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Physical and Chemical Defense Mechanisms in Aloe Barbadensis Against Insect Herbivores

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PHYSICAL AND CHEMICAL DEFENSE MECHANISMS IN ALOE BARBADENSIS AGAINST INSECT HERBIVORES

A Thesis

by

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ABSTRACT

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Chapter 1: This chapter provides an overview of insect herbivore impacts on plants and plant defense mechanisms to combat them. I particularly focused on physical and chemical defenses in the broader sense.

Chapter 2: This chapter describes the experiments that showed the effects of extracts from *Aloe barbadensis* on the growth and development of tobacco hornworm (*Manduca sexta*) and fall armyworm (*Spodoptera frugiperda*), model herbivores used to understand herbivore growth and plant defenses. This study shows that extracts from the *Aloe* differentially affected the growth and development of *M. sexta* and *S. frugiperda.* This chapter also gives insight on various growth and development traits used to accomplish these findings.

Chapter 3: This chapter is focused on discussing the role of these *Aloe* extracts in the growth of fall army worm (*Spodoptera frugiperda*) and tobacco hornworm (*Manduca sexta*) and wax content of *A. barbadensis*. In this study I show that the extracts, while had differential effects, on some extracts the caterpillars performed better during the larval and pupal stages, while others were neutral or inhibitory. I also shed light on the wax content and the effect it had on the feeding behaavior of *S. frugiperda.* This chapter will also show scanning electron microscopy images of *Aloe barbadensis* leaf surface and wax.

Chapter 4: This chapter highlights conclusions and possible future works for identifying the *Aloe barbadensis* defense phenotype. Our results shed light into the possible reasons why *Aloe barbadensis* is not predated on much by insect herbivores and defenses that deter them. Secondary metabolites paired with surface wax possibly prevents *Aloe barbadensis* from sustaining heavy insect herbivore damage.

DEDICATION

I would like to dedicate my thesis to my parents Ed and Leslie for jump starting my interests in the natural world, and my brothers Sam and Billy. I would also like to dedicate it to my beautiful loving girlfriend Carolina Moreno, whose love and support has powered me through my degree. I also thank Eric Morgan who has helped to guide me with lots of advice from my undergraduate years onward. Last but not least, Jeff Corwin, the late Steve Irwin, and Sir David Attenborough for showing me the natural world beyond the scope of America before I was able to travel it.

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CHAPTER I

THE ROLES OF MECHANICAL DEFENSES AND SECONDARY METABOLITES IN PLANT DEFENSE AGAINST INSECT HERBIVORES

Abstract

Aloe barbadensis, in a natural setting appears to be mostly untouched by insect herbivores, suggesting a possible role of secondary metabolites, combined with other leaf physical defenses defending against herbivores. To test this, compounds from *Aloe barbadensis* were isolated and mixed into a control diet, to determine the effects on two species of moth larvae, tobacco hornworm (*Manduca sexta*) and fall armyworm (*Spodoptera frugiperda*). The same species were also placed on leaves and caged, to study the potential effects of the waxy cuticle. Although most plant species contain waxy cuticle, it varies in composition and the quantity. And, we currently lack information on whetjer in *A. barbadensis* they act as a significant anti-herbivore defense. This thesis focuses on reviewing the physical and chemical defnses in *A. barbadensis* through a series of manipulated diet experiments and herbivore behavior assays, and we show that *A. barbadensis* is protected from herbivory through a combination of both physical and chemical defenses.

Introduction

Ever since the evolution of plants to be fit for life on land, they have become an extremely valuable resource for herbivores, and this relationship between the two has been an ongoing and evolving one ever since (Ehrlich & Raven 1964). The relationship between plants, and

specifically- herbivorous insects has been an ongoing tug of war where plants have evolved mechanisms to reduce the attacks from insects (Mello et al 2002; Howe and Jander, 2008), and the herbivores have evolved counter defenses to survive and flourish (Howe & Jander 2008; Singh et al 2021). It has been hypothesized that the tremendous diversity of secondary metabolites produced by plants constitutively, or post herbivory is the critical component of their continuous ability to resist herbivory. Clearly, this back-and-forth process has an immense impact in shaping ecosystems and their trophic cascades in both natural and agricultural habitats. Herbivory in agriculture and crop production on global scale has been estimated to reduce 18-26% of crop production This results in a loss of about $470 billion, with around 15% occurring in the field before harvesting of the crops (Culliney 2014)- exclusively by arthropod herbivores.

There are roughly 10,000 species of insects that damage crops worldwide (Dhaliwal 2010). In the past, control strategies were single use, short term solutions, such as pesticide use which gets the most attention, but also integrated pest management (IPM), which typically utilized multiple technologies such as biocontrol and crop rotation (Thomas 1999). Thomas also mentions that IPM methods are more popular with farmers as it is cheaper than pesticides, and when multiple methods are applied correctly in tandem, could also be more effective in controlling these pests that are causing damage crop production.

To protect against the wide range of herbivores and their feeding, plants have evolved defenses that can be broadly classified into two; structural (physical) defense and chemical defenses (Kaur et al 2020 A; Kaur et al 2020 B; Kariyat et al 2019; Tayal et al., 2020). Although plant chemicals are typically divided into two different categories, primary and secondary metabolites, only secondary metabolites are not always, but mostly involved in deterring herbivores, as primary metabolites are produced by the plant to promote growth and

development (Freeman et al 2008; Singh et al 2021). Secondary metabolites do not function in a plant's metabolic pathways; therefore, they are mainly, but not limited to enhance plant defenses. Secondary metabolites can be damaging to insect herbivores depending on species. For example., Tayal et al (2020A and B) and Singh & Kariyat 2020 showed that the polyphenol-rich purple corn pericarp rich in anthocyanins had adverse effects on the larvae of various moths. However, insect herbivores over millions of years of co-existence, have also co-evolved traits to circumvent or tolerate these defenses (Mortensen 2013). An example of this is the relationship between the monarch butterfly (*Danaus plexippus*) and plant family Apocyanaceae, with monarchs being able to metabolize the toxins created by milkweed, instead of it causing fatality, which is the case for the majority of insects (Agrawal et al 2012).

To be broken down even further, there are many classes of secondary plant metabolites. The largest class being terpenes, followed by, in no particular order of importance or abundance, phenols which are widespread between but not limited to Poaceae, Fabaceae, Rosaceae, tannins which are abundant in Fagaceae and Pinaceae, flavonoids present in Fabaceae, and sulfur and nitrogen containing which are present in Brassicaceae (War 2020; Singh et al 2021). The effects of tannins are very prevalent in the New Jersey Pine Barrens, a unique ecosystem that is dominated by *Quercus* and *Pinus*, which have altered the ecosystem with the tannins that they contain. For example., Tempel 1981 showed the relationship of herbivore damage and the concentration of tannins within this ecosystem in that there is good reason to believe tannins reduce herbivore damage.

Physical plant defenses on the surface are a first line of defense against herbivores. These act as a deterrent to prevent herbivory, or if it does occur, to shorten the length of time and limit

the extent of the damage caused by it (Freeman et al 2008; Kariyat et al., 2017; 2018; Kaur and Kariyat 2020A). Following these defenses, once breached, molecular signaling pathways are induced. An example of this was recently shown by Gandhi et al 2020, using *M sexta* caterpillar oral secretions to show that they act as an elicitor for ROS and has the potential to regulate the defenses of the host plant against herbivory. They also showed evidence that there was potentially crosstalk between Ca^{2+} and ROS signaling pathways, which could help show that the herbivore activity jump started these plant defenses. A suite of empirical studies and reviews (Reymond et al., 2000; 2004; Kaur et al 2020) also show how post feeding induces the activation of these pathways, the synthesis signaling of jasmonic acid, salicylic acid and ethylene combined with volatile organic compounds, and defense protein production aid in the plant defenses against herbivory as a second line of defense that could be both local and systemic.

Structural defenses on the surface start at the epidermis of the plant cell and are always present on the plant, formed before there is any sign of herbivore damage, as a preventative measure to deter herbivory before it takes place (Freeman et al 2008; Kariyat et al. 2013). Types of structural defenses range from trichomes and waxes, which are microscopic and not easily seen by the human eye, to very obvious structures such as thorns, modified stems, which are present in Fabaceae, prickles, extension of the epidermis, popular in Rosaceae, and spines, modified leaves, most famously known in Cactaceae (Freeman et al 2008; Mortensen 2013; Kariyat et al 2017). The latter three examples are typically plant deterrents from larger herbivores and smaller herbivores such as tiny insects can overcome these due to their size. This true in most cases, however Kariyat et al 2018 and Watts et al 2021 showed how larger Lepidopteran larvae movement could be hindered by spines on assorted *Solanum* species. Although this is true that larger structural defenses (thorns and prickles) are targeted more

toward larger animals, while smaller structures are aimed more toward smaller organisms; Kariyat et al in 2017 also showed that spines can deter herbivory by significantly increasing feeding time.

Trichomes serve as an important structural defense of plants that are on a microscopic level. They are a general defense system for plants, protecting from herbivores and biotic factors, but also are important in protecting against abiotic stressors such as temperature and UV damage (Emiko Harada et al 2010; Kaur and Kariyat 2020A). They extend out of the epidermis of the plant and aid in hindering insects' abilities to consume large amounts of plant material, reproduce on the plant, and even move among the surface of the plant. (Freeman 2008; Watts et al 2021). Serving as a first line of defense, there are two types of trichomes, glandular and non-glandular. Glandular trichomes essentially combine structural defenses with chemical defenses to ward off herbivores and even fire up signaling pathways for more defenses, while non-glandular trichomes rely mostly on physical defense (Kariyat et al 2017).

In studies that focus on plant defenses from herbivores, trichomes have been well studied, however waxes are important as well and can have an important role in protecting these plants from damage. Cell walls contain the epidermis of the plant, which is responsible for the formation of the cuticle which allows assorted material to pass through into the plant’s cells, such as water and gases. Harmful organisms also have the capabilities to pass through this layer as well. Wax is deposited on the outer edge of the cell wall of the epidermis (Reina-Pinto et al 2009). Wax serves the plant in aiding in the protection against pathogens which would have a negative effect on the plant, but also hindering the abilities of insects to maneuver on the slick surfaces, and any negative impacts those insects might have including but not limited to feeding

or ovipositing (Müller 2007). Another purpose of waxes, quite possibly the main purpose is to prevent water loss from the surfaces of the plant (Eigenbrode 1995). It has been shown by Blenn et al 2012 that brussels sprout (*Brassica oleraveae* var *gemmifera*) plants that had cabbage white butterflies (*Pieris brassicae*) lay eggs on them had a significant difference in the composition of the wax, compared to that of the control group. This further shows how wax can not only act as a physical barrier but have a chemical composition that deters herbivores as well. These waxes serve as a defense mechanism on the cuticle of most vascular plants in the world, however the amount and composition of waxes vary (Fürstenberg-Hägg 2013; Engelberth 2006). Some plants have species specific chemical compositions of wax, also known as epicellular lipids, and they have been shown to have more functions, other than water loss (Eidenbrode 1995). The chemical definition of a wax is comprised of esters of long chain acids and long chain primary alcohols, however there is a wide range of different chemical compounds that could make up these epicuticular waxes (Wilhelm et al 1998). Shown by Wilhelm et al 1998, most common but rarely dominant aliphatic compounds are hydrocarbons, wax esters, primary alcohols, fatty acids, and aldehydes. The less common but mostly dominant are ketones, β- diketones and secondary alcohols. More specifically, and directly relevant to this study, Radu et al 2015 isolated 11 compound classes out of the leaf epicuticular wax from *Aloe arborescens* which included typical aliphatic compound classes: alkanes, primary alcohols, aldehydes, fatty acids, methyl esters. 2- and 3- hydroxy functionalities were identified as unusual.

In the family Asphodelaceae, the genus *Aloe* contains roughly 420 species, the majority of which are native to Africa (Dagne 2000). Arthropod interactions with *Aloe* are not often documented and any documentation has shown minimal pest damage on the plant, or have been more focused on pollination. Kremer-Köhne et al 2020 documented arthropod associations in the

native range of *Aloe lettyae*, an endangered species of *Aloe* in South Africa, and only found an interaction between insects and the inflorescence and infructescence of the plant. A minimal amount of damage was documented on the leaves; however, the suspect of this damage was not identified. In the native range of *Aloe,* they suffer very little damage from insects, with the majority of the damage coming from mammal herbivores. However, in cultivation, common greenhouse pests do occasionally feed on them such as mealybugs, thrips and scale, but never in high enough numbers to cause any damage (Simmonds et al 2004). It is plausible to speculate that *Aloe* defenses against herbivores may be two-fold. Surface defenses through waxes, and the presence of toxic secondary metabolites that can reduce herbivore growth and feeding, as demonstrated in other systems (Tayal et al, 2020a,b).

Kremer-Köhne et al 2020 showed that in the field, *Aloe* does not get many herbivores on the foliage. When documenting the arthropod community, they did not identify any arthropod herbivores on foliage, and they were only able to document insignificant damage to minimal leaves. With the information above on waxes and secondary metabolites, we hypothesize that the lack of herbivory is due to a combination of secondary metabolic toxins, and wax. We suspect that this avoidance of pests from *Aloe barbadensis* stems from the surface wax inhibiting insects from being able to chew or maneuver on the leaves, or potentially having negative effects on the insects, as most if not all documented pests have piercing mouthparts that would help overcome through this waxy layer. We tested this with behavior assays of caterpillars on plant foliage, as well as feeding assays of isolated compounds. In our study we used *Manduca sexta* which is a specialist herbivore, that has a limited range of host plants that it will feed on, in its case, Solanaceae. We also use *Spodoptera frugiperda*, a generalist herbivore, that feed on a wide range of plants varies greatly between many different plant families. These two organisms were

utilized for their feeding response, ease to rear, and their readiness to eat an artificial diet. In this thesis we have shown that a *M. sexta* larvae can have a mass gain from first to fifth instar, infact they gain~600 times their initial body mass. Tayal et al 2020 (A) not only showed their feeding capacity by observing mass gain but also their readiness to feed on artificial diet and their ease to raise in a lab using petridishes even though they are Solanaceae specialist feeders. *S. frugiperda* is a generalist caterpillar, also with mass gain ranging in the hundreds, willingness to eat artificial diet, and the ease of raising in a lab (Singh 2020).

CHAPTER II

EFFECTS OF METHANOL, DICHLOROMETHANE, AND WATER EXTRACTS FROM ALOE BARBADENSIS ON MANDUCA SEXTA AND SPODOPTERA FRUGIPERDA GROWTH AND DEVELOPMENT

Introduction

Plants have many mechanisms that they use to overcome insect herbivore damage, and these defenses can be detrimental to the health of the herbivore (Singh 2021; Tayal 2020; Kariyat et al 2017; Mortensen 2013; Freeman et al 2008). In an effort to identify these effects on herbivores, we used compounds extracted from *Aloe barbadensis* using dichloromethane, methanol, and an aqueous water extract, and conducted experiments using mass, mass gain, pupal mass, pupal length, pupal width and time to pupate as parameters on interest. *Spodoptera frugiperda* and *Manduca sexta* were used as our study system, as they are ferocious eaters and have been demonstrated by (Singh *et al* 2021; Tayal *et al* 2020 (A); Kariyat *et al* 2019) to be excellent models to observe negative effects in lab based growth and development experiments. Previous work from our lab has also showed that in *S. frugiperda* and *M. sexta*, negative effects that the larvae were having when exposed to these extacts. When *M. sexta* were fed on polyphenol rich purple corn pericarp in their diet, Tayal et al 2020 (A) was able to show a significant difference between it and the rest of the diets including control and yellow corn pericarp added diet. The purple corn diet had significantly lower mass gain, showing negative effects on the larvae. They then showed the cascading effects in the adult moths, further showing these negative effects linger

to the next life stages (Tayal et al 2020 (B)). Singh et al 2020 showed these same effects in *S. frugiperda* using the polyphenol- rich purple corn pericarp extract to demonstrate the negative effects when added to diet compared to yellow corn and control. Colelctively, these studies showed that these polyphenols play a important role in mediating plant -herbivore interactions. These methods help us observe effects that extracts are having on the larvae overall, on an instar specific manner, and through the pupation stage.

Surface defenses of plants act as a first physical line of action toward the prevention of predation by insect herbivores. We wanted to identify any surface defenses in *A. barbadensis* by first looking at scanning electron microscope images of leaf samples. This would help determine if the surface of *A. barbadensis* contains any structural defenses such as trichomes or waxes. Waxes were identidied and we wanted to see if this influenced the feeding of *S. frugiperda* and *M. sexta*. Larvae were caged on leaves that had either wax left on or off, and allowed to feed for 48 hours. This helped shed light on weather the wax was preventing a feeding response in these larvae. Wax quantification was done to determine how much of the leaves surface contains wax. Lastly waxes extracted from the leaf were run on GC-MS in order to identify any volatiles from the wax. This is important because this chould shed more light on the defense phenotype of *A. barbadensis*. If there are chemicals that inhibit growth or prevent herbivory on this plant, this method would reveal that to add to the bank of defense mechanisms it uses. When *M. sexta* larvae were allowed to feed on *Solanum carolinense,* the volatile profiles of the plants directly influenced the interest of the larvae on those plants (Kariyat et al 2014). They showed that larvae constantly preferred inbred plants over outbred plants, even when visual cues were blocked. This type of host location influence could be helpful in predicting possible reasons for the the lack of herbivores on *A. barbadensis*.

We hypothesized that the *Aloe barbadensis* defense phenotype against insect herbivores is not one defense, but a combination of chemical and mechanical means. Furthermore, we hypothesized that these defenses are mediated by cuticular wax as well as secondary metabolites. We feel that one of these defenses is not enough and *A. barbadensis* uses a "hurdle effect" that would make an insect herbivore overcome many hurdles of defenses that may not be effective alone, but in numbers are effective enough for it to not have many herbivores.

Methods and Materials

Study System

Aloe barbadensis plants were donated by an organic certified nursery Hilltop Gardens (100 Lee Lane, Lyford, Texas 78569) in the Lower Rio Grande Valley. The plants were kept under green house conditions and watered as necessary (25C 16:8 day night cycle, and 65% RH).

Isolation of compounds for extract preparation

Aloe barbadensis leaf samples were obtained from the plants and were steeped in 100mL of deionized water in glass bottles. They were then incubated at 52 degrees celcius for 24 hours, where it was then stirred at 100 rpm for the liquid extracts to be prepared. Extracts where then centrifuged at 5000rpm for 5 minutes following steeping. The filtrates were collected and incorprated into diets individually, 5g each. These consisted of dichloromethane, methanol, and a water extract. Methanol is a weakly polar solvent which extracts some polar compounds and certain non polar compounds. Many bioactive compounds can be extracted using methanol. Dichloromethane is very polar as and is used to extract many organic products such as caffeine (in other plants).

Insect Colony - *Manduca sexta*

Manduca sexta eggs were purchased from a supplier (Great Lake Hornworm LTD. Romeo, Michigan, USA), and were reared under laboratory conditions. The eggs hatched on control artificial diet in petri dishes. After hatching, larvae caterpillars were moved to single petri dishes with a paper towel cutout to promote cleanliness, and a cubic centimeter of diet. A premass mesasurement was taken prior to moving to petri dishes.

Insect colony - *Spodoptera frugiperda*

Spodoptera frugiperda eggs were purchased from a supplier (Benzon Research. Carlisle, Pennsylvania, USA), and were reared under laboratory conditions. Eggs hatched on paper towel and after hatching, larvae were fed control diet until they were of measurable size, roughly 5 – 10 micrograms. Larvae caterpillars had their premass measurements taken, and they were moved to single condement cups with a cubic centimeter of diet.

Artificial Diet

The artificial diet used in this experiment as the control diet, is a soy- wheat germ diet that arrives in powder form. 875 mL of water is boiled, mixed with 19g of agar, and 144g of powdered diet is blended together. This mixture is then poured into a plastic container, solidifies at room temperature, and then placed in a refrigerator. This generates 1 liter of diet. Ingrediants of the artificial diet are sucrose, 50% soy flour, wheat germ, salt mix (Wesson; rich in Ca, Fe and NA), USDA vitamin premix, fiber, sorbic acid, methyl paraben and ascorbic acid.

Variable Diets

The variable diets were made in the same way as the control/ artificial diet. During the blending stage, 5g of either DCM, methanol, or water compound were added and blended for 1 minute. The mixtures were poured into plastic containers and allowed to solidify at room temperature before being added to the refridgerator. For details on diet preparation and experimental design see Tayal et al., 2020a, b; Singh and Kariyat, 2020.

Larval Mass

After hatching, 30 caterpillars per treatment for *M sexta* and 40 per treatment for *S frugiperda* were selected from the respective colonies. Mass of the caterpillars prior to the experiment was collected and the caterpillars were placed on one of the selected diets. Every 48 hours their mass was collected until they were ready for pupation (detailed methodology is described in Tayal et al., 2020a, b; Singh and Kariyat, 2020).

Mass Gain

We calculated three types of mass gain from caterpillar mass on each treatment; Mass gain differences over time = (Final mass - Initial mass)/Initial mass. Mass gain with initial mass = (current mass – initial mass)/ initial mass. Lastly mass gain with previous mass = (current mass- previous mass)/previous mass. Initial mass was the first mass of the caterpillar before placed on the diet. Current mass was the mass measurement just before calculating the mass gain (different for each instar).

Time to Pupate and Pupal Mass/Volume – *Manduca sexta*

M sexta pupae that reached the final, fifth instar stops feeding, and their aorta on their dorsal side will start to pulsate. At this time, they were transferred into falcon tubes individually and placed in the dark. The falcon tubes were cleaned daily until pupation occurred. The time it took to reach this stage with the pulsating vein, called wandering, was recorded. Once pupated, each pupae was weighed and measured, and treating them like a cylinder, volume was calculated using the equation, volume = 3.14 X R^2 H. 3.14 is the consyatnt (Pi), R is the radius (here half the pupal width), and H is height (here, length of the paupa). These pupae were then allowed to emerge as adults and raised.

Time to pupate and Pupal Mass/Volume – *Spodoptera frugiperda*

S frugiperda that reached their final instar will stop feeding and begin to shrink in size and lose weight. At this time, they will burrow into their diet and pupate within the chamber that they create. After pupation they were removed from the diet and mass was measured with a mg scale. Length and width was measured using calipers. Following this the pupae were frozen.

Mortality

The mortality rate per treatment was calculated as (dead/ initial population)x100. Mortality included all deaths, including post pupation stage but pre adult stage.

Leaf Damage

This experiment was conducted the same way for both *M. sexta* and *S. frugiperda* independently from each other. 12 *Aloe barbadensis* plants were selected, and the two newest grown leaves were selected from each. One pre weighed caterpillar was placed on each leaf for

48 hours, covered by a mesh bag, and allowed to feed. Bags were tied closed on the leaf and rubberbands were added for reinforcement. After 48 hours, caterpillars were removed, weighed, and leaves were cut off the plant. Using a microscope, the circumference of each bite mark was measured. (Figures 16, 17, 18)

Presence of Trichomes and Wax

To identify if there was a presence of trichomes or wax, scanning electron microscopy images were taken with (SNE- 4500 Plus Tabletop Scanning Electron Microscope). Leaf samples of *A. barbadensis* were cut off of the plant and the inner matrix was removed. A small leaf fragment was placed inside the SEM and following scanning, images were resolved to be sharper. Images were taken at 400x and 120x.

Wax Quantification

In order to quantify the wax from the leaves, one leaf tip from 10 plants was cut off, and the outer epidermis layer was thinly sliced, and any tissue from the inner leaf was removed. 1 gram per leaf was weighed of this plant material and placed in one of 10 pre-weighed tubes. 3 mL of chloroform was added into each tube, and vortexed for 1 minute each. Plant material was then discarded, and chloroform was allowed to evaporate for 24 hours. Following this, tubes were weighed again and the initial tube weight was subtracted from the final tube weight to get the total wax content per gram of epidermis leaf tissue.

Identification and quantification of volatiles

Wax was collected from the outer epidermis of six leaves of different plants of *A. barbadensis*. Waxes from outer surface was dissolved in chloroform. The chloroform was allowed to evaporate for 24 hours. After 24 hours, we got solidified wax. DCM dissolves at the

rate of 5 milligram per 1 milligram of wax . After mixing wax, solution was vortexed for 30 sec as to dissolve into DCM homogeously. Then the solution is centrifuges for 5000 rpm for 3 min to remove extra dust and unwanted particles. After centrifugation, 150 microlitre of the solution was inserted into glass vials. After that, 3 microlitre of internal standard (N-nonyl acetate - 80 ppm) was added to glass vials.A total of six samples were prepared. One microlitre was injected into the ISQ Single Quadrupole GC-MS (Thermofisher, USA) gas chromatography. HP 5 column (30m length; 0.25 mm internal diameter;0.25 mm film thickness) using Helium as carrier gas (1.2ml/L). The column temperature was kept at 500C for 2minutes., then it was raised to 300 with a flow rate of 100C / min, kept isothermally for 5 minutes. The from inlet temperature was 2800C, MS transfer line and ion source temperature were kept at 3000C respectively.

Results

Larval Mass – *Manduca sexta*

We recorded larval mass every other day at the same time each day, until the fifth instar when the caterpillars were ready to pupate. There were a total of ten mass recordings, which excludes premass (mass before diet treatment) throughout the larval stage from first to fifth instars. There was no significant difference between treatments during the first, early second or late second instars. Specifically, for first instar no significant differences were observed (Kruskal-Wallis test, Dunn's multiple comparison test, first, F= 4.149, P= 0.245), also for early second (Kruskal-Wallis test, Dunn's multiple comparison test, F= .336, P= 0.561), and late second (Kruskal-Wallis test, Dunn's multiple comparison test, F= 4.336, P= 0.037). Starting with early third and ending with late fifth instars, there was significant differences between the treatments. More specifically, in the early third instar, (Kruskal-Wallis test, Dunn's multiple comparison test, F= 4.760, P= 0.0291), for the mid third (Kruskal-Wallis test, Dunn's multiple

comparison test, F= 9.749, P= 0.0018), for the late third (Kruskal-Wallis test, Dunn's multiple comparison test, F= 7.44, P= 0.0064), for the early forth (Kruskal-Wallis test, Dunn's multiple comparison test, F= 9.92, P= 0.0016), for the late forth (Kruskal-Wallis test, Dunn's multiple comparison test, F= 8.95, P= 0.0028), for the early fifth (Kruskal-Wallis test, Dunn's multiple comparison test, F= 9.92, P= 0.0016), for the mid fifth (Kruskal-Wallis test, Dunn's multiple comparison test, F= 10.05, P= 0.0015), and for the late fifth (Kruskal-Wallis test, Dunn's multiple comparison test, F= 15.07, P= 0.0001). It can be seen that while DCM, methanol and control treatments are similar, water is an obvious outlier looking to be beneficial to the mass of the larvae (Figure 1).

Larval Mass Gain – *Manduca sexta*

A total of nine gains were calculated from first to fifth instar, which consited of mass gain over previous mass and mass gain over initial mass (premass). When mass gains were calculated using the initial mass, the three treatments other than control look to have a negative impact on growth, with methanol having the most negative impact, followed by DCM, and water. Significant values are marked with an asterisk and are as follows, first, (Kruskal-Wallis test, Dunn's multiple comparison test, F= 5.73, P= 0.0011) forth, (Kruskal-Wallis test, Dunn's multiple comparison test, F= 4.23, P= 0.0072) sixth, (Kruskal-Wallis test, Dunn's multiple comparison test, F= 2.97, P= 0.0350) seventh, (Kruskal-Wallis test, Dunn's multiple comparison test, F= 4.98, P= 0.0029) ninth, (Kruskal-Wallis test, Dunn's multiple comparison test, F= 3.63, P= 0.0152) and tenth mass gains, (Kruskal-Wallis test, Dunn's multiple comparison test, F= 3.08, P= 0.0306). There is one instance in the seventh mass gain that the water extract treatment mass gain is the greatest, however it gets in line with DCM and methanol.The remaining mass gains were not significant and are mass gain 2, (Kruskal-Wallis test, Dunn's multiple comparison

test, F= 2.11, P= 0.1023), mass gain 3 (Kruskal-Wallis test, Dunn's multiple comparison test, F= 1.89, P= 0.1353), mass gain 5 (Kruskal-Wallis test, Dunn's multiple comparison test, F= 1.24, P= 0.2988), and mass gain 8 (Kruskal-Wallis test, Dunn's multiple comparison test, F= 2.30, P= 0.0810). When mass gain is calculated with the previous mass, mass gains were significant early on up until the third instar, with mass gain 1(Kruskal-Wallis test, Dunn's multiple comparison test, F= 13.63, P= 0.0034), mass gain 2 (Kruskal-Wallis test, Dunn's multiple comparison test, F= 18.23, P= 0.0004), mass gain 3 (Kruskal-Wallis test, Dunn's multiple comparison test, F= 17.19, P= 0.0006) and mass gain 4 (Kruskal-Wallis test, Dunn's multiple comparison test, F= 12.09, P= 0.0071) and then they were no longer significant following the third instar excluding one instar which was mass gain 7(Kruskal-Wallis test, Dunn's multiple comparison test, F= 15.78, P= 0.0013). An interesting pattern was observed with the larvael appearing to be alternating their mass gains by skipping an instar or taking a "rest" period (Figure 2). In this calculation, it appears that compared to the control diet, the treatments had a negative effect on caterpillar mass gain when calculated with the initial mass, with methanol and water extracts looking to have a more negative impact. Other than mass gain 7 the rest were not significant including mass gain 5,(Kruskal-Wallis test, Dunn's multiple comparison test, F= 6.49, P= 0.0897), mass gain 6 (Kruskal-Wallis test, Dunn's multiple comparison test, F= 1.01, P= 0.7972), mass gain 8 (Kruskal-Wallis test, Dunn's multiple comparison test, F= 4.43, P= 0.2183), mass gain 9 (Kruskal-Wallis test, Dunn's multiple comparison test, F= 3.42, P= 0.3302), and mass gain 10 (Kruskal-Wallis test, Dunn's multiple comparison test, F= 0.25, P= 0.9687).

Days to Wandering- *Manduca sexta*

The number of days that it took *Manduca sexta* larvae to begin wandering, which is the stage before pupation but after feeding was recorded to determing effects of extracts. We found that the time it took larvae feeding on water treatment had a significant difference from methanol, DCM, or control (Figure 3). The time it took water treatment larvae to wander was less when compared to the other three treatments (Kruskal-Wallis test, Dunn's multiple comparison test, F= 5.83, P= 0.0010).

Pupal Mass and Pupal Volume- *Manduca sexta*

Once pupation occurred, pupal mass measurments were taken, and pupal volume was calculated. Within this data, there was a significant difference between DCM treated and water extract treated larvae, in the pupal mass (Kruskal-Wallis test, Dunn's multiple comparison test, F= 2.94, P= 0.0398). The larvae that fed on water extract had a significantly higher pupal mass than DCM treated larvae. In regards to the pupal volume, there was no significant difference between any of the treatments (Figure 4) (Kruskal-Wallis test, Dunn's multiple comparison test, F= 2.03, P= 0.1188).

Larval Mass- *S. frugiperda*

Larval mass was taken every 48 hours from when the larvae had a mass approximately 5-10 mg. The mass that was collected did not show a significant difference between any of the treatments (Figure 4). There were a total of 7 masses collected over a span of 14 days, and they are mass 1, (Kruskal-Wallis test, Dunn's multiple comparison test, F= 0.42, P= 0.8084), mass 2 (Kruskal-Wallis test, Dunn's multiple comparison test, F= 0.05, P= 0.9738), mass 3 (Kruskal-Wallis test, Dunn's multiple comparison test, F= 0.03, P= 0.9844), mass 4 (Kruskal-Wallis test,

Dunn's multiple comparison test, F= 0.01, P= 0.9911), mass 5 (Kruskal-Wallis test, Dunn's multiple comparison test, F= 0.14, P= 0.9312), mass 6 (Kruskal-Wallis test, Dunn's multiple comparison test, F= 0.78, P= 0.6755), and lastly mass 7 (Kruskal-Wallis test, Dunn's multiple comparison test, F= 1.20, P= 0.5482).

Larval Mass Gain – *S. frugiperda*

We calculated larval mass gain from initial mass between the different treatments, first through fifth instars. When calculated from initial mass, all mass gains were significant. Mass gain 1,2,3 and 5 had significant differences between the control with methanol and control with water with mass gain 1 having (Kruskal-Wallis test, Dunn's multiple comparison test, F= 14.30, P= 0.0001), mass gain 2 (Kruskal-Wallis test, Dunn's multiple comparison test, F= 13.35, P= 0.0001), mass gain 3 (Kruskal-Wallis test, Dunn's multiple comparison test, F= 9.31, P= 0.0002) and mass gain 5 (Kruskal-Wallis test, Dunn's multiple comparison test, F= 11.77, P= 0.0001). Mass gain 4 had significant differences between control with water (Kruskal-Wallis test, Dunn's multiple comparison test, F= 7.92, P= 0.0006), while mass gain 6 had significant differences between methanol with water (Kruskal-Wallis test, Dunn's multiple comparison test, F= 5.55, P= 0.0053). Mean larval mass gain calculated from previous mass. There was a significant difference between the treatments of mass gain 1, between the control with methanol and the control with water (Kruskal-Wallis test, Dunn's multiple comparison test, F= 14.30, P= 0.0001). Mass gain 3 also had a significant difference between the treatments, between methanol with water (Kruskal-Wallis test, Dunn's multiple comparison test, F= 5.34, P= 0.0061). Mass gain 5 and 6 both had significant differences and both had significant differences between control with methanol, and methanol with water (Figure 6) mass gain 5 (Kruskal-Wallis test, Dunn's multiple comparison test, F= 8.09, P=

0.0005), and mass gain 6 (Kruskal-Wallis test, Dunn's multiple comparison test, F= 39.69, P= 0.0001).

Larval Feeding Assay- *S. frugiperda*

Larvae of *S. frugiperda* were allowed to feed on sections of *A. barbadensis* for 48 hours (Figure 15). The bite mark circumference between leaves that had wax chemically stripped and controls with the wax left on was traced on a microscope (Figure 17). There was a significant difference between the two, with the wax stripped leaves having a greater circumference (Kruskal-Wallis test, Dunn's multiple comparison test, P= 0.0383). Though there was a significant difference between the circumference, there was not a significant difference between the number of bite marks that were made (Figure 7); (Figure 16) (Kruskal-Wallis test, Dunn's multiple comparison test, P= 0.8310)..

Mean Time to Pupate- *S. frugiperda*

The days that the larvae took to pupate was calculated as the day pupation was 100% completed. Between all of the treatments, there was no significant difference, as the mean days to pupate only differed slightly between them (Figure 8) (Kruskal-Wallis test, Dunn's multiple comparison test, F= 1.77, P= 0.1769).

Mean Pupal Mass- *S. frugiperda*

The mean pupal mass was calculated per treatment and there was a significant difference between the three treatments. Water had the highest mean pupal mass, followed by control and finally methanol had the lowest (Figure 9) (Kruskal-Wallis test, Dunn's multiple comparison test, F= 12.68, P= 0.001).

Mean Pupal Length- *S. frugiperda*

Following pupation, the length of the pupae was taken. The data shows a significant difference among the treatments. As with pupal mass, water had the highest mean pupal length, followed by the control and then methanol (Figure 10) (Kruskal-Wallis test, Dunn's multiple comparison test, F=11.72, P= 0.0001).

Mean Pupal Volume- *S. frugiperda*

Using the formula for volume of a cylinder ($V = \pi r^2 h$) and the measurements of length and width, pupal volume was calculated. There was a significant difference amongst all of the treatments and the common trend in mass and length stayed true. Water had the highest mean pupal volume, followed by the control and then methanol (Figure 11) (Kruskal-Wallis test, Dunn's multiple, F=51.48, P= 0.0001).

Presence of Trichomes and Wax

After viewing the SEM images of *A. barbadensis*, it was concluded that it does not contain any trichomes (Figure 12). There was a presence of wax on the leaf surface (Figure 13), and this wax was scraped off and rescanned to take images of samples with no wax as well (Figure 14).

Wax Quantification of *Aloe barbadensis*

Using one gram samples of *Aloe barbadensis* leaf epidermis, there was a range from 9% to 15% wax content. The mean wax content of ten, one gram samples was 12.4% wax content. Vial readings were taken once they were completely dry and only wax was the only remaining leftover product.

Identification and Quantification of Volatiles

For identification of volatiles, GC-MS comprising of 6890 GC with mass spectrometer (electron ionization mode). Peaks were identified from the NIST Spectral Library (National Institute of Standards and Technology). The samples were quantified by comparing the peak area relative to the internal standard (N-nonyl acetate - 80 ppm). Chemical compounds identified are as follows: Ethyl iso- allocholate; Ergosta- 5, 22- dien-3-ol; 24, 25- Dihydroxylcholecalciferol; 17- Pentatriacontene; Dodecanoic acid; 1, 2, 3- propanetriyl ester.

CHAPTER III

DISCUSSION OF EFFECTS OF ALOE EXTRACTS ON *MANDUCA SEXTA* AND *SPODOPTERA FRUGIPERDA*

Discussion

In this study, we examined how different compounds that were extracted from *Aloe barbadensis,* using methanol, DCM, and an aqueous water extract, effected the growth of *Manduca sexta* and *Spodoptera frugiperda.* We comprehensively tested the effects of these extracts on the larval stages of these two species, using mass, mass gain, larval survival and time to pupate as response variables. The surface content of the leaf was also examined under a scanning electron microscope to reveal the lack of trichomes and a predominance of wax. Larvae were allowed to feed on the leaves and it was shown that leaves with wax removed had significantly larger bite marks than those with wax. We then combined these results of chemical defenses of diet feeding assays with that of physical defenses of surface wax microscopy and leaf feeding assays to determine how efficient these strategies are at defending this plant against insect herbivores. Between these two pathways of defense, we found surprising results that the some of the extracts aided in the growth of the lepidopteran larvae. While our results cannot conclusively identify the complete defense phenotype, it sheds light on potential defense mechanisms in *A. barbadensis* for the first time.

It was shown that the mass of *M. sexta* larvae were not significant from eachother in the early stages (first and second instars), but became significantly different from the third through the fifth intar, with the water extract leading to a higher mean mass. This could be due to the factthat the water extract could contain carbohydrates and/or sugars which improved the larvae growth. Aqueous water extract was shown to negatively effect *M. sexta* larvae by Tayal et al 2020, using purple corn pericarp however we obtained quite the opposite result. This was not the case in *S. frugiperda* where there was no significance in any stage. Mass gains were also calculated using the previous mass, to show instar specific effects and the results were very interesting. In *M. sexta* there was a strong cyclical pattern with sharp peaks every 96 hours and low valleys in the same amount of time. This pattern could possibly be due to the molting pattern of larvae, as they empty their gut to molt and then eat a lot after (Tayal et al B; Singh 2020). Half of the mass gains were significant, showing that there is somewhat of an effect of the extracts that is instar specific, however each extract and the control took turns having significant beneficial results. Similarly with *S. frugiperda*, the same peaks and valley pattern was not observed, however, four out of six mass gains were significant, and the different extracts along with the control still took turns being the most beneficial. Lastly, mass gains were calculated, and it was shown in *S. frugiperda* that all values were significantly different than eachother, and water was most beneficial. In *M. sexta*, a majority of the values were significant from eachother with the control diet doing the best and the other diets having negative effects. These larval experiments were demonstrated to be beneficial in showing the negative effects of different diets in a paper by Tayal et al 2020, where they used *M. sexta* and purple corn pericarp extract. These extracts were shown, using similar experiments, to have detrimental effects on the larval caterpillars. Although we did not observe detrimental effects, we observed a combination of

positive and negative effects of these extracts. This may contribute to the number of the reasons why insects may not feed on *A. barbadensis*.

Our results regarding pupae align with what was observed with the larvae. The overall pupal mass in *M.* sexta was higher with the water extract, which could support the thought that water extract produced larger individuals from beneficial sugars or carbohydrates within it. *S. frugiperda* showed a significant difference with water influencing larger pupae and methanol having the smallest. Pupal volume in *S. frugiperda* pupae resulted in water extract having a beneficial impact, while methanol pupal volume was less than half that of water. Though there was no significant difference of pupal volume between treatments in *M. sexta*. Using pupal data when observing the effects of similar extracts has been proven by Singh 2021 where they followed these effects from the larval stage throughout the adult stage. It has also been found that through the adult stages, larval diet intake has had cascading negative effects on flight muscle metabolism, mass, and even flight metabolic rates of *M. sexta*, *S. frugiperda*, and *Spodoptera eridania* (Portman et al 2015)(Portman 2020). These are examples of how larval diet can have cascading effects that don't end at the larval or pupal stage, but follow through to the adult stage and beyond.

Using a scanning electron microscope, it was revealed that *A. barbadensis* did not contain trichomes, however it did contain wax. This method of using a SEM was shown to be an efficient way at observing surface plant defenses by Kaur & Kariyat 2020, where they looked at the trichome presence of *Solanum carolinense.* When *S. frugiperda* larvae were allowed to feed on leaves that did and did not have wax, the bite mark circumference was significantly higher on leaves that did not contain wax. This could be due to the wax containing compounds that are unfavorable to the larvae. Łukasz 2012 shows for the first time, the chemical composition of

Solanum macrocarpon cuticular waxes to reveal toxic compounds, and this could be the same case with *A. barbadensis*. However there was no significant difference between the amount of bitemarks between leaves that contained and did not contain waxes. This could be because once the larvae ingested the waxy epidermis they stopped feeding. It should be noted that all larvae that fed on the leaves lost mass.

Here we have a preliminary Gas Chromatography – Mass Spectometry analysis, however further work must be done in this field. Lavergne *et al* 2018 was able to use this method to evaluate the composition of plant cuticular wax in *Triticum aestivum* to identify 69 plant metabolites, and to show the function that compounds within the wax serve. This data could crack the case for what purpose waxes on *A. barbadensis* serve, as well as potentially determining what deters insect herbivores from feeding. Exudate composition has been documented, along with effects of the compounds and applications for humans and rats, but a more in depth investigation has not been conducted with insects (Hamman 2008; Nwajo 2006). We have shown that extracts have some positive and negative effects, but that is not enough. A look further into these vascular exudates could potentially reveal antiherbivore effects for insects. We have also shown that there is a lack of trichomes and a presenece of wax, and that larvae feed more on leaves without wax. As explained by Kariyat *et al* 2018, *Solanum lycopersicum* and *Nicotiana tabacum* both contain many different types of trichomes. This type of "hurdle" technology used by the plant potentially adds to the arsenal of defenses herbivores would need to feed on it. Now we need the content of the wax and the volatiles to help definitively identify the defense phenotype of *A. barbadensis*. Volatiles given off by the plant could be preventing herbivores from feeding, as they may potentially be toxic. Volatiles from within wax could potentially be the reason herbivores don't feed, and could be another hurdle

that insects would need to overcome, potentially making feeding not beneficial. In many insect groups including aphids and moths, volatiles play a major role in host location. It was shown in aphids that plant volatiles of infected cucumber plants with cucumber mosaic virus, were more attractive. Once the aphids fed on the plant they quickly emigrated, briging the virus with them (Mauck et al 2010). In *M. sexta* moths, it was shown by Kariyat et al 2013 that *Solanum carolinense* that are inbred alter the volatiles given off at night which would guide the oviposition of the adult moths. Identification of any volatiles is key in determining the full defense strategies of *A. barbadensis*. Toughness of the cell wall is something that could have major impacts on deterring herbivores. Two mechanical defenses are toughness and harndess, and the primary toughening of a plant is derived from a composit cell wall (Lucas *et al* 2000). Analyzing the toughness of the cell wall could be very useful, as it could help explain the low herbivory on leaves that had the wax removed. These data points combined with wax data could be the answer to the mystery of *A barbadensis* defense mechanisms, and why it is not often fed upon by insect herbivores.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS OF *ALOE BARBADENSIS* DEFENSE MECHANISMS

Through this thesis, it can be concluded that the combination of surface defenses, in this case wax, and secondary metabolites extracted from *Aloe barbadensis* leaves combine to deter, and negatively impact the feeding, growth and development two lepidopteran herbivores *Manduca sexta* and *Spodoptera frugiperda*. It has been shown by Kariyat et al 2017, 2020 A, 2020 B extensively that trichomes and spines in Solanaceae protect the plants from herbivory by acting as a barrier between the leaf surface and the herbivore. Feeding on some species is essentially like eating barbed wire. We have shown here that there is a significant difference between bite mark circumference of larvae that were feeding on *A. barbadensis* leaves that were chemically stripped of wax and ones that weren't. The leaves that were absent of wax had more herbivore damage than the leaves that had the wax left on. This information concludes that wax is an effective first line of defense for *A. barbadensis*, acting as a barrier between the leaf surface and herbivore, similar to trichomes.

In order to go beyond denoting wax to be only a physical defense, wax could potentially be shown as a double edged sword, having a hand in chemical defense as well. Liquid chromatography-mass spectrometry (LC-MS) could potentially be used to analyze chemical compounds that reside in, or make up the wax. In our study, we showed that when larvae of both *M. sexta* and *S. frugiperda* were given a no - choice assay and only permitted to feed on *A.*

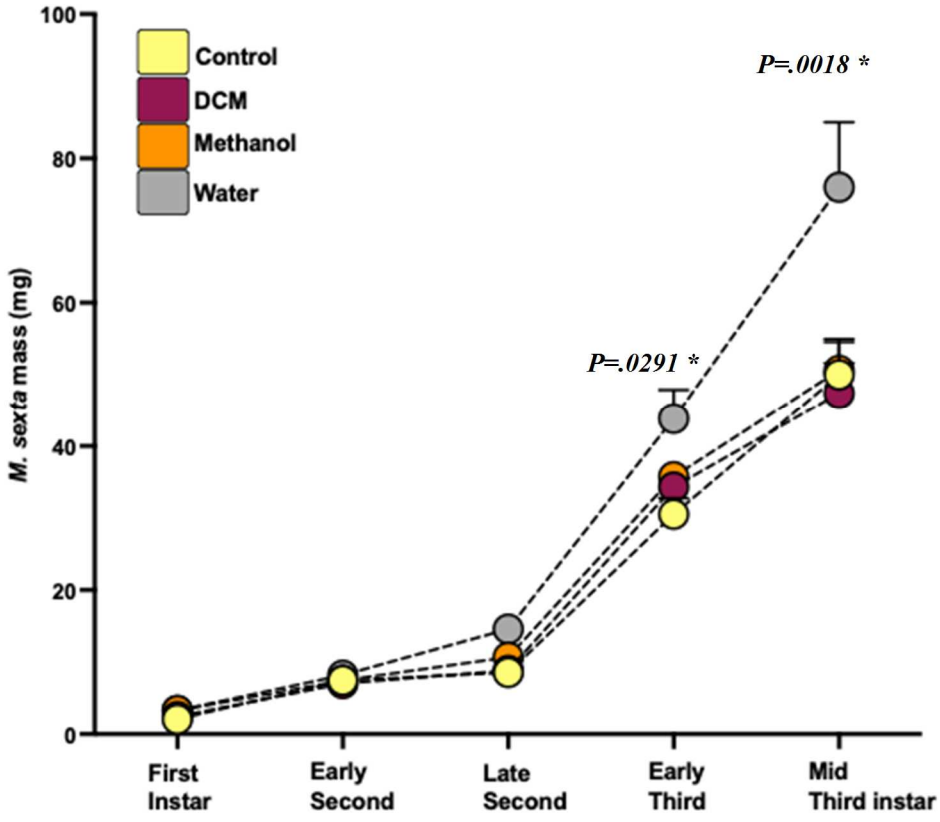
barbadensis, it had negative effects on their growth. *M. sexta* did not feed, which was most likely due to the fact that it is a specialist herbivore on Solanaceae, however *S. frugiperda* did feed and the results were clear. Though there was no significance of mass lost between the control leaves with the wax and the chemically stripped wax without wax, there was still a higher amount of mass lost within the control leaves. This could potentially show, if scaled up, that the larvae that ingested the wax had weight loss due to the wax, and if LC-MS was done, any inhibitory compounds. At the current moment we can only speculate this, and would need to run an analysis on wax components, and increase the sample size of the experiment of larvae feeding on leaves. Once any compounds are identified, high-performance liquid chromatography (HPLC) could be done to quantify and purify these compounds so that they could be fed to larvae to observe the effects.

Here we show the plant defense phenotype of *A. barbadensis*, which uses a combination of physical structural defenses in the form of waxes, and chemical defenses which include but are not limited to methanol, dichloromethane, and an extract of the water from within. To deter herbivory, the waxy epidermal layer serves as the first line of defense for *A. barbadensis*, preventing organisms from having direct access to the surface of the leaf. We have shown that when this layer is removed, feeding incidence remains the same, but the side of the feeding areas is significantly higher. Though it was not significant, there did appear to be more weight loss on individuals that ingested wax. If this wax layer is breached, compouds from within the plant effect the compounds extracted do appear to have an inhibitory effect toward larval growth. The inhibition is not severe, and did not appear to be deadly, but with the combination of the chemical nature within and the wax layer on the epidermis, *A. barbadensis* is well equipped to deter herbivory, to the extent that is is not commonly observed with extensive arthropod damage.

In this study, we looked at lepidopterans as the model to determine the defense phenotype of *A barbadensis*. Lepidopteran larvae chew on their food items, however this is not the only way insects feed on their source of nutrition. Other orders of insects such as hemipterans use piercing suching mouthparts, a stylet, to breach the epidermal layer and feed directly from the phloem. In future studies, to build off of this one, hemipterans such as aphids could be used as well to help fine tune the understanding of these defense mechanisms. In greenhouses and gardens, and even in the natural habitat of *A barbadensis*, if any herbivores feed on it, they are typically hemipterans, and are also typically not on the foliage. Thes experiments shed light using lepidopterans, on what the effect that chemical and structural defense mechanisms from this plant have on arthropods, using the larvae of a generalist and specialist. It would help to better understand this defense phenotype if assorded feeding strategies of arthropods was implemented into an experiment. Furthermore, it would be interesting to show what ways, if any, have insects in the native range of *A barbadensis* overcome these defenses. To date there has not been specifically any documentation of such origins, has been limited to other *Aloe* species, and has not specifically targeted defenses.

Figures

A


100
80
60
40
20
0
M. sexta mass (mg)
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DCM
Methanol
Water
P=.0018 *
P=.0291 *
First Instar
Early Second
Late Second
Early Third
Mid Third instar

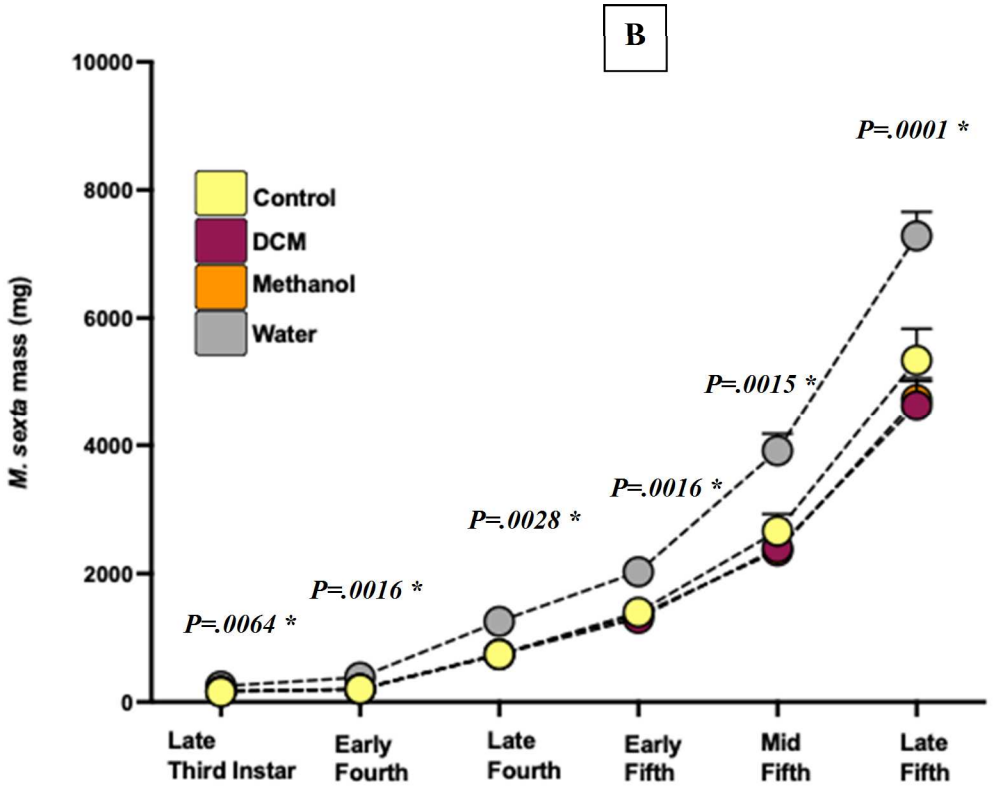
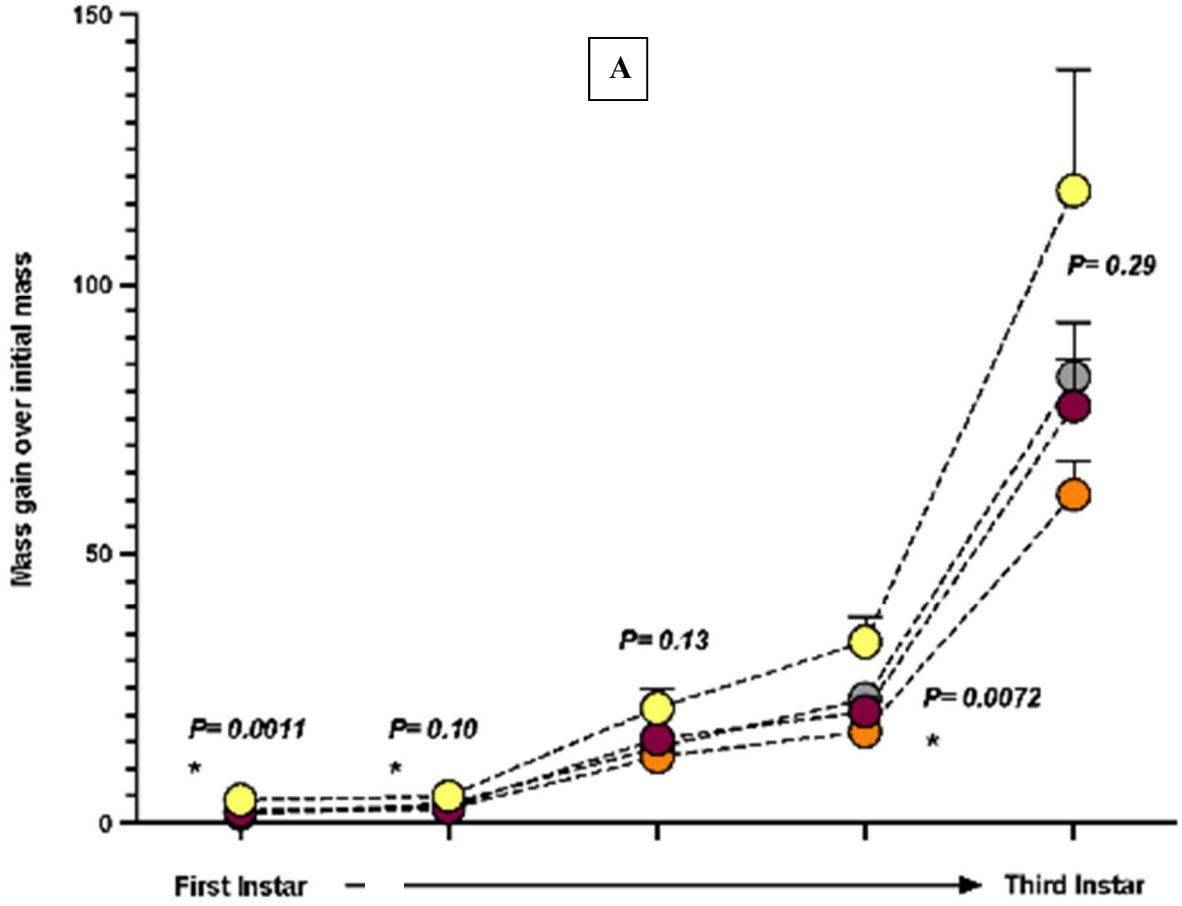


Figure 1. Represents the mean larval mass of *M. sexta* from first to last instar Mean first instar through mid third instar (**A**) and late third instar through late fifth instar larval mass (**B**) during different treatments. (Graphs split into two to represent an accurate visual, as the mass of larvae between first and fifth instars was drastic). All instars were significant excluding first, early second and late second instars. (Kruskal-Wallis tests, Dunn's multiple comparison test, $p<0.05$


A
150
100
50
0
Mass gain over initial mass
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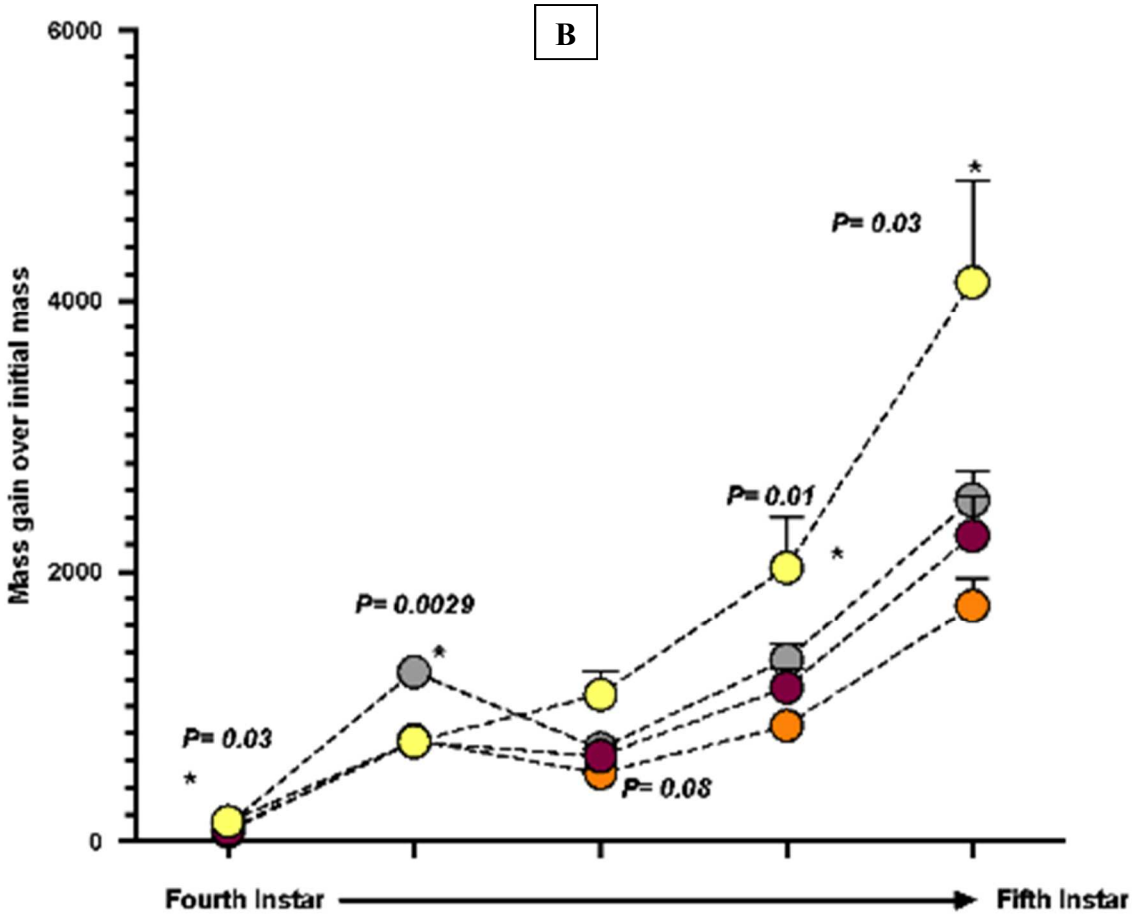
P= 0.10
*
P= 0.13
P= 0.0072
*
P= 0.29
First Instar
Third Instar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6000
4000
2000
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P= 0.0029
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Fourth Instar
Fifth Instar

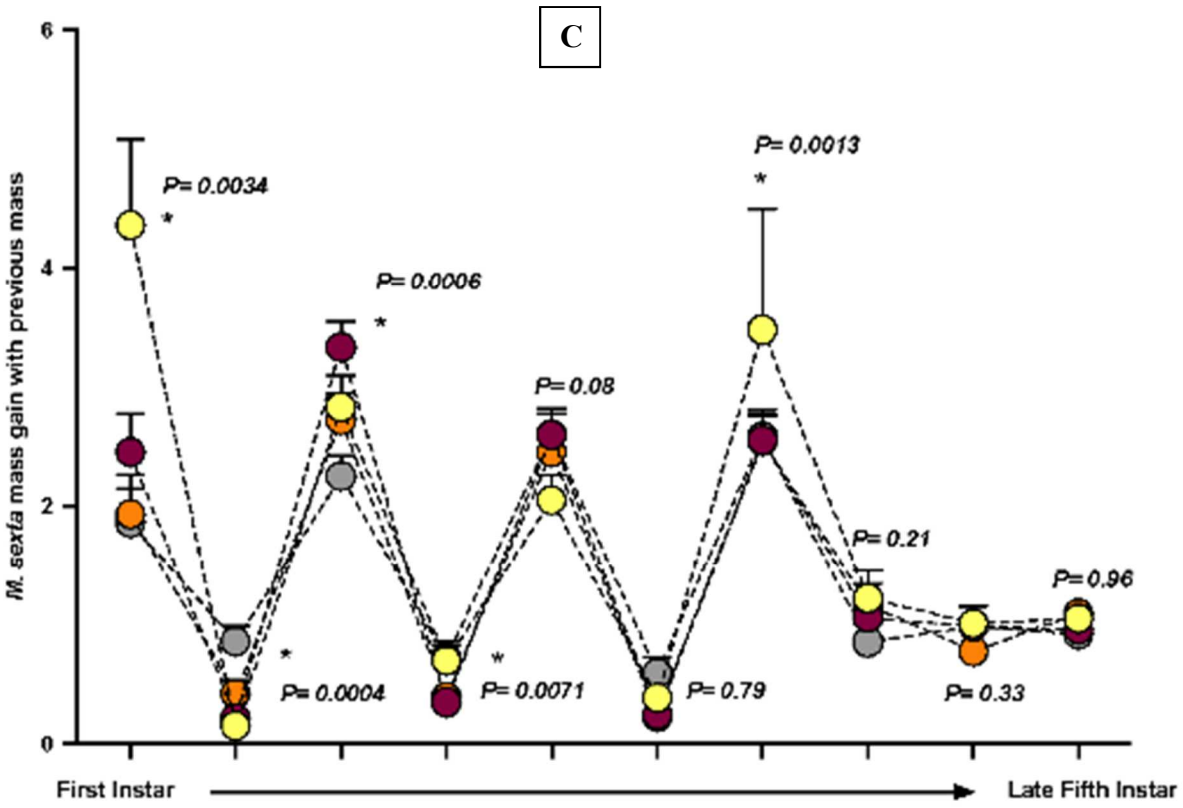


Figure 2. Represents mean mass gain calculated with initial mass (A, B) and mean mass gain calculated from previous mass (Bottom) of *Manduca sexta* . Mean larval mass gain calculated from initial mass between the different treatments, first through fifth instars (**A, B**) (divided into two to compensate for scale differences between early and late instars). Mean larval mass gain calculated from previous mass (**C**). Asterisk indicates means with significant differences between the treatments utilized. (Kruskal-Wallis, Dunn's multiple comparison test, $p<0.05$).

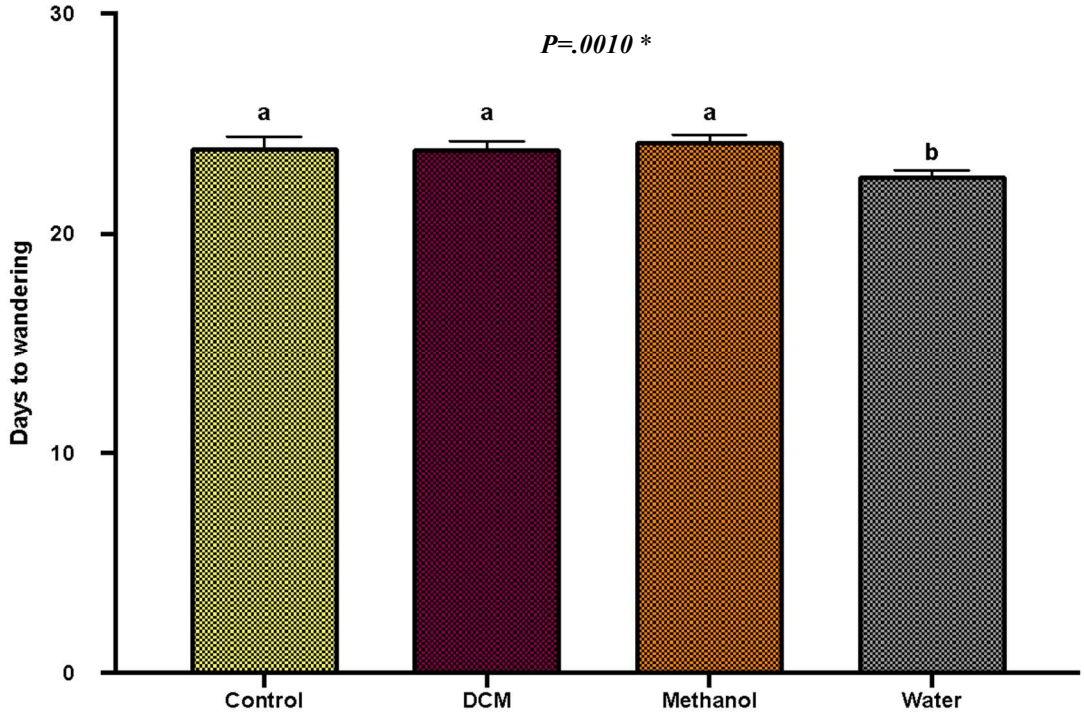
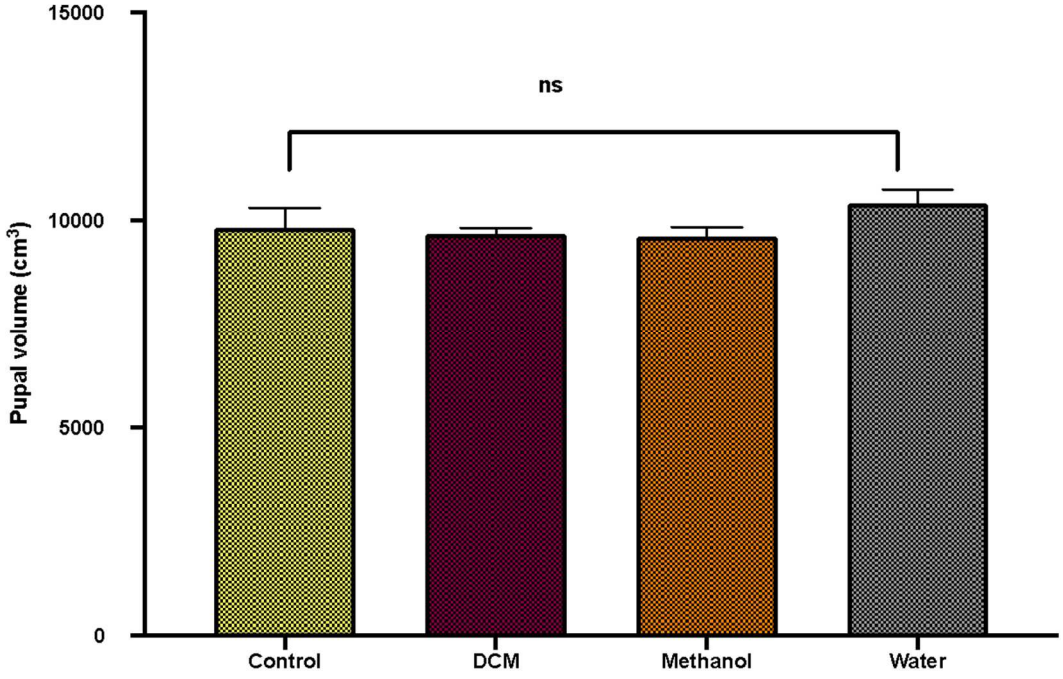


Figure 3. Represents the mean amount of days larvae took to wander. Mean number of days it took *M. sexta* larvae to begin wandering between the different treatments. Similar letters are not significant from eachother, whereas water extract has a significant difference from the other three (Kruskal-Wallis test, Dunn's multiple comparison test, $p < 0.05$).

A


15000
10000
5000
0
Pupal volume (cm3)
ns
Control
DCM
Methanol
Water

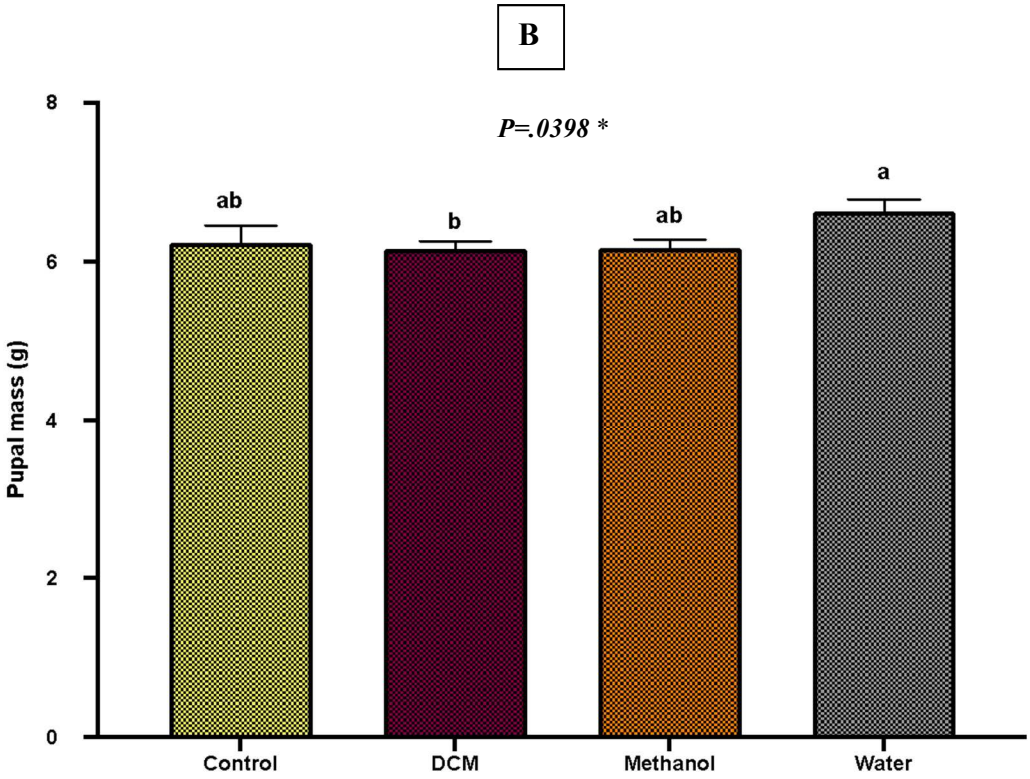
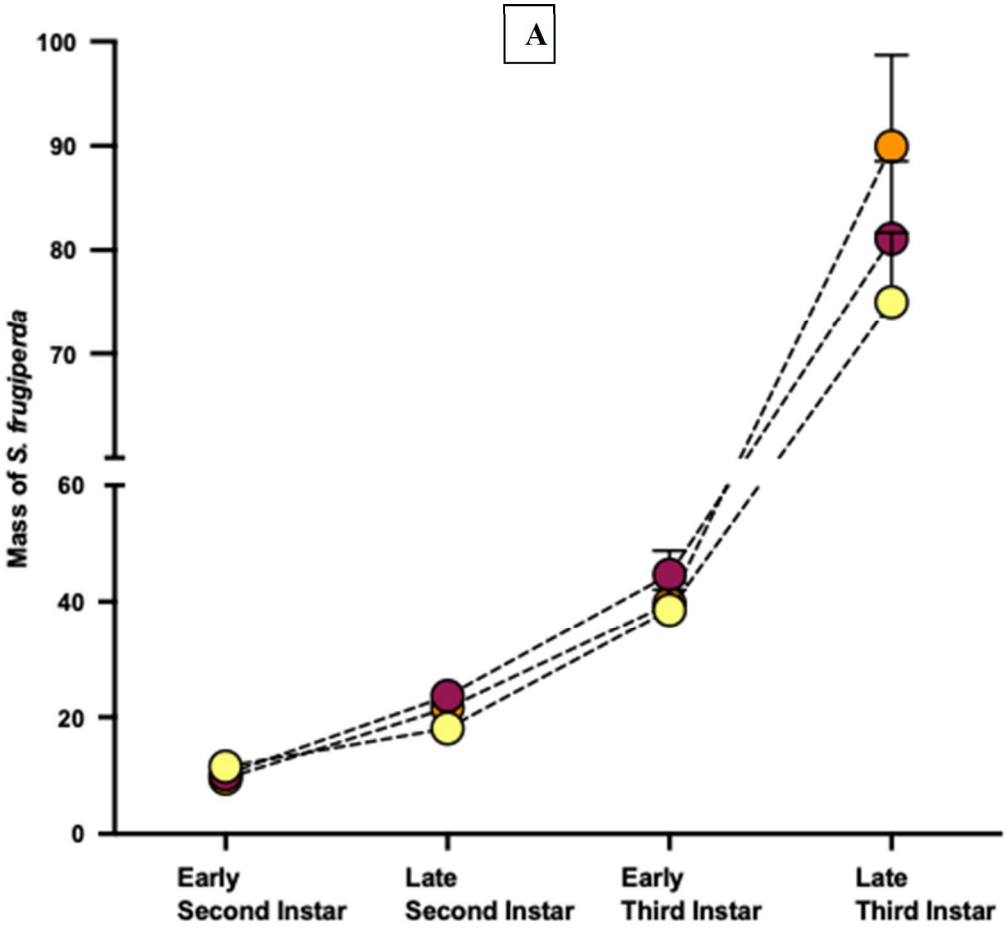


Figure 4. Represents the mean pupal mass per treatment. Similar letters represent not significant, whereas different letters represent significance to eachother (**A**). Mean pupal volume per treatment, there is no significant difference between treatments (**B**) (Kruskal – Wallis test, Dunn's multiple comparison test, $p < 0.05$).


A
100
90
80
70
60
40
20
0
Mass of S. frugiperda
Early
Second Instar
Late
Second Instar
Early
Third Instar
Late
Third Instar

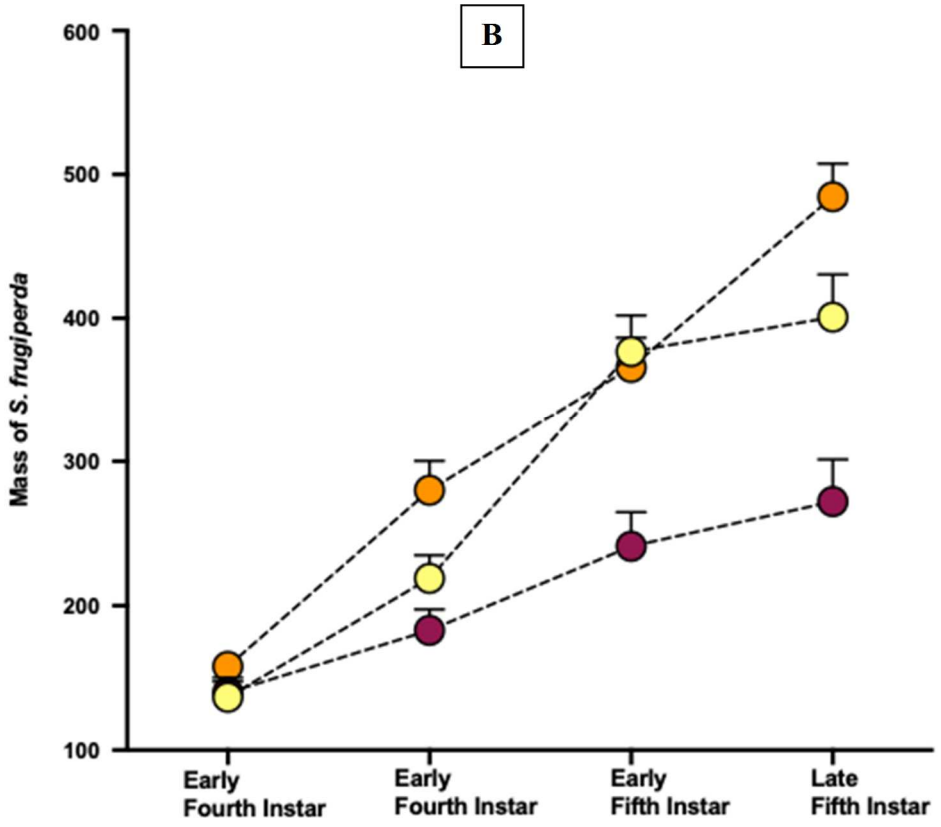
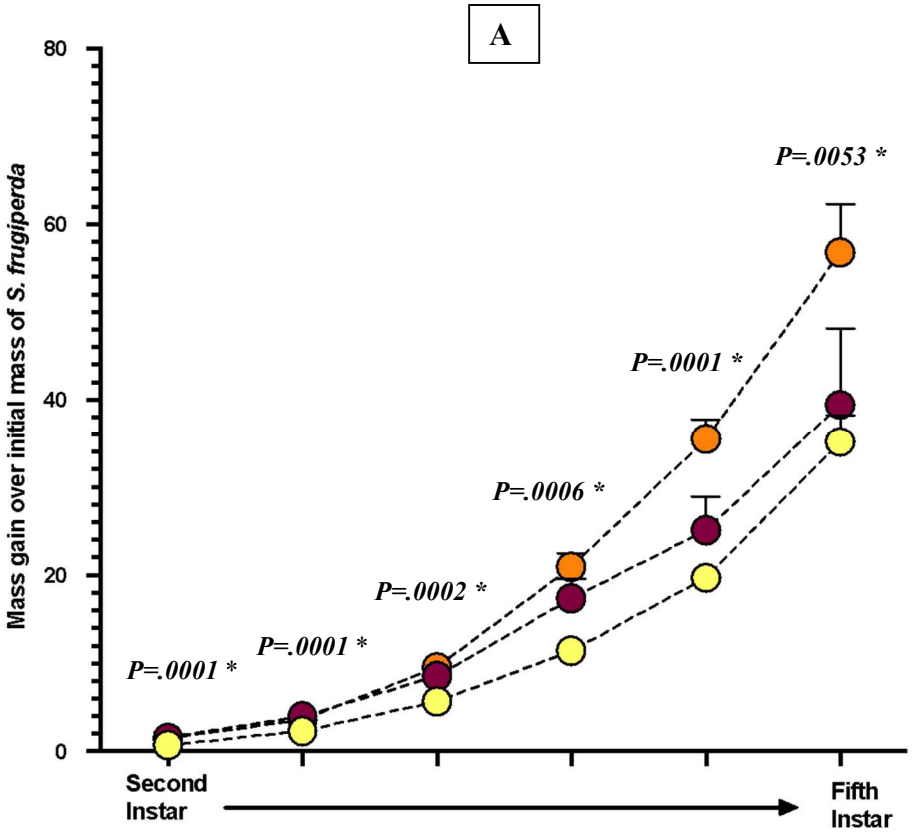


Figure 5. Represents the mean larval mass of *S. frugiperda* from first to final instar. Mean first instar through late third instar (**A**) and early fourth instar through late fifth instar larval mass (**B**) during different treatments. (Graphs split into two to represent an accurate visual, as the mass of larvae between first and fifth instars was drastic). No instars showed significant results. (Kruskal-Wallis tests, Dunn's multiple comparison test, $p<0.05$)


A
80
60
40
20
0
Mass gain over initial mass of S. frugiperda
P=.0053 *
P=.0001 *
P=.0006 *
P=.0002 *
P=.0001 *
P=.0001 *
Second Instar
Fifth Instar

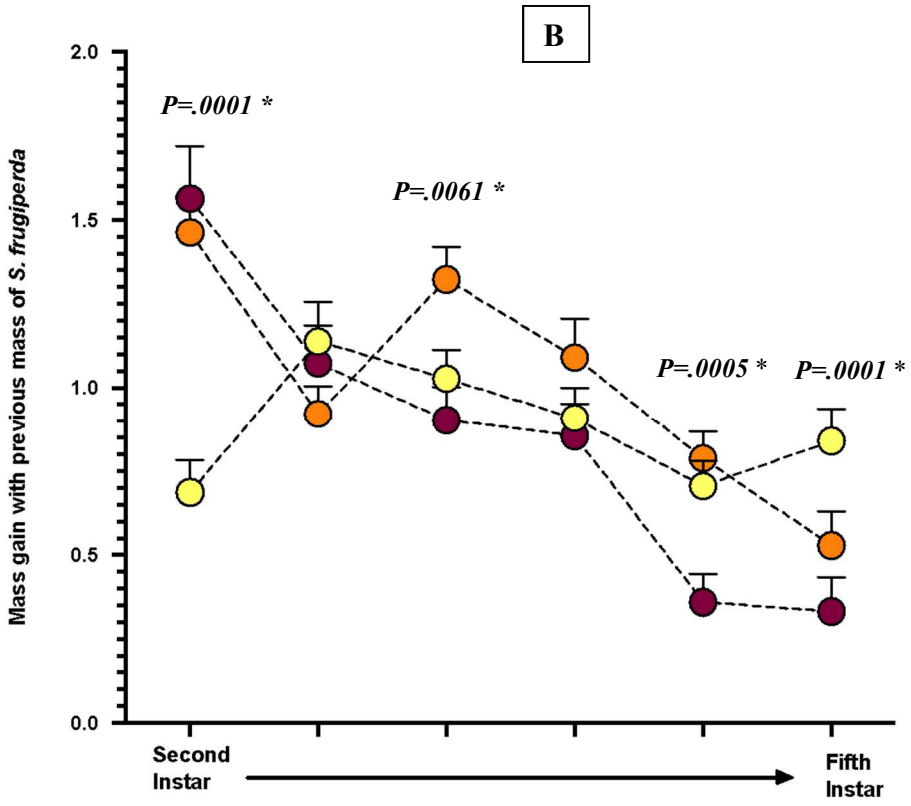
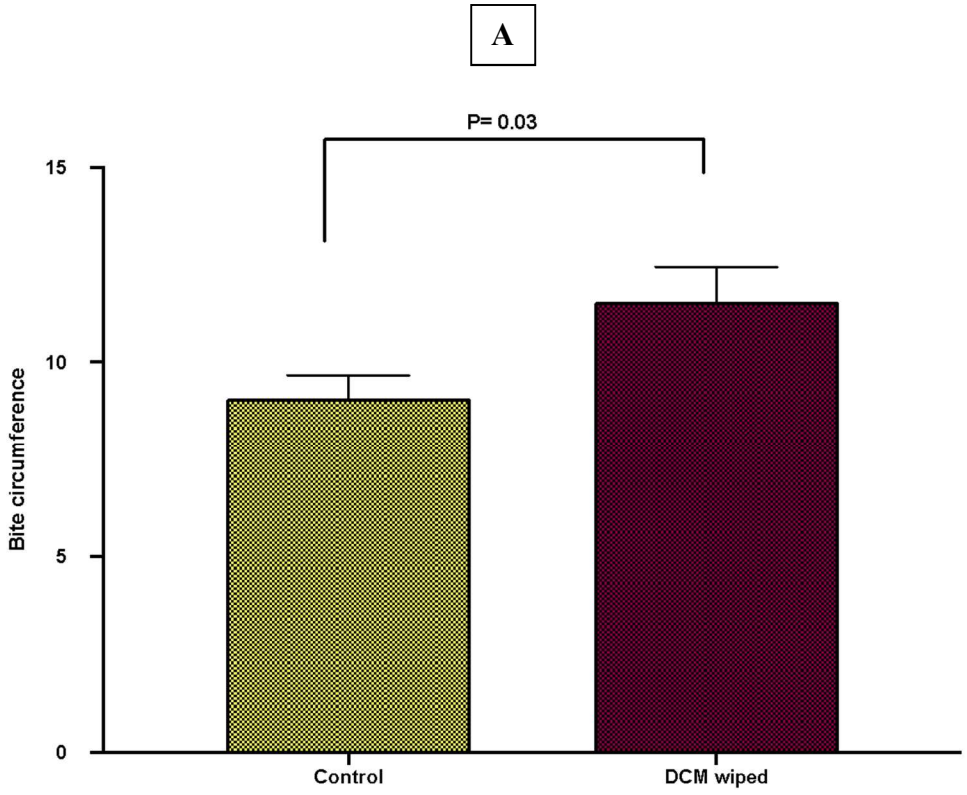


Figure 6. Represents mean mass gain calculated with initial mass (A) and mean mass gain calculated from previous mass (B) of *S. frugiperda*. Mean larval mass gain calculated from initial mass between the different treatments, second through fifth instars (**A**). When calculated from initial mass, all mass gains were significant. Mean larval mass gain calculated from previous mass (**B**). There was a significant difference between the treatments of mass gain 1, between the control with methanol and the control with water. Mass gain 3 also had a significant difference between the treatments, between methanol with water. Mass gain 5 and 6 both had significant differences and both had significant differences between control with methanol, and methanol with water.(Kruskal-Wallis, Dunn's multiple comparison test, $p<0.05$).


A
P= 0.03
15
10
5
0
Bite circumference
Control
DCM wiped

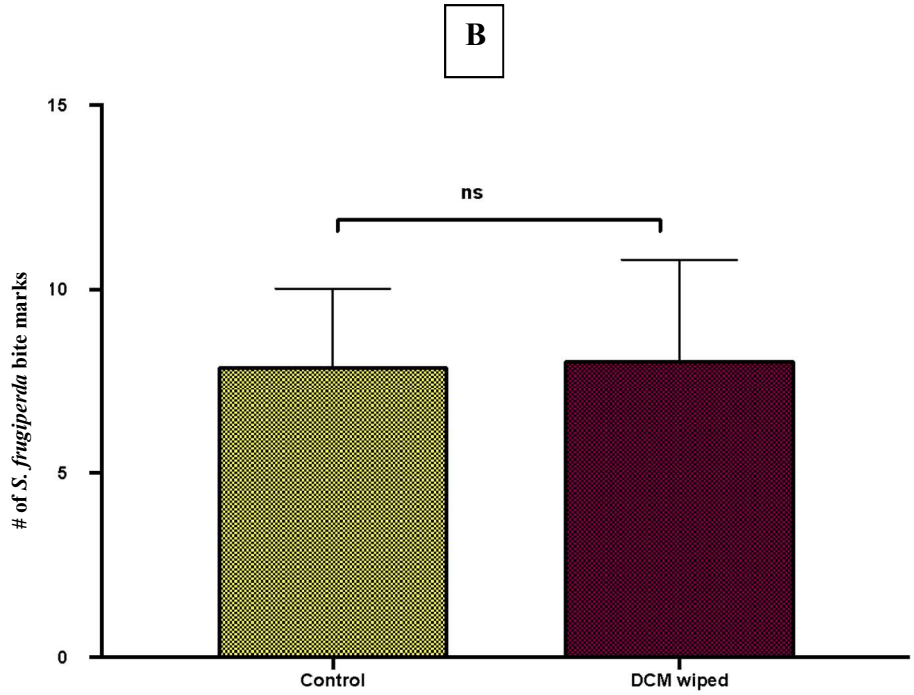


Figure 7. Represents the mean bite mark circumference (A) and the mean number of bites (B) on *Aloe barbadensis* by *S. frugiperda*. The bite circumference was significantly higher on leaves that were chemically stripped of wax than control leaves that had the wax left on **(A).** There was no significant difference between the number of bite marks between the control and the wax stripped leaves however (**B)** (Kruskal-Wallis, Dunn's multiple comparison test, $p<0.05$).

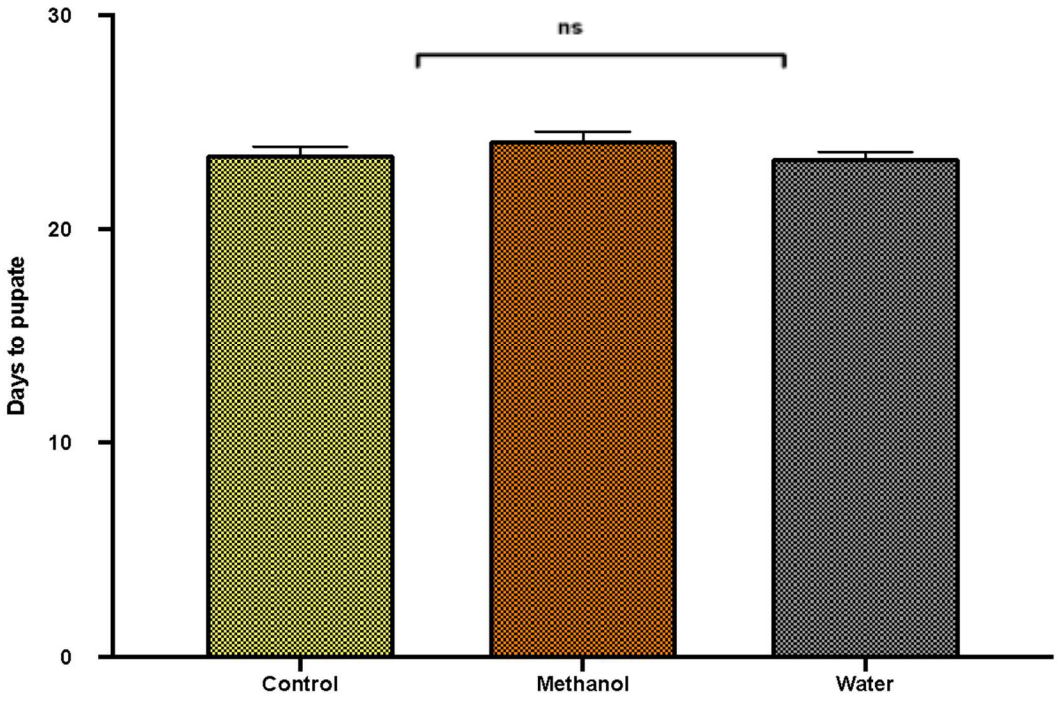


Figure 8. Mean amount of days *S. frugiperda* larvae took to pupate. There was no significance in the amount of days the larvae took to pupate between the treatments (Kruskal-Wallis, Dunn's multiple comparison test, $p<0.05$).

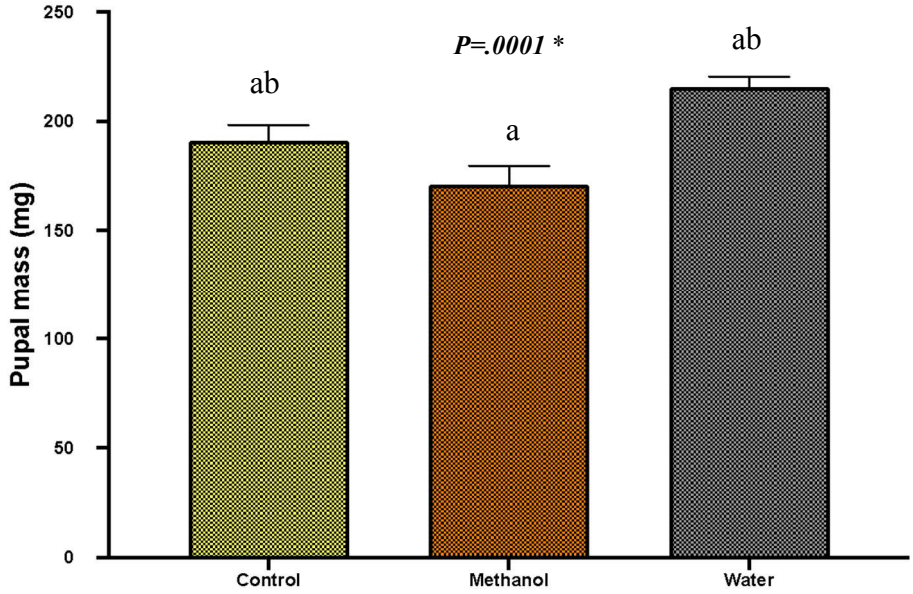


Figure 9. Mean pupal mass per treatment of *S. frugiperda*. There was a significant difference between the treatments on pupal mass (Kruskal-Wallis, Dunn's multiple comparison test, $p<0.05$)

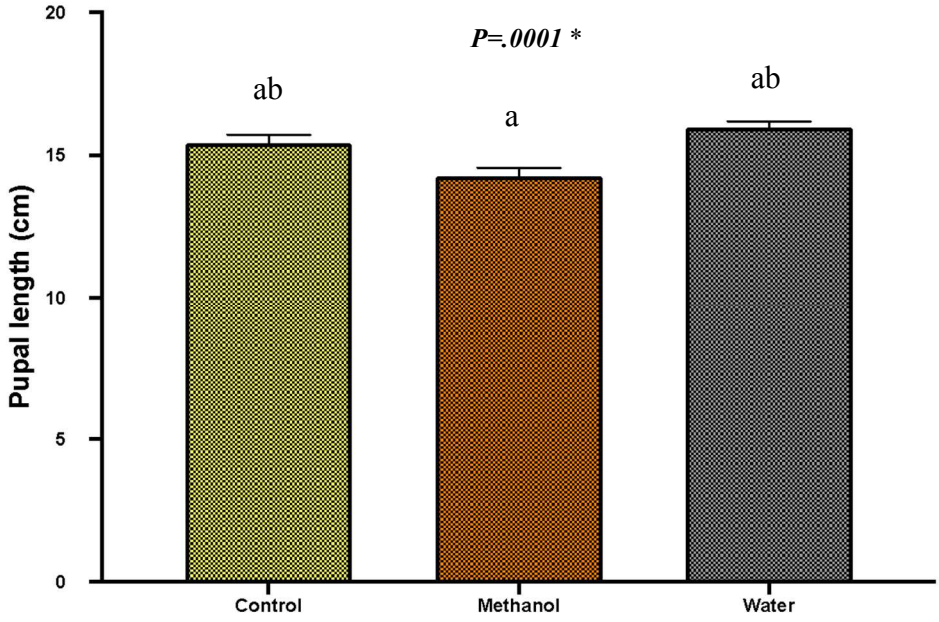


Figure 10. Mean length of *S frugiperda* pupae. There was a significant difference between treatments in regards to the length of the pupae (Kruskal-Wallis, Dunn's multiple comparison test, $p<0.05$).

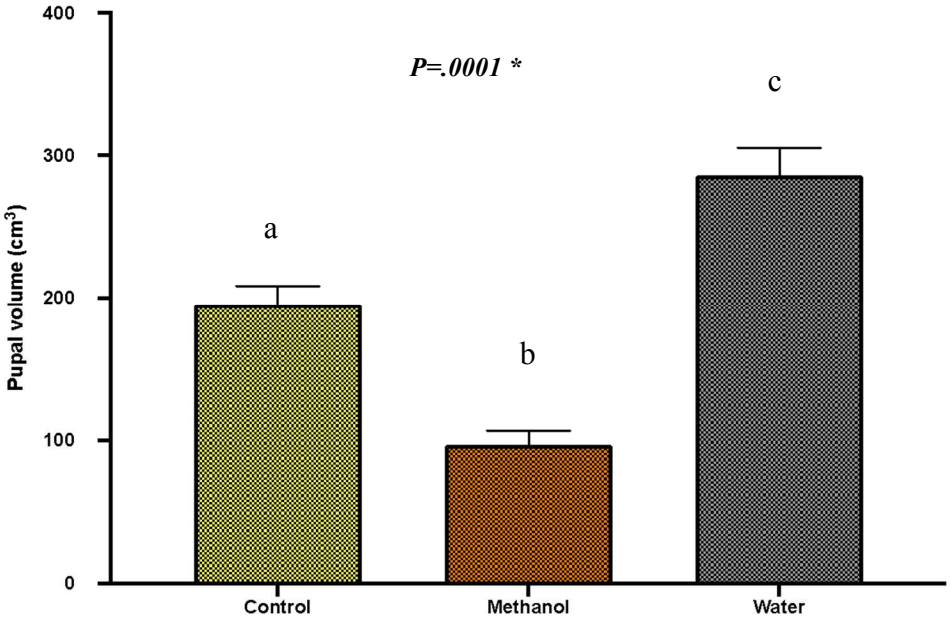


Figure 11. Mean pupal volume of *S. frugiperda*. There was a significant difference between the mean pupal volume among treatments (Kruskal-Wallis, Dunn's multiple comparison test, $p<0.05$)

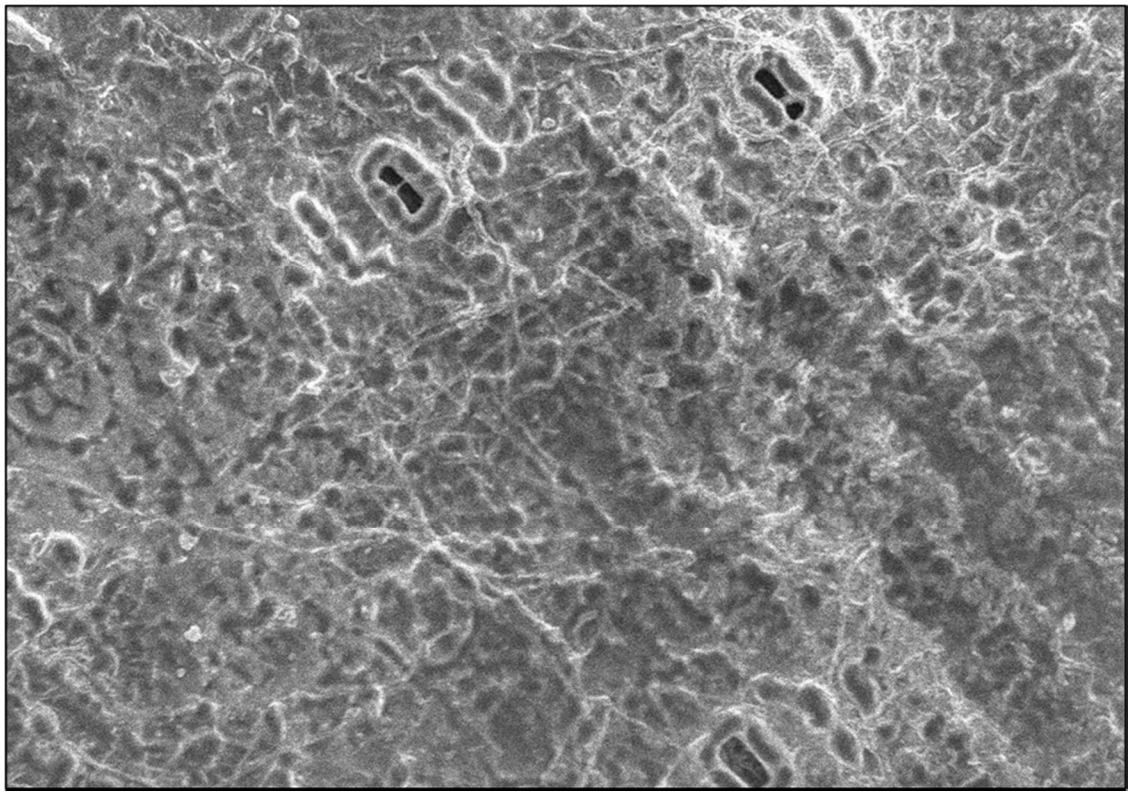


Figure 12. ***A. barbadensis*** **leaf magnified on SEM at 400x.** This image reveals that there are no trichomes on the leaves.

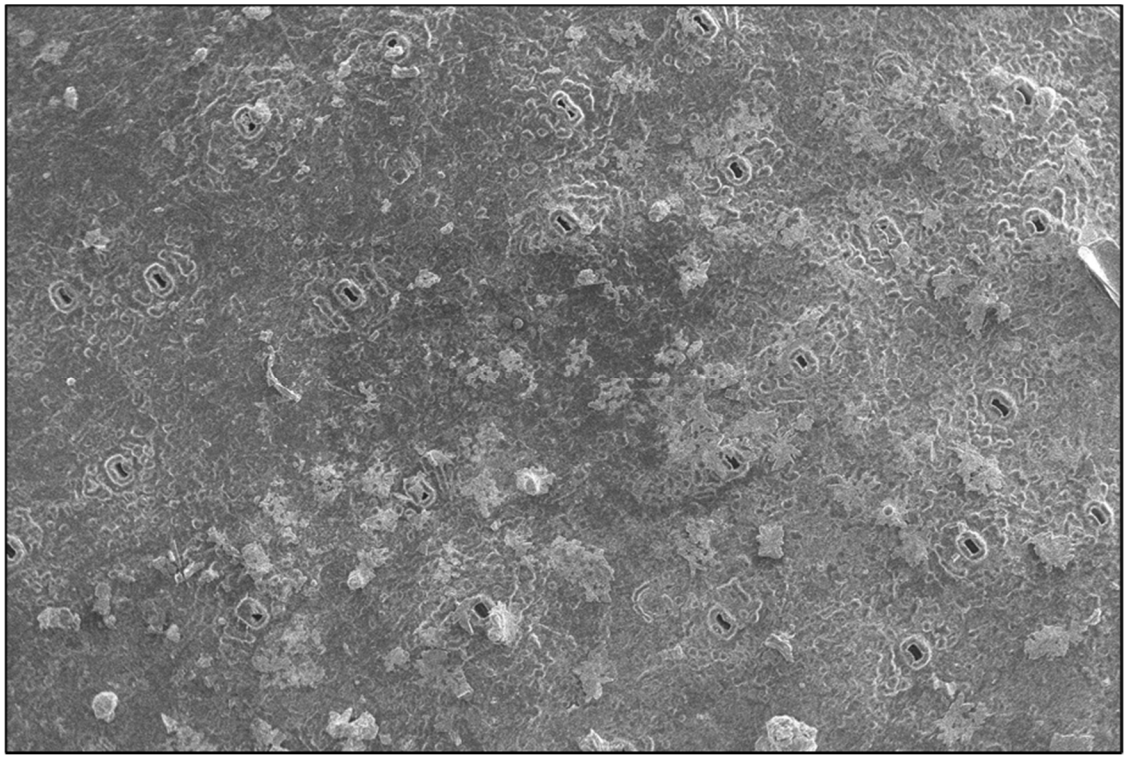


Figure 13. *A. barbadensis* leaf magnified at 120x with wax on. This image reveals the leaf that has wax left on and shows a lack of trichomes

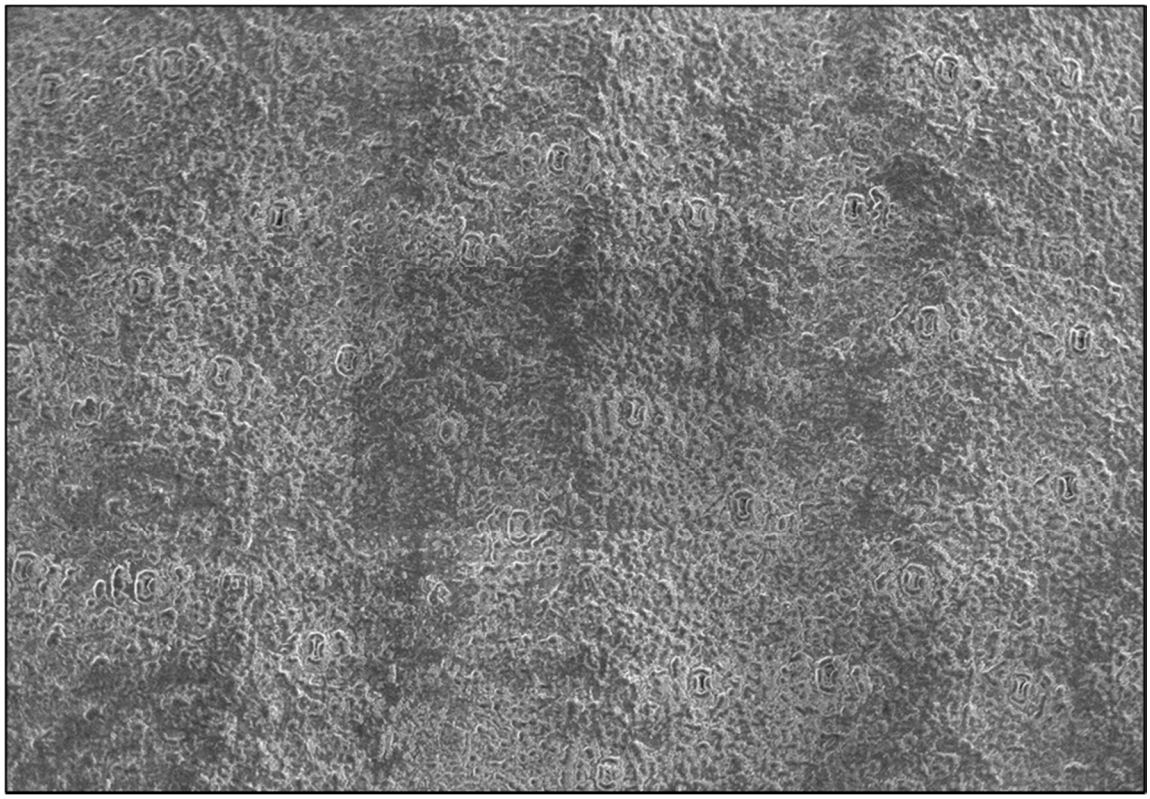


Figure 14. The same *A. barbadensis* leaf sample as above with the wax material removed at 120x. Here we show the same leaf as above when all of the waxy material is removed.

Figure 15. *A. barbadensis* feeding assay. *S. frugiperda* or *M. sexta larvae were* placed on leaves that either had wax removed or wax left on the leaves and left to feed for 48 hours

Figure 16. Bite marks made by *S. frugiperda* during the feeding assay on *A. barbadensi*

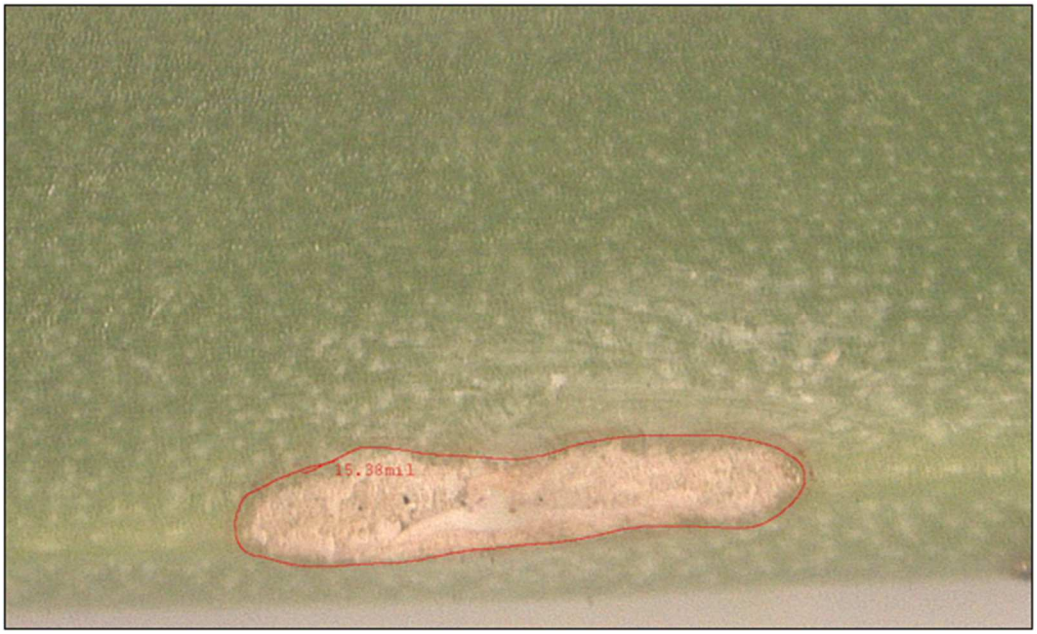


Figure 17. Measurement of *S. frugiperda* feeding mark during feeding assay. This feeding area was measured at 15.38 millimeters

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