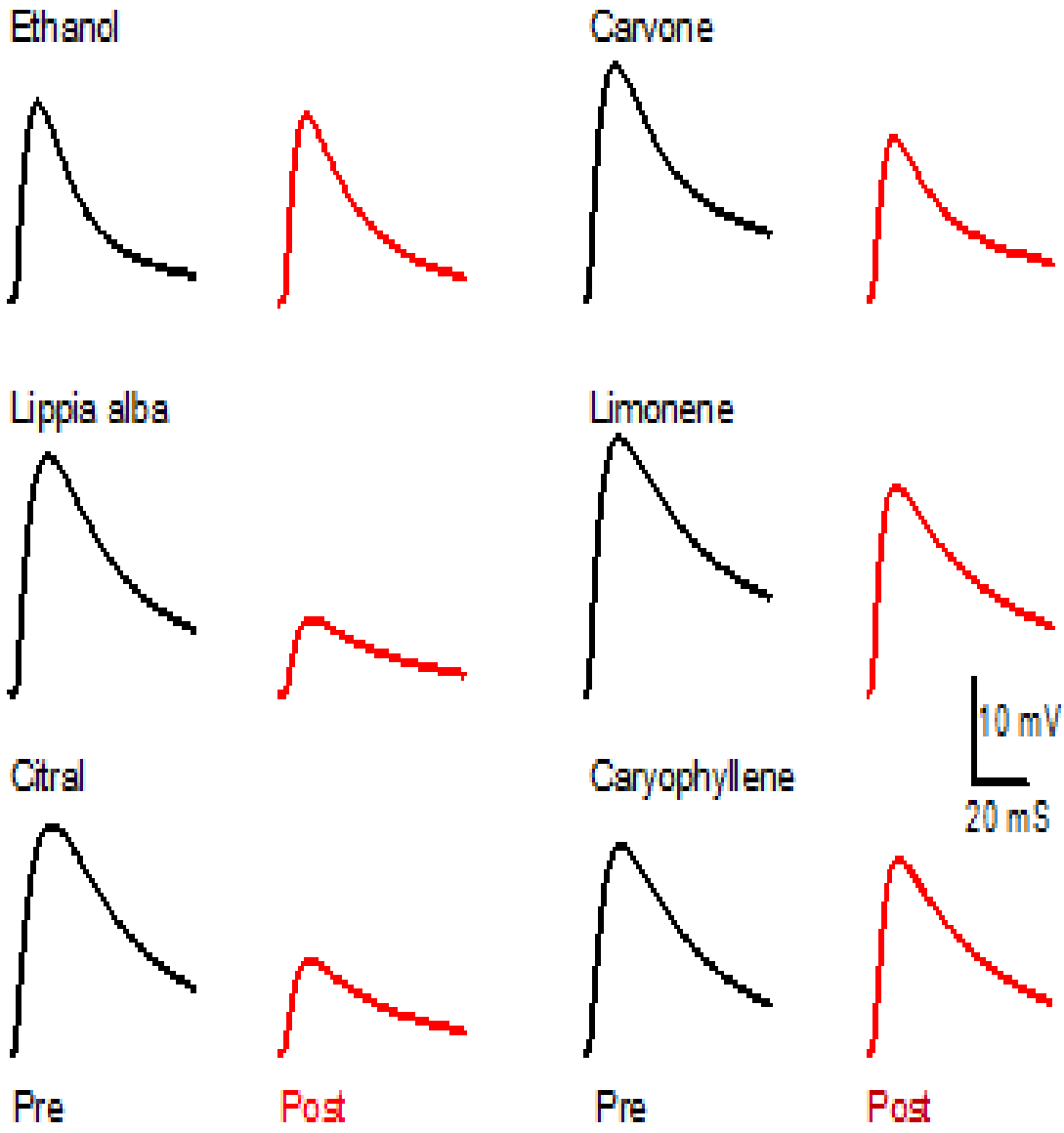

Figure 8: Shows the change in EPSP amplitude in terms of examining the EPSP before and after administrating either LA or its components in *Drosophila* larvae.

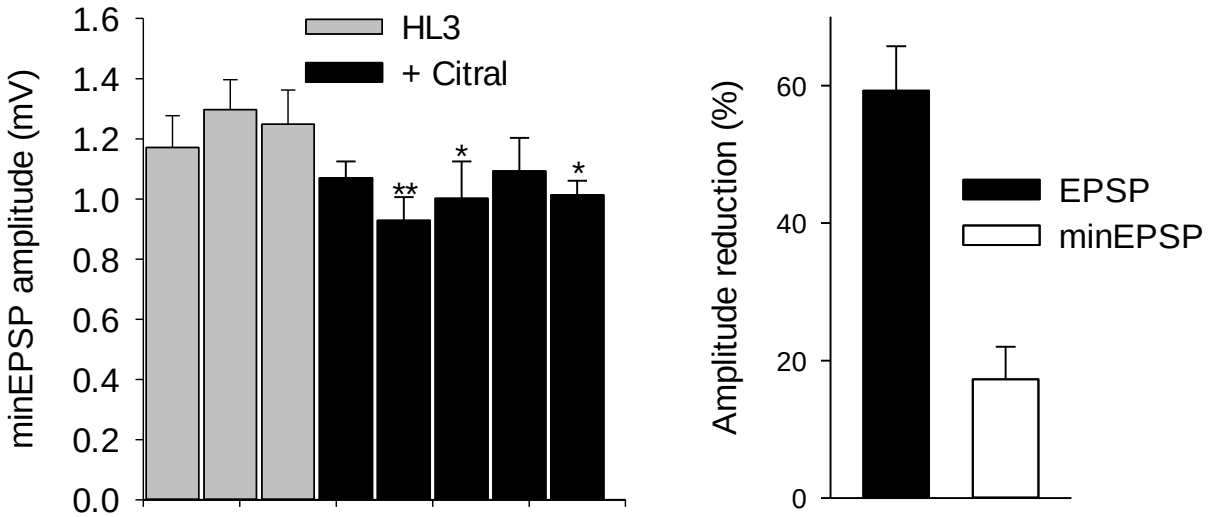

Figure 9: Shows the change in EPSP amplitude and miniEPSP amplitude. This can be used to calculate the reduction of transmitter release due to Citral.

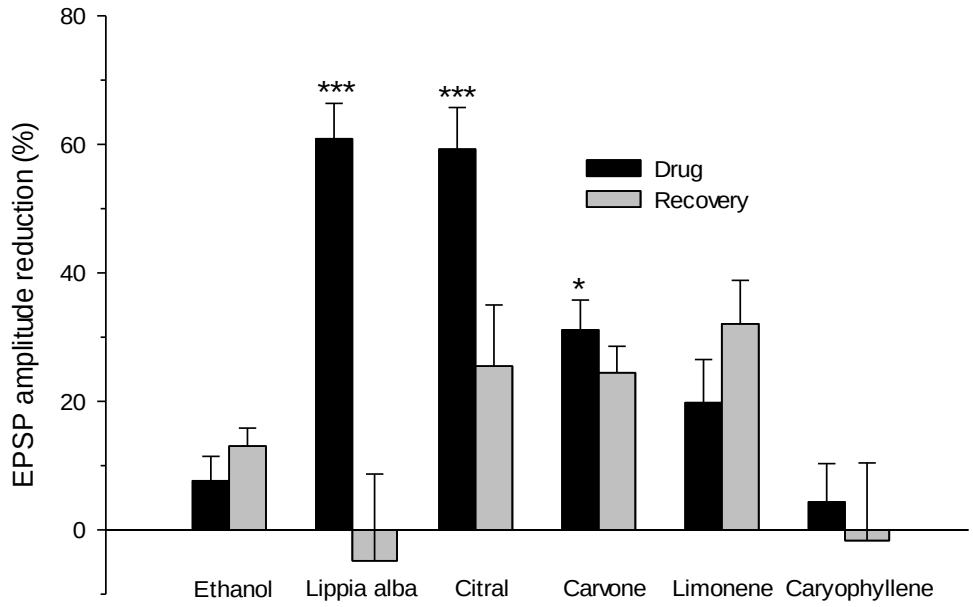

Figure 10: Shows the change in EPSP amplitude. This can be used to calculate the reduction of transmitter release due to Citral. * indicates a p value less than 0.05. *** indicates a p values less than 0.001.

These results were obtained using SigmaPlot. The graphs as shown illustrates the effects the essential oil and its components on half width and decay time. The half width is fairly consistent also before the drug was administered and during the wash phase. The Saline-Ethanol is used as the control for this experiment. First the Saline would be administered. Then either LA or a component would be added. Finally saline would be used to wash away any of the experimental drug. For the results of the decay time constant they seem to be gradually increasing as exposure to the essential oil increases. Carvone, Caryophellene, and Ethanol had the largest increase in decay time in the larvae.

The results of Figures 7 and 8 were derived from *Drosophila* larvae. The ethanol was used as the control in these experiments. LA and its components were dissolved in ethanol. Before the physiology experiments were conducted the EPSPs were recorded. The larvae would first be administered with saline. Next either LA or its components would be administered. After Saline would be used to wash out any LA or the components and the EPSP would be measured again. This recording would be considered the post EPSP. The comparisons between pre and post EPSP can be seen in Figure 8. All the components and LA caused a reduction in EPSP. However, the reduction of EPSP in LA is most similar to that of Citral. In Figure 7 when LA was administered the drop in EPSP was most similar to the drop in EPSP of Citral. Next in Figure 9 we examined the reduction in miniEPSP and EPSP. When Citral was administered, there was a significant drop in miniEPSP. However, the drop in miniEPSP isn't as large as the drop in EPSP. EPSP has a 60% reduction which is about 3 times larger than the reduction of mini EPSP. The purpose of this experiment was to examine the reduction in transmitter release due to Citral. Citral shows a 50% reduction in transmitter release.*

***Transmitter release= EPSP**
Mini EPSP

Discussion:

The first major conclusion that can be made from this project is that LA is an anesthetic. There is direct relationship that is observed in Figure 2 and this is expected because the increase in volume of LA will affect the geotaxis of the flies. As seen in Figure 2 as the volume of LA increases the immobilization % also increases. There is also another direct relationship between exposure time and immobilization %. As exposure time to LA increases, the immobilization % increases as well. It can be concluded that LA functions as an anesthetic because the *Drosophila* are able to make a full recovery within six hours. Geotaxis is the motion of an organism due to gravity. *Drosophila* will always want to be moving upwards, and prolonged exposure to high concentrations of LA as shown in figure 2 will disrupt the geotaxis. As a result, the flies will be on the bottom of the test tubes lying on their backside and unable to crawl upwards on the sides of the test tubes. In 2014 Cunha conducted a very similar experiment, except in Cunha's experiment flasks were used instead of test tubes, but the results were very similar to the results found in Cunha's experiment.

The next part of the experiment was to measure the physiology of LA along with its components. The purpose of this particular part of the experiment was to see which component of LA had similar physiological effect as LA. We were able to examine the physiological effects of LA and its components using electrophysiology on *Drosophila* larvae. To determine the

effects of the oil on synaptic physiology, we examined its effect on peak, half width, decay, and baseline. Half width determines the amplitude and then shows the duration of the EPSP. Decay is when the EPSP decays from its peak to 37% of its original value. Baseline is the resting membrane potential. The results from half width due to LA is most similar to the half width results of Citral. Next we examined the results of decay time in Figures 5 and 6. It can be seen that the change in decay time in LA is most similar to that of Citral. Based on these two results we were able to conclude that Citral and LA have similar physiological effects.

LA and Citral appear to produce their anesthetic effects by reducing transmitter release. For example, a reduction in EPSP amplitude with no reduction in mini EPSP shows a reduction in transmitter release. This was examined by comparing changes in EPSP amplitude to miniEPSP amplitude. Transmitter release can be calculated by dividing the size of EPSPs and size of the mini EPSPs. Figure 9 shows that there is a significant decrease in miniEPSP. There is also much larger decrease in EPSP. Since there is such a large decrease in EPSP it causes a decrease in transmitter release. As seen in Figure 9 there is a 50% reduction in transmitter release due to Citral. This reduction in transmitter release is attributed largely due to the reduction in EPSP, which can be seen in Figures 7 and 8. Figure 8 displays EPSP before and after administration of the drug. It can be seen that the change in EPSP in LA is most similar to that of Citral. This again further shows that the effects of LA can primarily be contributed to Citral. Figure 7 shows EPSP amplitude reduction over the course of 800 seconds. The reduction in EPSP in LA is most similar to that of Citral. Figure 10 shows that EPSP amplitude reduction is significant in LA, Citral, and Carvone. EPSP reduction is most significant in Citral and LA. With all this data we can confidently conclude that the effects of LA can be largely attributed to Citral. This makes sense because Citral has the highest %area in LA.

Next in Figure 11 we examine the immobilization % of each compound. Citral and Carvone have a very similar immobilization % and we were able to conclude based on these results that Citral is responsible for this affect more than Carvone because Citral acts much quicker in the *Drosophila* than Carvone does. Carvone and Citral both function as anesthetics because the flies are able to make a full recovery after 6 hours. Also on Figure 11 we also examined the effect of Limonene on *Drosophila*. Limonene at 1 MF is how it is naturally found. The results show that it causes 100% immobilization. There is also very little recovery and we were able to conclude that this occurs because when Limonene interacts with the *Drosophila* there will be an irreversible reaction. The reason we had to lower the MF of the Limonene was that its vapor pressure is much larger than the other components. In fact it is about 20 times larger than the other components. After dissolving the Limonene in Propylene glycol we were able to lower its vapor pressure so that it would match that of Citral's and Carvone. This occurs at 0.05 MF. On Figure 11 it can clearly be seen that 0.05 MF of Limonene has very little effect on immobilized flies. Based on these results we were able to conclude that the strongest anesthetics in LA were Citral, Carvone, Limonene, and Beta-caryophyllene respectively.

On Figure 12 we used the specific MFs that each component would have naturally in LA. Again based on the results we were able to conclude that Citral and Carvone were the two most potent anesthetics in LA and Limonene had no effect. We then combined the various MFs in Figure 12 to form a reconstituted form of LA. The purpose of this was to compare the effects of natural LA to our reconstituted form of LA. The results show that the reconstituted LA had a similar immobilization % to that of the natural LA. Since our results between the natural LA and the reconstituted LA were similar we can conclude that Citral is the most potent and the MFs we used were correct.

Even though this experiment showed great results there could've been some flaws. The flies used for the experiment are at least 7 days old. In the future we may want to find a way to better control the age of the flies and see what factor age plays. Some of the flies that may have emerged from the larvae before or after other flies. In a future experiment the best way to avoid this problem is to separate the flies each day from the bottles so it can be easier to identify how old each fly is. In the future we can take this experiment further by examining various ion channels in the *Drosophila* larvae. For example, we can use Calcium imaging.

LA compunds	%Area
ni	0.09
Isobornyl acetate	0.09
Copaene <α>	0.09
Sesquiterpene hydrocarbon	0.14
Bourbonene <β>	0.2
Cubebene <β>	0.2
Elemene <β>	0.39
Sesquithujene	0.09
Caryophyllene (E-)	0.56
Copaene <β>	0.15
Guaiene <β>	0.16
Humulene <α>	0.19
Aromadendrene <allo>	0.16
Muurolene <Γ>	3.37
Zingiberene <α>	0.62
Muurolene <α>	0.15
Bicyclogermacrene	0.14
Bisabolene <β>	0.12
Amorphene <Δ>	0.25
Cadinene <Δ>	0.28
Elemol	3.24
Nerolidol <E>	0.38
Guaiol	0.32
Atlantol <β>	0.12
Eudesmol <Γ>	0.24
Torreyol	0.09
Eudesmol <α>	0.58

LA compunds	%Area
α-thujene	0.23
α-pinene	0.13
abinene	0.42
hepten-2-one 6-methyl-5-	1.29
α-terpinene	0.16
cymene <ortho>	1.26
Limonene	7.32
ocimene-β	0.47
γ-terpinene	3.02
monoterpene hydrocarbon	0.1
Linalool	0.78
Geijerene	0.23
oxygenated monoterpene	0.1
Citronella	0.11
cis-Chrysanthenol	0.5
Isocitral <E>	1.02
Myrtenol	0.15
cis-sabinene hydrate acetate	0.27
Citronellol	0.29
Neral	24.16
Carvone	7.41
Geraniol	0.36
Piperitone	0.32
Geranial	34.38

Table 1: Displays the various components in LA and their respective composition in LA. This table was derived using Mass spectrometry.

References

Cunha, Fransico et al. "Eugenia Uniflora Leaves Essential Oil Induces Toxicity in Drosophila Melanogaster: Involvement of Oxidative Stress Mechanisms." *ResearchGate*. Toxicol Res, 12 Dec. 2014. Web. 5 Dec. 2015.

Lee, Yuk-Lan, et al. "A systematic review on the anxiolytic effects of aromatherapy in people with anxiety symptoms." The Journal of Alternative and Complementary Medicine 17.2 (2011): 101-108.

López, Molkary et al. "Chemical Composition and Antigenotoxic Properties of *Lippia Alba* Essential Oils." *Genetics and Molecular Biology*. Sociedade Brasileira De Genética, 1 July 2011. Web. 5 Dec. 2015.

Peixoto, Magna et al. "Acaricidal Activity of Essential Oils from Lippia Alba Genotypes and Its Major Components Carvone, Limonene, and Citral against Rhipicephalus Microplus." *Science Direct*. Science Direct, 12 Mar. 2015. Web. 5 Dec. 2015.

Peixoto, Magna et al. "Toxicity and Repellency of Essential Oils of Lippia Alba Chemotypes and Their Major Monoterpenes against Stored Grain Insects." *Science Direct*. Science Direct, 27 Mar. 2015. Web. 4 Dec. 2015.

Toni, Cândida, et al. "Fish anesthesia: effects of the essential oils of Hesperozygis ringens and Lippia alba on the biochemistry and physiology of silver catfish (Rhamdia quelen)." *Fish physiology and biochemistry* 40.3 (2014): 701-714.

Unsicker, Sybille B et al. "Protective perfumes: the role of vegetative volatiles in plant defense against herbivores." Current opinion in plant biology 12.4 (2009): 479-485.