THE UTILIZATION OF NATURAL PRODUCTS FOR AGRICULTURAL BENEFITS

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Dedication:

This dissertation is dedicated to my family and friends who have worked tirelessly and unconditionally to support me throughout this arduous process. Thank you, parents, Glennis and Byron, for your love and fighting spirit, putting the burning passion into me to stand up for myself. Thank you, siblings, Nikki and Josh, for being my bedrock, keeping me afloat even as events continued to hit roadblocks and reinforcing to me that family always looks out for each other even when oceans apart. I hope to support you as you have supported me through your own higher education life journeys. Thank you, Papa, Glenn Oura, for showing me the bigger picture and reminding me to find the beauty in all victories, big and small. Lastly, thank you Melinda Ho, for being my sunshine during the darkest times and giving me hope for a brighter tomorrow.

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List of Publications

Published papers

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Ooka, J., Owens, D., *Allelopathy in tropical and subtropical species.* Phytochemistry Reviews, November 2018

Presentations (excludes UH unless awards)

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Ooka, J., Hara, S., Owens, D., *The Search for Herbicidal Natural Products from the Plants of Hawaii.* Poster, WSSA/WSWS 2020, March 2020

Ooka, J., Hara, S., Owens, D., *The Search for Herbicidal Natural Products from the Plants of Hawaii.* Poster, AG HI 2019, October 2019

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- Frank and Mary Loewus Student Travel Award

Ooka, J., Owens, D., *Strawberry Guava as a Source of Natural Herbicides: Utilizing the Chemical Warfare Capacity of an Invasive Species for Human Benefit.* Presentation,

CTAHR Student Research Symposium, April 2019

Ooka, J., Owens, D., *The Search for Novel Herbicidal Natural Products in Strawberry Guava*. Poster, CTAHR and CoE Student Research Symposium, April 2018

Ooka, J., Owens, D., *The Search for Novel Herbicidal Natural Products in Strawberry Guava*. Poster, CTAHR and CoE Student Research Symposium, April 2018

Ooka, J., Dayan, F., Owens, D., *The Search for Novel Herbicidal Natural Products*. Poster, Phytobiomes for Sustainable Agriculture Plant Sciences Symposium, March 2018
- 1st Place Poster Presentation

Ooka, J., Dayan, F., Owens, D., *The Search for Novel Herbicidal Natural Products*. Poster, ASPB Plant Biology 2017, July 2017

Abstract

The invasive species *Psidium cattleianum*, termed strawberry guava, poses a major problem for the State of Hawaii. It outcompetes native plants, with the dominance of strawberry guava potentially due to allelopathy, which is the biochemical inhibition of one plant by another. To isolate potential allelochemicals, water extracts from both varieties of strawberry guava (red and yellow), were tested and found to reduce dicot and monocot growth. The yellow variety of strawberry guava produced an Inhibition constant of 50% lethality (IC_{50}) of 10.74mg/mL for lettuce and 12.2 mg/mL for green onion, while the red variety produced a IC_{50} of 9.57mg/mL for lettuce and 6.54 mg/mL for green onion. In soil trials, it has been found that *Psidium cattleianum* leaf chemicals reduced the germination rate of the monocot (Echinochloa sp.) more relative to the dicot (*Amaranthus retroflexus*). High performance liquid chromatography bioassay mediation fractionation and tandem mass spectroscopy found that the phenolic acid, gallic acid, is a constituent for *Psidium cattleianum's* allelopathy. Additionally, *in-silica* molecular docking was conducted with the enzymatic molecular target site, hydroxyphenylpyruvate dioxygenase, to create a predictive model. The trends showed that, based upon 2-acyl-cyclohexane-1,3-diones with simple aliphatic side chains, the perfect R_1 tail length is an undecyl 11-C. Too short a tail did not fully utilize the binding pocket, while too long of a tail did not fit within the binding pocket properly, showcased by having a more positive binding energy and a less efficient IC_{50} value. This class, along with cyclohexane-1,3-diones with phenyl side chains or phenylene side chains also followed another trend, where the addition of an R_2 methyl group again made a more positive binding energy and a less efficient IC_{50} value, possibly due to sterics. This showed proof of concept that it is possible to correlate binding energy and measured IC_{50} values to create this predictive model that can be adapted to other molecular target sites and ligands. In addition,

while only strawberry guava was investigated within the course of this work, there remains multiple suspected allelopathic species than can be further analyzed following strawberry guava's path as a blueprint for future studies.

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List of Abbreviations

ATP, Adenosine Triphosphate; GA, Gallic Acid; HPPD, Hydroxyphenylpyruvate Dehydrogenase; HPLC, High Performance Liquid Chromatography; IC_{50}, Inhibition Constant 50; LC/MS, Liquid Chromatography/Mass Spectrometry; MS/MS, Tandem Mass Spectrometry; QTOF, Quadrupole time-of-flight; US, United States.

Chapter 1. Literature Review

Plants are living beings that can produce ATP from sunlight via photosynthesis while accumulating a distinctive complement of natural products and other metabolites. They are also unable to physically run or fight when faced with confrontation, instead having developed a multitude of alternative methods to engage their opponents in an endless quest for sunlight, water and other essential elements. One of these approaches is allelopathy, sometimes informally referred to as plant chemical warfare. Allelopathy is the ability to produce metabolites that disrupt the germination, growth, and reproduction of other organisms. It was first studied with modern techniques in Austria in 1937 by Hans Molisch, who focused on negative plant-on-plant effects (Willis et al., 2007). Since these initial studies, the definition of allelopathy has developed to a more global scale including not only land plants, but also ocean corals, seaweed and algae. Growing strategies utilizing allelopathic plants for weed suppression and pest control (such as intercropping and borders) have grown popular as a result of the public outcry over the amount and varieties of synthetically derived chemicals being used for pest control on the food they consume (C and P, 2015). While allelopathy-based strategies were traditionally used by indigenous groups and early settlers, much of modern-day research has focused on synthetic pesticidal compounds. Another aspect of allelopathy is its use in the description of the strategies for how invasive species successfully colonize and dominate foreign ecosystems (Sangakkara et al., 2008). Allelopathic chemicals are often produced in the leaves (Keating, 1999), but can also be produced in stems, roots and seeds. Due to the varying modes of action and chemical composition of the allelopathic complement and the morphological and physiological difference between plants, what affects a plant in one soil type may or may not affect another plant in a different soil type (Kaur et al., 2014; Sodaeizadeh et al., 2009). In addition to having detrimental effects on plants, allelopathic chemicals can also inhibit nematode reproduction (El-Rokiek et al., 2012). While one goal of weed science-based herbicide discovery studies is to determine the chemical compound or compounds that cause or result in allelopathy, potentially utilizing them for agricultural usage, research is a multistage process that requires a variety of approaches in identifying, characterizing, and developing the usage of active compounds. From towering trees to the weeds beneath our feet to those that live beneath the sea, allelopathic organisms come in a

variety of shapes and forms, with many having the potential for human utilization in various capacities.

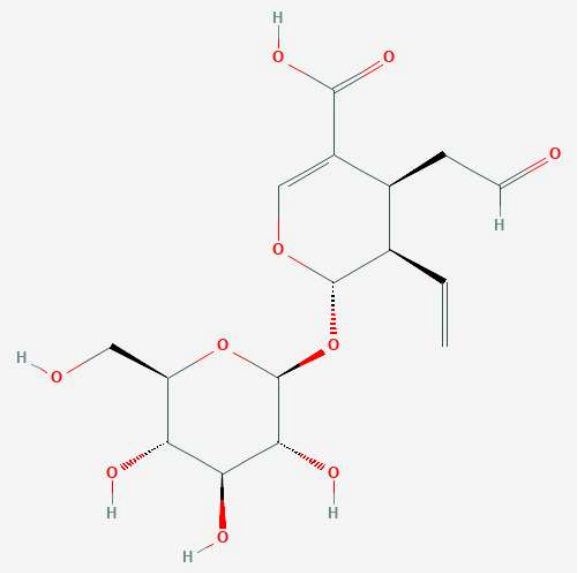


Figure 1. Secologanic acid, an allelochemical of *Sphenoclea zeylanica* (chicken spike) (Hirai et al. 2000). National Center for Biotechnology Information (2021). PubChem Substance Record for SID 274057467, 22864-93-3, Source: Japan Chemical Substance Dictionary (Nikkaji).

1.1 Allelopathy in Land Plants

1.1.1 Herbaceous Shrubs

Many of the weeds that agriculturists struggle to combat in their fields and plots today are not native to the surrounding land. Those that are the most aggressive often produce allelopathic chemicals that can harm commercial production of crops and native species. This aggression is often compounded by the presence of herbicide resistance and/or phenotypical avoidance. Humans have artificially selected for evolution of resistance to various herbicides by applying the same selective pressures time and time again, and not taking into account integrated pest management strategies, resulting in weeds that have a greater fitness (Norsworthy, 2012). Herbicide resistance, a trait where a plant can survive application of a herbicide that would otherwise kill a non-resistant member of the same species, currently exists globally in over 200 different species for a variety of herbicides, including 2,4-D, glyphosate, and atrazine (Heap, 2011). For example, rice, a crop cultivated worldwide, has been under attack by allelopathic and herbicide resistant weeds, often costing farmers millions of dollars in lost yields. *Sphenoclea zeylanica* (chicken spike), is native to Africa but plagues rice paddies in Asia and America. It has a life cycle that parallels rice's own and is resistant to the commonly used herbicide 2,4-D (Esterno) (Hirai et al., 2000). This combination makes it almost impossible to eliminate before and during the growing season except by costly manual removal. In addition, its 9 allelochemicals (isomers of cyclic thiosulfinate, secologanic acid (Figure 1), and secologanicside) depress other weeds and inhibit the growth of rice seedlings, making each additional growing season the weed is not addressed less productive and more costly to remedy.

Parthenium hysterophorus (famine weed) is an herbaceous weed native to Mexico but has now spread widely impacting farmers around the world. This weed has several properties that make it an ideal invader, including its highly regulated uptake of nutrients and water,

production of 15,000-28,000 small, light seeds that can lay dormant for up to 2 years, and the plant's potent allelopathy. In addition to agricultural fields, this weed can also grow in salty and dry conditions, often overtaking natural boundaries that normally inhibit weed proliferation (Tanveer et al., 2015). By releasing phenolics into the soil and reducing nitrogen levels, germination and growth rates of crop plants (such as major cereals, pulses, oilseed crops, forage crops, and assorted vegetables) suffer a reduced yield of up to 40-90%. Afflicted trees (such as *Acacia leucophloea, Casuarina equisetifolia, Eucalyptus tereticornis*, etc) and even other weeds (*Najas graminea, Eleusine coracana, Vigna sinensis,* etc.) also experienced reduced germination and growth rates, some by aqueous extraction concentrations of as little as 6% (Narwal, 2003; Swaminathan et al., 1990). Somewhat surprisingly, some 20 other plants have been identified that themselves have allelopathic activity against famine weed, including crop plants such as *Oryza sativa* (Tanveer et al., 2015). While untested, this could provide potential for alternative herbicide development or intercropping strategies which can be used to combat famine weed.

Echinochloa colona (jungle rice), originated in Asia but can now be found worldwide. It is considered one of the most widespread and damaging weeds among 60 countries, including the US and Australia, in agricultural crops such as cotton, cassava, maize, peanut, rice, sorghum, soybean and sugarcane. Jungle rice has the same appearance as rice in the early stages of planting and is a host of a variety of crop diseases (Zhang et al., 1996). The use of herbicides, including the broad-spectrum and widely used glyphosate (e.g. Roundup), has been occurring in greater concentrations every growing season because the weeds have become more resistant to initial application concentrations (Peerzada et al., 2016). Biological control with the introduction of fungus or disease (Peerzada et al., 2016) as well as control utilizing other allelopathic plants such as sorghum, rice and mango either directly (intercropping) or indirectly (mulching or leaf detritus layering) have proven successful (Peerzada et al., 2016). This suggests that allelopathy may be most effective when used in combination with other techniques such as traditional herbicide treatment, hand-based weeding, and natural crop plant-based defenses against even the most aggressive weeds.

1.1.2 Ferns

Ferns are often the first pioneer plants to reinvigorate burned or newly formed land such as those found on volcanic soil. However, it is not only their unique physiology including creeping weather durable rhizomes that allow them to be such good colonizers. Several ferns have allelopathic activity that allows them to immigrate and maintain control of nutrient rich soil (Russell et al., 1998). For example, within Hawaii, there exists the fern *Dicranopteris linearis*, known as "uluhe" in the Hawaiian language. This fern can often be found in large patches of pure colonies. Two weeds often found in the same environment as uluhe are *Echinochloa colona* and *Avena fatua* (wild oat). Wild oat can often be found plaguing wheat fields, where this weed can reduce the yield of the crop plants by up to 80% (Sharma, 2017). It was found that within the soil surrounding uluhe, the aqueous allelochemical cinnamtannin B-1 (Figure 2) was in concentrations 20x greater than those required for 50% growth inhibition of *Echinochloa colona* and *Avena fatua* (Kato-Noguchi et al., 2012). This concentration was deadly to both weeds and provided a plausible explanation for the aggressive expansion and dominance of uluhe in tropical environments.

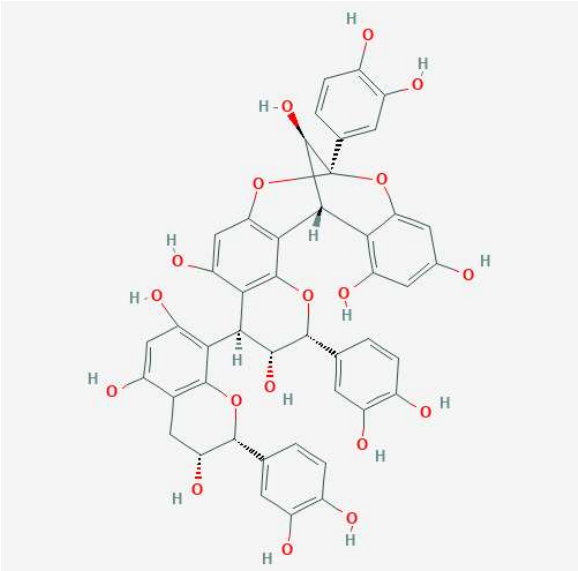


Figure 2. Cinnamtannin B-1, an allelochemical of fern *Dicranopteris linearis*, known as "uluhe" in the Hawaiian language (Kato-Noguchi et al. 2012). National Center for Biotechnology Information (2021). PubChem Compound Summary for CID 475277, Cinnamtannin B1.

Another fern forming large monospecific colonies is *Gleichenia japonica* (urajiro). Urajiro is found across multiple areas of Asia where it is the dominant fern in the region. Aqueous methanol extractions of the leaf were found to contain two novel compounds (3-O-B-allopyranosyl-13-O-B-fucopyranosyl-3B-hydroxymanool and 18-O-a-l-rhamnopyranosyl-(1→2)-B-D-glucopyranosyl-13-epitorreferol) that inhibited the growth of garden cress (*Lepidium sativum*), lettuce (*Lactuca sativa*), ryegrass (*Lolium multiflorum*) and timothy (*Phleum pratense*). In addition, soil levels of these two compounds were found to be almost double what was required for 50% growth inhibition of the test species, showing how the colonies established their presence within the soil. (Kato-Noguchi, 2013). *Gleichenia japonica* can also be found

growing alongside *Dicranopteris linearis*, where the presence of one or both species, shown as allelopathic, have resulted in reduced biodiversity when compared to non-fern-dominated or intermediate type secondary forests of Miyajima Island in southwest Japan (Kuroda et al., 2006).

1.1.3 Trees

It is often assumed that the most damaging and invasive plants are weeds or shrubs that are small in stature, have rapid growth rates, and can produce a bountiful amount of seeds in a short amount of time. However, the significance of invasive trees is often overlooked. *Ailanthus altissima* (tree of heaven) is native to China and Taiwan and was initially established in North America and Canada for its use as a road median foliage, but has since become an invasive species that has spread widely, bringing with it other invasive species such as the spotted lantern fly. The properties of having the ability to grow in dry, nutrient-deficient soil, being able to produce 300,000 seeds that can travel up to 200m by wind power alone and still maintain almost 100% germination rates, and its allelopathic arsenal that can be delivered from its roots, leaves or bark, have allowed this tree to form dominant colonies in the most unexpected places (Small et al., 2010). Allelochemical experiments of this tree have been found to reduce seed germination and growth rates of 10 species of woody angiosperms, 35 species of gymnosperms, and 7 crop and weed plants (Lawrence et al., 1991). In a native vs invasive weed species case study, the allelochemicals were tested against Southeast America native *Verbesina occidentalis* (yellow crownbeard) and invasive *Dipsacus fullonum* (wild teasel) (Small et al., 2010). Yellow crownbeard is a helpful weed that can often be found in hayfields and is used in home gardens. Its yellow flowers attract soldier beetles, a type of insect that preys upon other insects that can be harmful to vegetable plants. Introduced from Europe, wild teasel heads were once used in the textile industry. However, since the industry's decline, it has become an invasive species, crowding out native species. The study found that, while soil from around trees of heaven were found to inhibit native yellow crownbeard seed germination rates, seedling length, and leaf production, the invasive wild teasel was not affected (Small et al., 2010). This demonstrates an example of different responses to allelochemical exposure, where some plants may have effective defense mechanisms due to similar competition in their region of origin while other plants are unaffected.

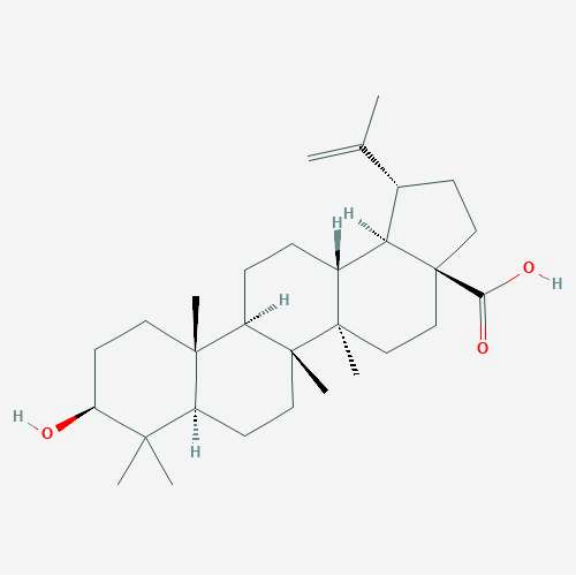


Figure 3. Betulinic acid, an allelochemical of *Casuarina equisetifolia* (Australian pine tree) (Long, F., et al 2018). National Center for Biotechnology Information (2021). PubChem Compound Summary for CID 64971, Betulinic acid.

A common tree in Hawaii is the *Casuarina equisetifolia* (Australian pine tree), one of the many trees grouped into the general category of "ironwood". This tree produces a plentiful amount of leaf litter (recorded at over 1 cm deep) and has been a known threat to native forests for multiple generations. It displaces species with important cultural value to Native Hawaiians such as *Chrysodracon halapepe* ("hala pepe" in the Hawaiian language) and *Schenkia sebaeoides* ("'Āwiwi" in the Hawaiian language) from their traditional range ("Hawaii Ecosystems at Risk Project. 'Ironwood, Australian pine' bulletin," 1999). It has been shown that the Australian pine tree creates areas of reduced biodiversity underneath its canopy due to the rich complement in phenolics of its leaf leachates (Batish et al., 2001). A fresh leaf litter leachate solution of 2% was able to reduce the germination rate of *Medicago sativa* (alfalfa) by 32.5% and *Ageratum conyzoides* (chickweed) by 68.8% (Batish et al., 2001). In addition to inhibiting other species, it was also found that this tree inhibits the growth and germination of its own seedlings, ensuring its own survival. It was found that five chemicals (12, 13-dihydromicromeric acid, betulinic acid (Figure 3), 3-Ocaffeoyl lupeol, catechins and epicatechin) extracted from the roots were able to inhibit *Casuarina equisetifolia* germination and young plant growth, with 12, 13-dihydromicromeric acid being the most inhibitory (Long, 2018). This consistent application of leeching into the ground creates an area of inhibition where only established *Casuarina equisetifolia* can continue growing, forcing young *Casuarina equisetifolia* to create their own area of inhibition and resulting in their prolific and dominant groves across the islands.

1.1.4 Epiphytes/Vines

An epiphyte is defined as a non-parasitic plant that grows on another plant that does not need a root connection to soil to survive (Benzing, 2012). Their ability to grow in nontraditional places (on treetops, along bushes, on telephone poles, etc) makes them difficult to control with

traditional pesticides without killing everything around them. Some vines also have a chemical arsenal that enables them to integrate into an area already full of vegetation. The epiphyte *Mikania micrantha* (bitter vine) is native to Central and South America, but in recent years has invaded and caused the degradation of multiple protected forests in China, including a 10% total smothering and death of co-existing species in Shenzhen (Zhang, 2004). Aqueous leaf extracts and volatiles of the vine inhibit the growth of *Raphanus sativus* L*, Lactuca sativa, Chrysanthemum coronarium,* and *Abutilon theophrasti* by up to 35% and have been shown to have a unique affect upon soil nutrients (Chen et al., 2009; Ma, 2021). Additions of this leaf extract to topsoil have been shown to increase carbon, NO_3^- and NH_4^+ levels, suggesting that the invasive species facilitates enriching its own soil for further expansion (Chen et al., 2009). It also has the potential to be used as an alternative herbicide to clear fields and enrich the soil, reducing the need for crop rotations and resulting in off season nitrogen fixation by causing complete elimination of the existing vegetation. Landscapes smothered by *Mikania micrantha* can be 90% cleared by hand removal with occasional upkeeping during the growing season or the utilization of herbicides including glyphosate, 2,4-D and atrazine for a blanket clearing, although planting with traditional ground cover is recommended (Zhang, 2004).

Vines are also climbing plants, but they continue to maintain their connection to the soil. They are popular in the landscaping community for their properties of large flowers and vertical growth patterns. One such vine is *Ipomoea cairica* (Cairo morning glory), a canoe plant (a plant brought by ancient Polynesians to Hawaii for its use as a food) (Chock, 1968). Part of the morning glory family, *Ipomoea cairica* produces large white/lavender flowers and has been found to contain allelopathic chemicals within its leaves. Leaf extracts were able to inhibit the germination and growth of crop weeds *Echinochloa crus-galli (L.) Beauv., Euphorbia heterophylla L*. and *Ipomoea*

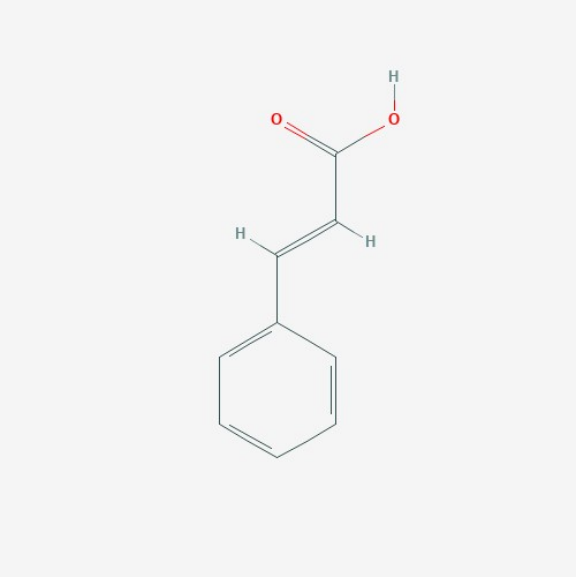


Figure 4. Cinnamic acid, an allelochemical of *Ipomoea cairica* (Cairo morning glory) and *Mikania micrantha* (bitter vine) (Ma et al 2020). National Center for Biotechnology Information (2021). PubChem Compound Summary for CID 444539, Cinnamic acid.

grandifolia (Takao et al., 2011). Both *Mikania micrantha* and *Ipomoea cairica* can be found in the state of Hawaii and on other tropical islands in the pacific. A comparison of their inhibitory compounds on *Chrysanthemum coronarium* (garland chrysanthemum) found that two main constituents in the plant leaf extracts [cinnamic acid (Figure 4) and benzoic acid], while in different quantities in each plant, were the main inhibitors of growth and resultant reduced chlorophyll content (Ma, 2021). This shows how two invasive vines can overtake the same area with a different mixture of the same chemicals and achieve dominance of the environment.

1.2 Allelopathy in the Ocean

The sea is full of sessile creatures that cannot physically run or fight when danger approaches, similar to the situation discussed with plants in section 1.1. As a result, many have developed chemical-based methods including various toxins, deterrents, and inhibitors, in order to compete for resources and survive. Predation and competition for space are two of the most impactful forces determining the composition and distribution of coral reef communities. For these reasons, many secondary metabolites isolated from sponges, seaweeds, and algae often have anti-predation or anti-encroachment uses, with some metabolites functioning in multiple roles (Bonaldo and Hay, 2014). Due to the constant movement of the ocean occurring because of the tides, allelochemicals cannot be released in constant concentrations directly into the environment as is common in plant-based allelopathy, where they can be released through roots or leaf litter/detritus. Rather, they are distributed as either being water-born or through physical contact, with both manners being in response to environmental signals. However, just like on land, ocean allelopathy is dependent upon a variety of factors including predation, touch and nutrient availability to name a few, resulting in many different interactions between species (Bonaldo and Hay, 2014).

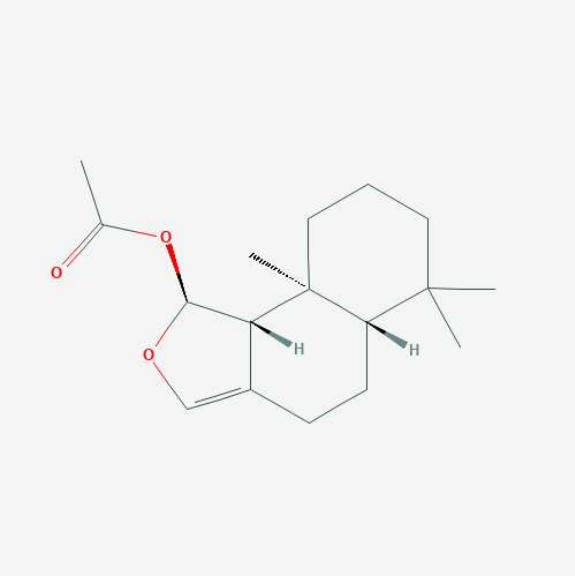


Figure 5. 7-deacetoxyolepupuane, an allelochemical of the sponge *Dysidea sp.* (Thacker et al. 1998). National Center for Biotechnology Information (2021). PubChem Compound Summary for CID 126180, 7-Deacetoxyolepupuane.

One example of ocean allelopathy is the interaction between two sponges *Dysidea* sp. (order Dictyoceratida, family Dysideidae) and *Cacospongia* sp. (order Dictyoceratida, family Thorectidae). Both sponges are found within the tropics often in close proximity to each other, where it was observed that *Dysidea* is taking over areas covered by *Cacospongia* in a one-sided offensive. This is surprising because, although *Cacospongia* has two terpenoid toxins called scalaradial and desacetylscalaradial that allow it to defend itself from other invaders, they do not appear to be alleopathic against *Dysidea*, even when under increased concentrations by stressed *Cacospongia.* Along with crude chemical extracts

from *Dysidea*, it was found that a specific chemical that is utilized for anti-predation, 7-deacetoxyolepupuane (Figure 5), also causes necrosis of target *Cacospongia*. However, since *Dysidea* is not induced to produce this chemical in physical contact with *Cacospongia*, its evolutionary purpose may be elsewhere, as lab tests have also shown it provides antimicrobial properties (Thacker et al., 1998). In the ocean, where allelopathy first evolved, its function of aggression or defense requires multi-use chemical, resulting in complex tradeoffs and shifting antagonistic/mutualistic relationships between surrounding species.

Algal blooms produce some of the deadliest natural products, often impacting fisheries and other ocean-based businesses causing the US government to invest $79 million over the 2012-2016 legislative session to combat and provide relief for such occurrences (Congress, 2014). *Alexandrium tamarense* is a dinoflagellate that produces paralytic shellfish poisoning (PSP) toxins, neurotoxins (including saxitoxin or STX) that are filtered out of the water by shellfish and, if consumed by humans, can cause nervous system damage by blocking sodium ion channels of the Na_v subtype, inhibiting action potentials (Thottumkara et al., 2014). *Prorocentrum donghaiense*, *Chattonella marina,* and *Heterosigma akashiwo* are three species that constitute the red tides that can appear suddenly, destroying entire coastal collections of fish, and then disappear suddenly. However, while these algae produce some of the oceans most destructive biochemicals, they themselves are not resistant to each other's chemical complement of toxins. *A. tamarense* successfully inhibited the growth rates and completely killed *H. akashiwo* and *C. marina*, but proved to be able to have a co-existence with *P. donghaiense* at certain levels. Filtrate (representing chemicals washed off of *A. tamarense*) also proved to inhibit the growth of all 3 algae, with chemical levels corresponding to the levels of PSP toxins being produced concurrently (Yin et al., 2010). Even within the same region, different blooms of the same species can hold different concentrations of

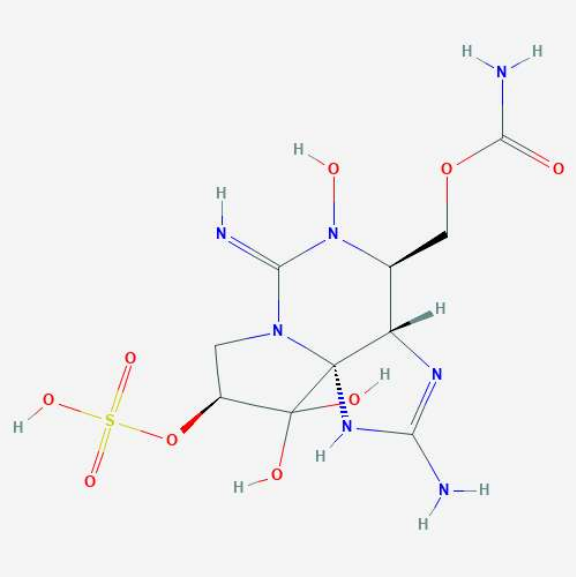


Figure 6. GTX4 (Gonyautoxin-4), a paralytic shellfish poisoning (PSP) neurotoxin produced by the dinoflagellate *Alexandrium tamarense* (Ichimi et al 2002). National Center for Biotechnology Information (2021). PubChem Substance Record for SID 440699577, Source: NORMAN Suspect List Exchange.

PSP toxins and their analogs (ex. C2 vs GTX4 (Figure 6) inverse ratios in northern Japan), indicating that a third factor, including nutrient availability or the other members of the surrounding planktonic population (Ichimi et al., 2002). This shows how red tide alga become dominant in nutrient rich areas and how competition between algae is strongly mediated by allelopathy.

Lobophora variegate inhabits reefs in the tropics from shallow waters down to mesophotic depths (30-150m) and is considered critical in macroalgal phase shifts. This occurs when reefs previously dominated by coral and sponges are suddenly overtaken by algae. For example, in the Bahamas and "the Caribbean", algal composition of reefs have increased to greater than 50% following the invasion of the carnivorous fish *Pterois volitans*, otherwise known as the lion fish. These fish prey upon herbivores that typically control brown algae levels. While the loss of herbivores would perhaps eventually induce a phase shift, the observed rapidness of the transition is likely a result of physical contact allelopathy expressed by brown algae (Slattery and Lesser, 2014). The coral *Montastraea cavernosa* (great star coral) and the sponge *Agelas clathrodes* (orange elephant ear sponge) are beneficial members of a healthy tropical reef community that are also affected negatively by brown algae's allelopathic effects. As reef communities are further dominated by invasive species and macroalgal populations, there are clear adverse effects on reef health. Therefore, allelopathic interactions between seaweed, coral, and sponges are critical components to be considered in the maintenance of reef health.

Another problematic seaweed in the tropics is the chemically rich red seaweed *Galaxaura filamentosa*. It contains within its chemical arsenal an anti-herbivore compound that makes them unappetizing to grazing fish (Rasher and Hay, 2010). Commonly seen on the same degraded reefs (reefs overrun with seaweed) as the red seaweed is the brown seaweed *Sargassum polycystum*, which is usually enjoyed by grazing fish as this seaweed has no particular chemical defense against herbivores (Rasher and Hay, 2014). One of the corals that make up healthy reefs being encroached upon by the red and brown seaweeds is the coral *Porites cylindrical*, common name hump-coral, whose home to a variety of sea animals. While *S. polycystum* saw no change in grazing and had no display of allelopathy by being in contact with *P. cylindrical*, when *G. filamentosa* was placed in physical contact with *P. cylindrical*, *G. filamentosa* produced allelopathic chemicals that caused reduced growth and death of *P. cylindrical*. However, due to

the nutrient and energy cost of producing the allelopathic chemical, the seaweed was forced to reduce its chemical defense against herbivores and experienced increased levels of grazing (Rasher and Hay, 2014). This is an example of seaweed-herbivore-coral interaction, where secondary metabolites did not have overlapping functions and the cost of aggression was reduced defense against an aggressor. While this project does not directly touch upon allelopathy found in the ocean, it is important to highlight the diversity of allelopathy and how multidimensional the interactions between different species can be in the realms of chemical interactions from competing species, herbivory, environmental abiotic factors, and unexpected interactions from a unconsidered third party.

1.3 Allelopathy for Weed Control and Management

1.3.1 Weed control in Crops

The continued rise of herbicide resistant weeds has and continues to cost farmers an increasing amount of production cost per hectare, with increases ranging from $28 to $64, cutting into profits as they struggle with the currently available weed management methodologies and using herbicides with modes of action that are slowly losing their effectiveness (Norsworthy, 2012). As a result, rather than adopting best management practices including mulching (plant detritus + soil), intercropping (alternating rows of crop plants with anti-weed or anti-insect plants), or homeland security planting (surrounding crop plant fields with anti-weed or anti-insect plants in a barrier), many farmers have cemented their dependence upon herbicides for weed control, causing the general public and surrounding farmers to become concerned with the environmental impact of such high chemical usage on local ground water and soil quality (Gianessi, 2013). Additionally, many traditional non-chemical methods of weed control are not able to be efficiently scaled for the modern farm or have problematic consequences themselves. For example, while hand weeding is one of the most environmentally favorable weed management techniques, it is not economically feasible to modern farmers who care for many acres of land often with minimal staffing due to economic considerations. Additionally, mechanical solutions such as tilling have come under fire in developing countries for contributing to global warming, where purchasing a petroleum burning green house gas emitting tractor is no longer an environmentally friendly solution (Gianessi, 2013; Komatsuzaki and Ohta, 2007). Alternative means of weed control, such as utilizing allelopathy, continue to be a growing field with potential sources of alternative weed control being less of a focus than conventional chemical herbicides in current pesticide research. However, there are a number of current reports in the research that show promising findings on small farms and during environmental restoration efforts that have the potential to have impacts on the future of weed control in agriculture.

Argemone mexicana (Mexican poppy) is a native to Central America that is slightly toxic to grazing animals. Due to its hardy and drought tolerant nature, it is often the first and longest lasting weed in Mexico found by roadsides. Aqueous soluble leaf extract of Mexican poppy was tested upon tomato, an important commercial crop with Mexico City alone producing 2.9 million

tons annually (U.S.D.A., 2016), in an attempt to control the *Meloidogyne javanica*, a nematode that impacts tomato production with losses ranging from 25-100% depending on the tomato cultivar (Seid, 2015). Composting done with 10g of Mexican Poppy per kg of soil reduced *M. javanica* population levels by 30% while increasing tomato plant height by 18%. A 50% aqueous shoot extract promoted tomato plant growth 21% and reduced the levels of *M. javanica* 13%. However, a 100% aqueous shoot extract reduced the tomato plant growth by 14% (Shaukat, 2002). In trials with 50g of Mexican poppy per kg of soil, mortality of the tomato plant was 80%, indicating that this weed also has allelopathic affects. Mexican poppy, which can be easily apprehended, harvested in great numbers, and grown at little cost, has the potential to provide a potential alternative nematicide for tomato plants infested with *M. javanica* and facing competition from other weeds.

A vine whose fruit and leaves have become more popular and can now be found in grocery stores and in medicinal cabinets is called *Passiflora edulis*, common name passion fruit. Besides producing nutrient rich fruit, this native to Brazil has many chemicals containing medical properties found in its leaves, including as a sedative, diuretic, and a tonic to treat hypertension, menopause, and various skin diseases. In focusing on its agricultural uses, aqueous leaf extracts of this plant were successfully able to reduce patty weeds (one of which was *Monochoria vaginalis*) by 70% and subsequently increase rice yield by 30% (Khanh et al., 2008). *Monochoria vaginalis* (oval-leafed pondweed) is a noxious weed that inhabits and quickly overtakes rice fields in Asia. Passion fruit may be a possible alternative to herbicides by being intercropped with rice plants or if the leaves are used as detritus for mulching.

Besides cultivating plants specifically for their allelopathy, there are also industry byproducts that could be reused to help in the fight against weeds. *Brassica juncea*, of the variety ‘Pacific Gold’, and *Sinapis alba*, of the variety ‘Ida Gold’, are two mustard plants grown worldwide that can be utilized in the production of biofuels. The byproduct of the fuel production, mustard seed meal (MSM) has been shown to have herbicidal, insecticidal, nematocidal, and fungicidal effects affects against a variety of plants. Two worldwide weeds that the MSM was tested upon were large crabgrass (*Digitaria sanguinalis*) and palmer amaranth (*Amaranthus palmeri*). Large crabgrass can be utilized as a grain crop in Eurasia, but is considered a weed in the western world, often seen overtaking lawns and golf courses if left

unattended. Palmer amaranth is a weed often found within soy and cotton fields that has become a larger problem as the weed has developed herbicide resistance to glyphosate. When MSM was mixed into the top portion of a crop plant's soil, it was found that both MSMs successfully partially inhibited both crabgrass and palmer amaranth emergence and growth rates. However, a trend was seen where Pacific Gold had a greater inhibition effect on large crabgrass while Ida Gold has a greater inhibition effect upon palmer amaranth (Wang et al., 2015). While MSM is a waste product and has been proven to control several weed types, the large amount required per application and the requirement for multiple applications through the growing season limits the economic feasibility of using it as an alternative to traditional herbicides.

The traditional practice of incorporating legume vines, be it through intercropping or detritus, into crop plants is being studied as natural herbicides in addition to their ability to provide nutrients to the soil. Four legumes commonly used in this purpose, *Mucuna deeringiana* (velvetbean) and *Canavalia ensiformis* (jackbean) as living crop cover and *Leucaena leucocephala* (haloe koa) and *Lysiloma latisili* (wild tamarind) as leaf litter, were found to have allelopathic effects against barnyard grass, amaranth (*Amaranthus hypochondriacus)* and tomato. Both velvetbean and jackbean, at lower concentrations, had negative affects against nematodes infesting tomato plants while still allowing the tomato plants to survive. When incorporated with corn after germination and sprouting, all four legumes inhibited weed growth and enhanced corn biomass, with velvetbean producing the greatest reduction in weeds and all legumes providing nitrogen to the soil through their symbiotic associations with nitrogen fixing rhizobia. However, if the legumes were sowed at the same time as the corn, they also inhibited the growth of the crop plant (Caamal-Maldonado et al., 2001). This shows the potential of incorporating either live crop cover or leaf litter as an alternative herbicide with other additional benefits such as soil nitrogen restoration, but also highlights the difficulty of the balancing act to ensure that the allelopathy of the additional plant does not negatively affect the growth and production of the crop plant (Tanveer et al., 2015).

1.3.2 Land Management

Barriers to environmental restoration, renewing the environment to conditions to where native plants and animals have reacquired dominance, include invasive seed dispersal, abiotic conditions (such as soil nutrient levels and water flow) and the removal/reduction/control of current invasive species. Using the homeland security hypothesis, where invasive plants may be susceptible to allelopathic chemicals produced by native plant species and vice versa, restoration efforts are now focusing on bolstering the natural defense of native areas by reintroducing plants that have allelopathic properties to invaders (Cummings et al., 2012). In a case study in Panama, a weed called *Saccharum spontaneum* (wild sugarcane), rapidly took over abandoned or cleared agricultural land and caused frequent flash wild fires harming the local economy and young trees that were introduced for reforestation.

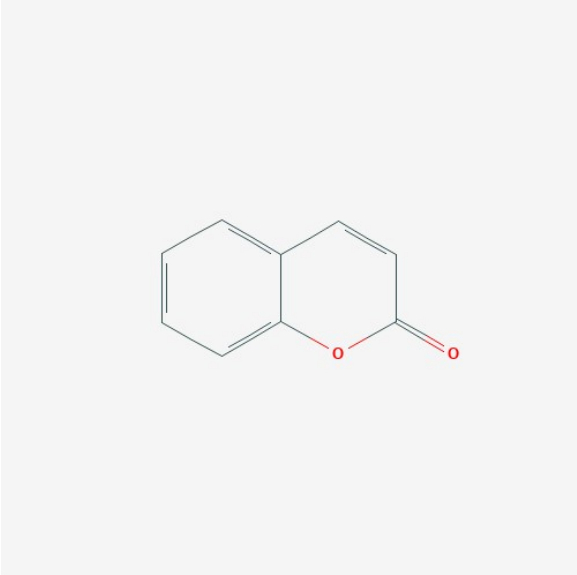


Figure 7. Coumarin, an allelochemical of *Gliricidia sepium* (gliricidia) (Takemura et al 2013). National Center for Biotechnology Information (2021). PubChem Compound Summary for CID 323, Coumarin.

While different rates of inhibition occurred between species, it was found that leguminous trees, those that produce seeds rich in protein often housed in pods, *Gliricidia sepium*, *Diphysa americana*, and *Inga punctata* have on average a minimum estimated 20% greater allelopathic ability to inhibit the growth of *S. spontaneum* as compared to non-legume trees (*Tectona grandis* and *Terminalia confuse*) (Cummings et al., 2012). When looking at other studies of these leguminous trees, *Gliricidia sepium's* predominant allelopathic chemical was found to be coumarin (Figure 7) with a IC_{50} of 23.3 µmol per liter when tested upon lettuce (Takemura, 2013). While the legume tree's allelopathic ability has yet to be tested upon other types of local monocotyledonous weeds growing in the area, its ability to inhibit the growth of the weed considered the most damaging to this local area helps environmentalists determine which trees should be cultivated for sustainable long-term reforestation.

Contrary to the homeland security hypothesis, there exists the novel weapons hypothesis (or the allelopathic advantage against resident species hypothesis), which explains how invasive

species proliferate compared to their resident counterparts because of their novel (at least to the new and unadapted residents) allelopathic agents (Callaway and Ridenour, 2004). These agents can act in a variety of ways, but include direct inhibition of neighboring plant growth and development and the indirect methods of affecting soil-microbe interactions, nitrogen and other nutrient/mineral availability, affecting pH or even remaining behind after the production plant has been removed (Callaway and Vivanco, 2006). For example, *Myrica faya* (fire tree) invaded the slopes of Hawaii Volcanoes National Park by displacing *Metrosideros polymorpha* (ohia lehua) and created regions of nitrogen fixation 2-4X greater than native sources, changing the overall ecosystem (Vitousek and Walker, 1989). Dieback of *Myrica faya* has shown that, even with the increased nitrogen levels, native species, without human introduction and assistance, may not be the primary replacement long term (Seibold, 2000). While native ground coverage (majority *Carex wahuensis* (O'ahu sedge)) increased by 10.4% and introduced grasses (*Andropogon virginicus* (broomsedge bluestem) and *schizachyrium condensatum* (Colombian bluestem) increased by 11.8%, *Carex wahuensis* is in danger of being overrun in the long term as both grasses grow more aggressively and taller than the sedge (Adler et al., 1998). This is a concern to restoration managers because not only is the removal of the invasive a priority, but ensuring recovering native plants are not overrun by other invasives with greater fitness in a healthy nitrogen environment poses a long term problem to be considered as many native plants are not as aggressive as their invasive counterparts (Corbin and D'antonio, 2004; Seibold, 2000).

Another restoration concern that occurs upon removal of the invasive allelopathic plant is the lingering effects that soil leachate may have on the transplanted native species, commonly known as the legacy effect (Grove et al., 2012). For example, in areas overrun by the allelopathic *Fallopia japonica* (Japanese knotweed), whose rhizome contains allelopathic stilbenes glucoside piceatannol (Figure 8) and resveratroloside and the proanthocyanidins catechin and epicatechin (Fan et al., 2010), *Fallopia*

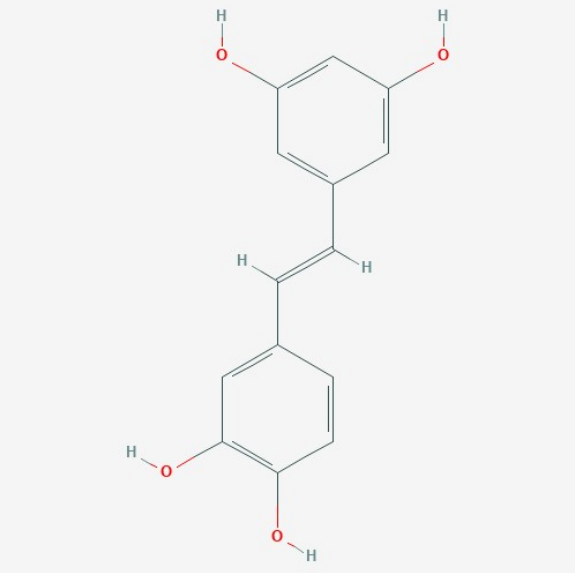


Figure 8. Piceatannol, an allelochemical of *Fallopia japonica* (Japanese knotweed) (Fan et al 2010). National Center for Biotechnology Information (2021). PubChem Compound Summary for CID 667639, Piceatannol.

japonica soil leachates were tested upon cuttings of potential restoration plants *Populus nigra* (black poplar), *Salix viminalis* (basket willow) and *Salix* atrocinerea (grey willow). It was found that *Populus nigra* and *Salix viminalis* had vegetative growths reduced by 20% while *Salix atrocinerea* was not statistically affected (Dommanget, 2014). This shows how allelopathic chemicals can be released through elevation waterflow downstream without direct plant-plant or plant-plant detritus contact and the importance of choosing your restoration plant to help maximize restoration success chance.

Certain vines, called parasitic epiphytes, typically survive by acquiring photosynthetic products, water, and often nutrients from a host plant while using it as a structural support. *Tillandsia recurvate* (ball moss) is a vine that grows in balls and does not usually have a negative effect upon host tree health. However, preferred host tree bias is shown towards trees such as *Bursera copallifera*, which have rough, non-peeling bark. To combat this threat, certain trees have developed allelopathic chemicals that inhibit epiphyte growth and development. Three of these trees are *Ipomoea murucoides*, *I. pauciflora* and *Lysiloma acapulcense*. All three trees are native to the tropics and are seen with decidedly less epiphytes growing upon them. Aqueous and dichloromethane extracts of the tree barks significantly reduced the germination rates of *T. recurvate* (Valencia-Díaz et al., 2010). This provides an alternative method for reducing levels of *T. recurvate* within invaded forests and also gives reforestation experts a baseline to which trees are best suited to be planted in areas where there are lots of destructive epiphytes.

1.4 Sources of New Herbicidal Modes of Action

Herbicide discovery has often taken inspiration from nature for a starting molecule, with human derived analogs and other chemical modifications made to this template to ensure shelf-life stability, increased effectiveness, and other commercially required attributes (Hachisu, 2021). These chemicals come in a variety of commercial brands and application formulas, but their core function derives from their mode of action, or how a herbicide affects a plant biologically/enzymatically, resulting in plant injury and death (Duke et al., 2019). Different herbicides with different chemical families can have the same mode of action, as their active ingredients can affect the same biochemical location and may result in the same manner and display of injury and death (Armstrong, 2009). Currently, there are 26 recognized modes of action sub-categorized into 3 major divisions, including light activation of reactive oxygen species, cellular metabolism, and cell division and growth (H.R.A.C., 2021). The most well-known herbicide is glyphosate (aka Roundup) (Figure 9), which is a aminophosphonic analogue of the amino acid glycine, and was synthesized by Monsanto chemist John Franz in 1970 as a water softening agent (Franz et al., 1997). Glyphosate, a nonselective herbicide, targets the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase) as a competitive inhibitor, resulting in shikimate accumulation and the deadly waste of carbon components and energy as the shikimate pathway is deregulated (Shaner, 2006). This blockage results in the plant being unable to synthesize three critical amino acids, tyrosine, tryptophan and phenylalanine and is most effective on post-emergent targets. Plants absorb glyphosate generally through foliage and, while growth stops within hours of application, desiccation and overall death of the plant occurs over the course of several days (Hock and Elstner, 2004). Glyphosate has a varying half-life depending upon where it is found, with soil being 2-197 days compared to water being 2-91 days

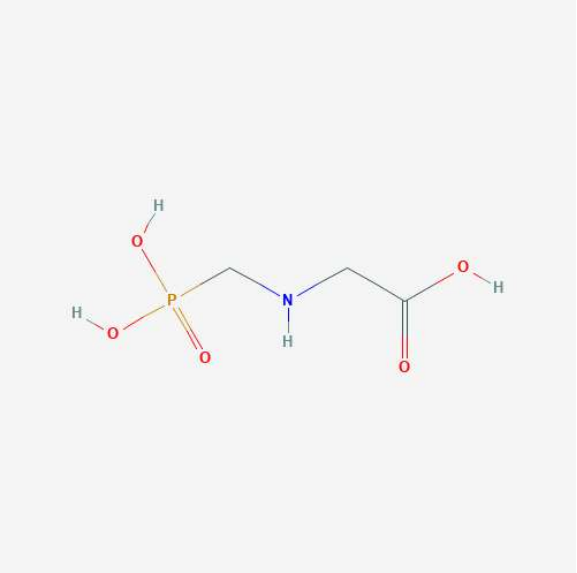


Figure 9. Glyphosate, a herbicide that targets the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSP Synthase) (Franz et al 1997). National Center for Biotechnology Information (2021). PubChem Compound Summary for CID 3496, Glyphosate.

("Glyphosate Technical Fact Sheet," n.d.). While animals do not have the EPSP protein nor the shikamate pathway (and thus cannot affect them in the same manner as plants), there have been several controversial lawsuits tying glyphosate and assorted Roundup co-formulants to cancer in humans. Regardless of its side effects, glyphosate containing products are registered in 130 countries and approved for use on over 100 crops (Valavanidis, 2018). There has also been the production of Roundup Ready crops, such as soybean and corn, which utilize genetically modified crop plants incorporating a bacterial EPSP gene resistant to roundup, allowing farmers to spray Roundup without having to worry about crop plants being affected (Green and Owen, 2011).

Another popular herbicide, from DuPont, is called Glean. This nonselective herbicide's active ingredient is chlorsulfuron. (2-Chloro-N-((4-methoxy-6-methyl-1,3,5-triazin-2-yl)aminocarbonyl)-benzenesulfonamide; Figure 10), It is classified as a sulfonylurea herbicide as it contains a central S-arylsulfonylurea structure with a phenyl ring off of the sulfur group (connected to a variable group) and a variable group branching off of the nitrogen. Application of chlorsulfuron to corn has resulted in the inhibition of growth in 2 hours and the reduction of growth by 80% in 8 hours with IC_{50} values of 18-36 nanomolar (Ray, 1982). It was shown that chlorsulfuron targets the enzyme acetolactate synthase by radiolabeled chlorsulfuron and protein purification trials (Ray, 1984). Acetolactate synthase (also known as acetohydroxy acid synthase) is important to plants because it catalyzes an early step in essential branched chain amino acids biosynthesis. Isozymes are not evident for this enzyme in corn and maize (Durner et al., 1991). Kinetically, it was shown to have a slow binding inhibition, where non-competitive (mixed type inhibition) binding eventually increases in potency to have a tightly bound complex with a maximum rate constant estimated at $0.074min^{-1}$ (Durner et al., 1991). The binding site itself overlaps with a pyruvate binding domain

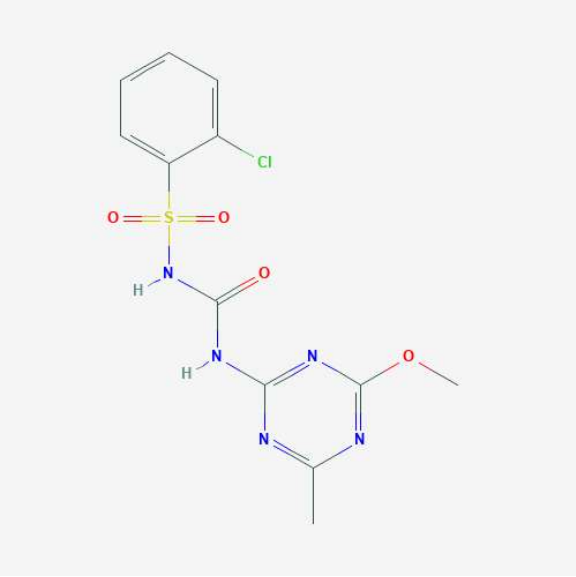


Figure 10. Chlorsulfuron, a herbicide that targets the enzyme acetolactate synthase (Ray, 1982). National Center for Biotechnology Information (2021). PubChem Compound Summary for CID 47491, Chlorsulfuron.

and binding in this secondary domain is not permanent. The half-life for dissociation of the complex is 3.5 hours, but results in the permanent inhibition of the enzyme even with lower than necessary levels of chlorsulfuron (Durner et al., 1991). Thus, the loss of the ability of plants to grow is because of the inability to produce essential amino acids valine, leucine and isoleucine (Ray, 1984). As with glyphosate, plant death can come about slowly. Without certain amino acids, proteins cannot be produced, and thus cellular division (initially) and cellular functions (over time) shut down as proteins cannot be replaced and energy spent trying to resolve the amino acid deficiency is wasted, leading to a downward spiral. In terms of carbohydrates, after application of chlorsulfuron there was an initial loss of source/sink dynamics (carbohydrate accumulation in leaves and roots due to potential lack of usage as cellular division is inhibited) and also loss of chloroplast structural organization. In addition, some plants experience a delayed inhibition of carbon fixation (and photosynthesis), resulting from the secondary effects of reduced stomatal conductance, loss of chloroplast structural organization and notable carbohydrate accumulation (Zabalza, 2004). The EPA has found minimum risk to human and aquatic life due to long lasting or acute toxicity to chlorsulfuron ("US-EPA 'Chlorsulfuron RED factsheet' Prevention Pesticides And Toxic Substances (7508C," 2005).

Nature is never one to sit still and, when presented with a challenge such as an herbicide, provides eloquent solutions in an attempt to survive. Plants have developed resistance to a number of popular herbicides in a variety of ways. The first common method arises from mutations to the molecular target site. These are genetic mutations that typically change an amino acid, resulting in a protein structural change due to a charge or hydrophobicity difference. This in turn, renders the enzyme less sensitive to the herbicide while maintaining moderate function. For example, resistance to glyphosate occurs in a proline to serine mutation at amino acid 106 or a proline to threonine mutation at the same amino acid in EPSP. A closer look at the genome reveals a C319-T mutation replaces Pro 106 with Ser, resulting in a conformational change due to the polarity and bulk difference of the two amino acids and increasing the LD50 for *Eleusine indica* (goosegrass) approximate 2-4x greater than the control (Baerson, 2002). In another example, glyphosate resistant weed species most likely occurred naturally through chance, but was accelerated with human help, as the consistent spraying of glyphosate being the selective pressure that brought about the "sudden explosion" of glyphosate resistant weeds. While early studies suggested development of resistance should be rare (Bradshaw et al., 1997;

Komoba et al., 1992; Padgette et al., 1991), current studies and empirical evidence have shown that consistent application of glyphosate to field weeds, without integrated pest management strategies being taken into account, can bring about glyphosate resistance (Powles and Preston, 1998). In addition, it has been shown that a stepwise mutation in *Daucus carota* (carrot) resulted in increased EPSP activity, and thus glyphosate resistance can be gained without extrachromosomal interference (Shyr et al., 1992). For example, in Malaysia, goosegrass was shown to have gained glyphosate resistance quite rapidly after 1994, the year in which glyphosate prices were a fraction of their usual cost and usage spread across the country and through the seasons, increasing to 6 to 8 applications per plantation per year (Lee and Ngim, 2000). As such, any and all mechanisms that can increase a plant's fitness and survival will be selected for, especially when exposed to a single selective pressure such as a single target herbicide (Powles et al., 2006).

Another nonselective herbicide is atrazine (Figure 11), which works on plants by binding competitively at the plastoquinone B binding site of the D1 subunit of PSII. This blocks the flow of electrons from plastoquinone A to plastoquinone B and results in an increase of free radicals and the reduction in the production of ATP and NADPH, ultimately leading to cell death (Hess, 2000). Resistance for this occurs in the *psbA* gene, where an A to G mutation at nucleotide 790, results in codon 264 Serine to Glycine change, cumulating in a conformational change due to the polarity and size difference between the two amino acids (Goloubinoff et al., 1984). This mutation results in a minimum two fold increase in the LD50 of *Palmer amaranth* (Kohrt, 2017).

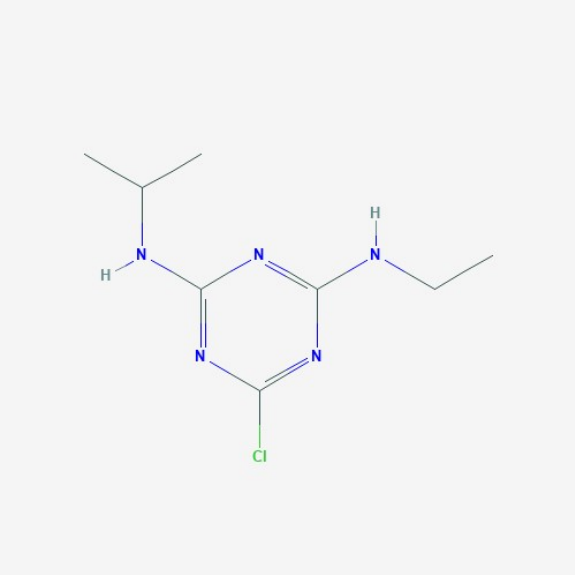


Figure 11. Atrazine, a herbicide that targets the D1 subunit of photosystem II (Hess, 2000). National Center for Biotechnology Information (2021). PubChem Compound Summary for CID 2256, Atrazine.

Another common mode of resistance comes from inhibiting the herbicide's movement through the plant or relocating it to one section of the plant so that effects are limited. For example, Lorraine-Colwill et al. in 2002 showed in *Lolium*

rigidum (rigid ryegrass) using radioactively labeled glyphosate that at 48 hours after application, resistant plants had up to 50% of the glyphosate accumulated in the leaf tips. This is in comparison to the susceptible plants, which accumulated the glyphosate in their still growing leaf bases and roots. This is suspected to be caused by glyphosate being transported by the transpiration stream to the leaf tips which causes the glyphosate to stay away from meristems and leaf bases, increasing plant survival (Lorraine-Colwill, 2002).

Another common mode of resistance is degradation. In *Amaranthus palmeri*, it has been noted that resistant plants concentrate atrazine in their leaves for degradation (Chahal et al., 2019). A method of atrazine degradation (in atrazine resistant plants) was shown by Anderson and Gronwald in 1991 in *Abutilon theophrasti* (velvetleaf) where atrazine, concentrated in the leaves, resulted in increased glutathione S-transferase activity by 4.4 fold compared to the control, including a 3 fold higher Vmax (Anderson and Gronwald, 1991). Degradation is also one method for producing glyphosate resistant crop plants (ex, soybean, canola) via glyphosate oxidoreductase activity, but has yet to become a common evolutionary trait in wild weeds (Duke, 2011).

One of the most efficient methods of weed and other pest control is to develop and follow an integrated pest management (IPM) strategy. The United States Council on Environmental Quality defined IPM as "An approach that employs a combination of techniques to control the wide variety of potential pests that may threaten crops. It involves maximum reliance on natural pest population controls, along with a combination of techniques that may contribute to suppression – cultural methods, pest-specific diseases, resistant crop varieties, sterile insects, attractants, augmentation of parasites or predators, or chemical pesticides as needed." In terms of weeds, this implies looking at chemical control means, like herbicides, as one part of, not the entire, method of treatment. It can include incorporating old world techniques (hand weeding, traditional farming practices, crop rotations, etc) with new world solutions (chemicals, smart crop management, GMOs, drones, etc). Another example is intercropping with alternative crops such as the allelopathic velvetbean (*Mucuna deeringiana*) and jackbean (*Canavalia ensiformis)* as living crop cover or utilizing jumbiebean (*Leucaena leucocephala*) or wild tamarind (*Lysiloma latisili*) as leaf litter to help keep weeds suppressed in crop fields in addition to rotating herbicides (Caamal-Maldonado et al., 2001). Another utilized theory is the homeland security hypothesis. In this strategy, invasive species are susceptible to native allelopathic

chemicals and vice versa, thus, to protect a native restoration area against invasive species it is surrounded by allelopathic plants to keep weeds away (Cummings et al., 2012). These are all more holistic approaches to "weed control" and can help alleviate the constant herbicide resistant selection pressure put on weeds every growing season or crop rotation to delay the development of herbicide resistant weeds.

Utilizing harvested and minimally processed plant extract mixtures, those that contain a variety of compounds, compared to a single individual chemical is another potential approach to addressing weeds. For example, while the traditional Western standard continues to be one chemical - one molecular target, "in tune with nature" applications of natural products, mixtures and extracts are gaining popularity for their multifaceted modes of action to address a complex problem. Due to the varying complexity and often elastic approach of botanical extract's health, herbicide and pesticide affects, regulation of such mixture products has been shown to be less stringent, less expensive, less costly and overall commercialization can be less arduous (Wallace, 2015; Wu, 2020). While pesticides and herbicides continue to have additional regulation requirements before their commercial application is approved for widescale crop usage, "traditional" natural product plant mixtures, utilized either on small home-grown scale or within indigenous communities, continues to provide working potential in its current form that is often overlooked (Atanasov, 2021; Cordeau, 2016). While acceptance of such mixtures in commercial agriculture is a growing field, the rise of herbicide resistant weeds combined with the enthusiasm and effectiveness of holistic mixtures in human health points to the expanded utilization of natural product mixtures in the future (Raveau et al., 2020; Westwood, 2018).

While studies have shown the potential for the use of allelopathy via intercropping or having extracts sprayed upon crop plants for weed control and increased yield, there are limitations on the implementation of such techniques (Bhowmik and Inderjit, 2003). The high costs associated with the extraction and/or synthesis of complex natural products, combined with often short environmental lifespans requiring reapplications, make large scale applications of most allelopathic chemicals currently unfeasible in large scale agriculture (Bhowmik and Inderjit, 2003). In addition, the property of being toxic to not only the target species but also to other organisms makes applying the allelopathic chemicals to crop plants potentially more damaging than beneficial. For example, there are allelopathic chemicals that are harmful not only to plants,

but also to animals. The toxins AAL-toxin and fumonisin disrupt sphingolipid metabolism in both animals and plants, creating a dangerous situation if the poisons were to be sprayed around humans or pets. In addition, certain plant-based chemicals can also cause allergic reactions within humans, such as seen in the mango tree (*Mangifera indica*) (Chou, 1999). However, when faced with the extraordinary cost and potential environmental backlash due to the increasing concentrations of herbicides currently being used, an alternate and greener solution may be a better option for sustainable agricultural practices. This tradeoff between production and lowered potency of natural product herbicides is being balanced with new research into the very core of how herbicides work, the mode of action. While the most common herbicide on the market, glyphosate and its commercial spinoffs, work by inhibiting the plant enzyme 5-enolpyruvylshikimate-3-phosphate synthase, many weed species have begun to obtain herbicide resistance to such a mode of action (Beckie et al., 2019). Thus, in the search for alternatives to glyphosate, one of the most recently described modes of action, which was discovered by investigating natural product herbicidal compounds, is targeting the enzyme 4-Hydroxyphenylpyruvate dioxygenase (HPPD).

1.4.1 Hydroxyphenyl Pyruvate Dioxygenase (HPPD)

HPPD (4-Hydroxyphenylpyruvate dioxygenase) is an iron containing oxygenase found in the cytoplasm of plant cells and in the liver of animals (Garcia et al., 1999). This enzyme facilitates the oxidation of 4-hydroxyphenylpyruvate (HPP) to homogentisate (HGA) (Figure 12), a process important in the catabolism of tyrosine, the production of tocopherols and plastoquinone, as well as, indirectly, carotenoids. This enzyme is unique from other dioxygenases in that it does not utilize alpha-ketoglutarate in its reaction, catalyzing a NIH (1,2-hydride) shift (oxidative decarboxylation of the alpha-keto acid and an aromatic ring hydroxylation) without an additional acid cofactor or substrate. A crystal structure of this enzyme has been determined with the active site containing an iron atom (Fritze et al., 2004).

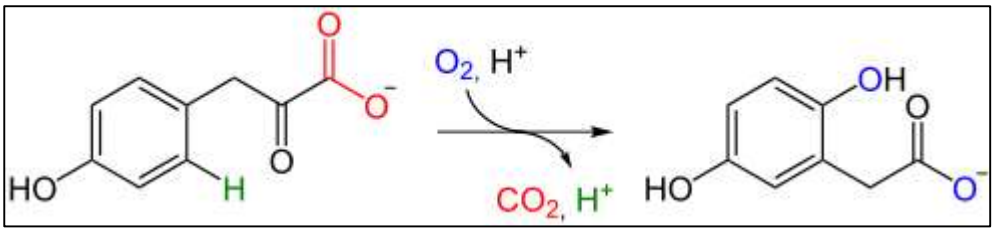


Figure 12. The conversion of 4-hydroxyphenylpyruvate to homogentisate by HPPD.

HPPD inhibitors are the most recent discovered herbicidal mode of action that have been widely developed for agricultural usage. They can be grouped into three classes based upon their structure: pyrazolones, triketones, and diketonitriles. All 3 classes have had herbicides developed from natural product base structures; Pyrazolones have a characteristic 5 membered ring containing 2 adjacent nitrogen groups, triketones starting with 3 ketone groups and diketonitriles with two ketone groups and nitrile group. Pyrazolones were the first commercially developed HPPD inhibitors in the 1980s with sulcotrione (Figure 13), derived from the natural product leptospermone and isoxaflutole subsequently introduced in 1990 and 1996, respectively. HPPD inhibitors (Figure 14) competitively bind to HPPD and inhibit the conversion of 4-hydroxyphenylpyruvate into homogentisate, resulting in the downstream inhibition of multiple pathways and eventual death of the plant. An indication that a plant has been affected by a HPPD inhibitor is bleaching resulting from inhibition of chloroplast production and resultant oxidative damage buildup due to inhibition of the biosynthesis of the protective carotenoids or tocopherols. It has been found that the HPPD inhibitors that have triketone base structures are often situated in the active site of the HPPD protein and interact with the ferrous ion along with three conserved resides (Glu 373, His287, and His205) (Dayan, 2007). As the rise in glyphosate resistant weeds have continued unabated, research has returned to HPPD and have produced topramezone (2006) and tembotrione (2007), along with the more recent creation of HPPD resistant crops such as soybean, oat and maize (Dreesen et al., 2018; Hawkes, 2019; Liu, 2020; Siehl, 2014).

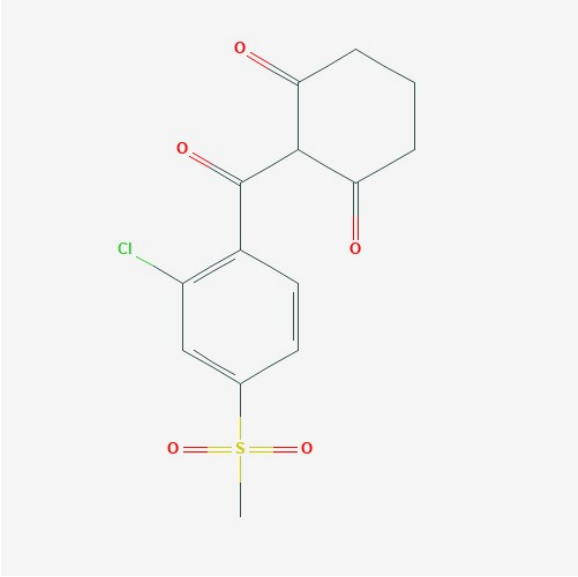


Figure 13. Sulcotrione, a herbicide that targets the enzyme 4-hydroxyphenylpyruvate dioxygenase (HPPD) (Kim et al 2002). National Center for Biotechnology Information (2021). PubChem Compound Summary for CID 91760, Sulcotrione.

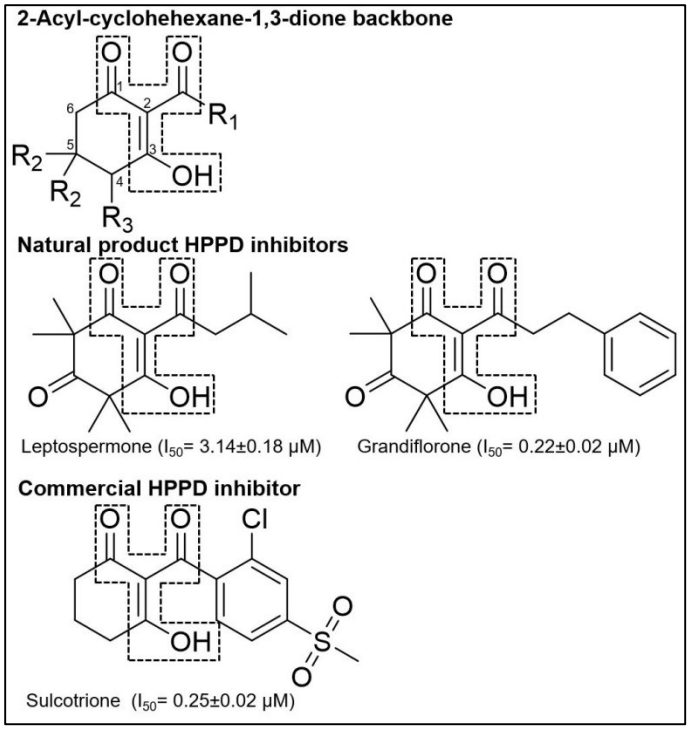


Figure 14. Comparison of natural and synthetic compounds containing the 2-acyl-cyclohexane-1,3-dione backbone with known HPPD inhibitory activity. The region within the dashed line represents the triketone containing the 1,3 dione feature required for HPPD inhibitory activity. This interacts with the iron in the active site of the protein. The IC50 are values as tested against recombinant *Arabidopsis thaliana* HPPD.

Although humans have HPPD, HPPD herbicides are non-toxic to humans; although slightly elevated tyrosine levels have been reported to occur rarely with prolonged usage (Lewis and Botham, 2013; Shaner, 2004). In fact, triketone compounds can be utilized to help reduce the severity of Type 1 tyrosinemia and are approved for human usage. In individuals with this hereditary disease, the catabolic pathway of tyrosine is inhibited by a non-functional fumarylacetoacetate hydrolase (FAH), and often results in death at an early age along with other deliberating symptoms. HPPD precedes FAH in the catabolic pathway and its inhibition reduces toxic succinyl acetone buildup by preventing formation of maleylacetoacetic acid and fumarylacetoacetic acid (McKiernan, 2006). Another HPPD inhibitor drug, nitisinone (triketone class), has been developed and since helped reduce liver transplants and increased quality of life for diagnosed patients by being prescribed to treat hereditary tyrosinemia type 1 under the brand names Orfadin or Nityr (Das, 2017).

Chapter 2: Project Overview

2.1 Introduction

The identification, characterization, and ultimate utilization for human benefit of natural products is a long and arduous process. Studies and experiments can span decades. For example, natural product identification and development of triketone herbicides from the bottle brush tree, (*Callistemon citrinus)* was over a 30 year timeframe (Beaudegnies, 2009; Cornes, 2005). However, the extensive potential for these compounds to aid in overcoming problems, particularly to aid in weed control, makes this process a worthy investment for the future. It is also of note that as this work does require significant investments of time that for future generations to see any benefits, the work must begin now. Herbicide discovery is already seeing a significant decline due to decreases in funding and research as glyphosate was once saw as the "magic bullet" to end the issues caused by weeds.

Our research interests focus on Hawaii, its tropical biota, its high levels of biodiversity and has implication on the local community's fight to maintain the land for future generations against the rise of invasive species. As the first student to initiate such a program in MBBE at UHM, my overarching goal was to discover and develop natural products for potential agricultural use, such as in weed control, from tropical and subtropical plants that have evolved in the biodiverse Hawaiian environment. Our approach to identifying compounds of interest is that natural products with weed killing activity are likely to be identified in plants related to those with previously analyzed allelopathic activity. My central hypothesis is that allelochemicals that have evolved in a natural environment have greater potential for the discovery of compounds with unique, novel herbicidal target sites. The herbicidal activity of identified and/or related active compounds can then be improved through QSAR (quantitative structure activity relationship) modeling and chemical modifications to improve herbicidal activity for agricultural usage. This work is important in that current agricultural practices are unsustainable with the available weed control methods and identification and development of new target sites is essential for the future. New molecular target sites will aid in combating the growing proliferation of herbicide resistant weeds. I have made noteworthy progress towards this long term goal and have aided

significantly in pushing the field of natural product identification/development and herbicide discovery forward.

2.2 Objectives and hypothesis

2.2.1 Objective 1

Develop a bioassay to determine allelopathy.

Hypothesis: If a plant has allelopathic properties, then test plants grown in the allelopathic plant's leaf extracts will have reduced growth/germination compared to a control.

Activities undertaken in Objective 1

1. Equipment sourcing
2. Assay plant selection
3. Assay growth timing
4. Assay data selection metrics
5. Assay volume analysis
6. Assay material preparation
7. Dilution Series

2.2.2 Objective 2

Determine which weeds and to what degree growth and germination rates are affected by the allelopathy.

Hypothesis: If the plant's allelopathic properties do not differentiate between monocots and dicots, both treated seeds and sprouted plants will receive an adverse effect similar to the effect seen in objective 1 by application of the plant's extract solution.

Activities undertaken in Objective 2

1. Bioassay strawberry guava and other plants (18) for both water and acetone extracts for comparative analysis
2. Leaf soak time trial (1 Day, 2 Day, 4 Day, 8 Day, 16 Day, 32 Day) bioassay
3. Root and shoot soil analog bioassay
4. Agricultural weed bioassay for pre-germination activity
5. Agricultural weed bioassay for post-germination activity

2.2.3 Objective 3

Potential allelopathic compounds will be characterized.

Hypothesis: If the allelopathic chemicals are released by or transported by rainwater, then the chemicals should be water soluble.

Activities undertaken in Objective 3

1. Ethyl acetate fractionation
2. Waters Sep Pak C18 Gravity Flow Non-polar chromatography
3. Waters Silica Gravity Flow Polar chromatography
4. HPLC Analysis of bioactive fractions
5. MS/MS Analysis of bioactive fractions

2.2.4 Objective 4

QSAR analysis of molecular target site HPPD

Hypothesis: Synthetic analogs that have the lowest binding energy will fit best into the active site pocket and reduce Hydroxyphenylpyruvate dioxygenase protein activity.

Activities undertaken in Objective 4

1. Build synthetic analogs in Avogadro
2. Test binding affinity in AutoDock
3. Create molecular modeling for 4-Hydroxyphenylpyruvate dioxygenase.

Chapter 3: Developing a Bioassay to Test for Allelopathy

3.1 Introduction

While it would be quite appropriate for the establishment of an allelopathy version of Koch's Postulates, modern science has yet to agree on a consensus as established as Koch's criteria in the medical field. In the most organic of definitions, a display of allelopathy requires application of the suspected compound(s) or mixture under natural field conditions, when the plant that produces the compounds is absent or removed, to ascertain if the symptoms or suspected effects are recreated in other plants (Keating, 1999). However, many studies have started with *in-vitro* studies of bioassays, where a test plant is grown in a petri dish with the suspected compound(s) or mixture under standardized conditions, due to the many compounding factors (soil type, light, temperature, weather, etc) that can arise when working on field or soil trials (Harper, 1975). Even before growing plants in a bioassay, there is the problem of how best to extract the compounds in question, with many variables (leaf age, cellular components of salt, amino acids, nitrogen, method of intended release vs laboratory extraction, etc.) again bring doubt into this procedure (Inderjit and Dakshini, 1995). Some consensus that has been formed as the basis for bioassays is that a control for the variable of allelopathic mixture/compound(s) to be tested upon a plant is most often distilled water. This control being able to separate the effects of resources provided to each test plant compared to the compound(s) in question (Harper, 1975). While soaking the leaves (or other plant parts such as stems and roots) is the preferred strategy of allelochemical release as this method of extraction avoids cellular debris found in grinding the leaves, not all allelopathic chemicals are designed to be released by having water run over the surface of the leaves, and thus grinding is a viable first look at potential allelopathy in a plant species (Chou and Muller, 1972; Harper, 1975; Inderjit and Dakshini, 1995; Inderjit and Nilsen, 2003). While the argument for what test species to utilize for allelopathic bioassays varies by region, for ease of convenience, consistency in germination, categorization as a crop plant, and susceptibility (non-allelopathic), many studies utilize lettuce *Lactuca sativa* (Antonelli et al., 2020; Chapla and Campos, 2010; Inderjit and Dakshini, 1995; Nilsen, 1999). Thus, this project begins with the design and testing of a bioassay for determination of allelopathy.

3.2 Materials and Methods

3.2.1 Plant Material

Mature, healthy native and non-native plant leaves (*Acacia confuse, Acacia koa, Atriplex semibaccata, Bauhinia blakeana, Bauhinia galpinii, Bauhinia monandra, Bauhinia purpurea, Erythrina abyssinica, Erythrina crista-galli, Jatropha integerrima, Metrosideros polymorpha, Pachira aquatica, Platymiscium stiuplare, Plectranthus amboinicus, Prosopis pallida, Pseuderanthemum carrutherrii, Sesbania tomentosa, Tamarindus indica)* were collected from the UH Manoa campus arboretum.

Psidium cattleianum leaves of both variety (red and yellow) were collected from private residences (with landowner permission) located within and around Manoa valley, Hawaii, USA. Manoa lettuce (*Lactuca sativa* var.) and Green Onion – 'Koba' (*Allium fistulosum*), species that are representative of dicots and monocots respectively, were purchased from the University of Hawaii – Seed Lab.

3.2.2.1 Isolation of Plant Growth Inhibitory Compounds – Maceration of Leaf Material

Harvested leaves were cleaned by hand to remove any large particles and then ground to a light confetti utilizing liquid nitrogen, mortar and pestle. Either water or acetone in a 1:1 ratio (50g leaf : 50mL solvent) was added to the pestle, soaking the macerated leaf material. This mixture was ground again with the pestle and filtered with a cheesecloth to remove plant leaf debris. The liquid was collected and lyophilized until dry. The dried extracts were resuspended in their respective solvent (water or acetone) to obtain a uniform concentration (350mg/mL for water extracts, 250mg/mL for acetone extracts). Resuspended extracts underwent a bioassay to determine allelopathic potential.

3.2.2.2 Isolation of Plant Growth Inhibitory Compounds – Leaf Soak

Harvested leaves were cleaned by hand to remove any large particles and placed into a plastic 1L bottle with a 1:1 ratio (50g leaf : 50mL solvent) of water. The bottle was lightly shaken by hand and sealed with a screw top lid. Water was recovered at time intervals 24 hours (1 Day), 48 hours (2 Days), 96 hours (4 Days), 192 hours (8 days), 384 hours (16 days) and 768

hours (32 days) and replaced with new water to be harvested at the next time interval. Recovered extracts underwent bioassay to determine allelopathic potential.

3.2.3 Bioassay

Costar 24 cell well plates (Figure 15) were layered with Whatman #1 filter paper circles on the bottom of each well. To test for allelopathic compounds, experimental solutions in each well were diluted to 10% v/v with water (180uL water + 20uL water extract) or 5% v/v with acetone (190uL water + 10uL acetone extract). To each well, 5 Manoa lettuce (dicot) or 3 green onion 'Koba' seeds (monocot) were added (numbers chosen due to the size of the seeds). Each set of plates were harvested when the first cotyledon hit the cover of the plate (~5 days for water extracts, ~7 days for acetone/methanol extracts at 22 °C with 24H light) and the mass of the fresh weight of the germinated seedlings were measured and compared, experimental to control utilizing Anova single variable for statistical differences. In order to determine if there was an herbicidal effect, a dose response curve was created, where the concentrated extract was diluted in a series from 100%, 80%, 60%, 40%, 20%, final dilution to 10% and then applied to the 10% or 5% v/v water well dilution as described previously. Each experimental condition was replicated utilizing at least 6 wells/plate. Six control wells were also used per plate using only water or a 5% acetone dilution. A 5% acetone concentration does not statistically affect the test plant's growth compared to a water control. Leaf soak recovered water, which was not concentrated, was not diluted in a 10% v/v but was plated directly to the wells in a dilution series manner as previously described.

15A

15B

Fig 15A. A overview of the bioassay developed utilizing Costar 24 cell well plates layered with Whatman #1 filter paper circles on the bottom of each well. On the left side of the plate, 5 Manoa lettuce seeds per well were grown in a 200uL volume 10% water leaf extract solution while the right side of the plate was a water control. **15B:** This is a comparison showing the effects of strawberry guava upon Manoa lettuce growth (a water control (left) compared to a ~10mg/mL yellow strawberry guava leaf extract (right). Affected plants have stunted development in both root and shoot formation, with roots appearing burned and twisted.

3.3 Results and Discussion

The UH Manoa campus is a living arboretum containing a wide variety of plant life from across the globe. Utilizing this variety, in addition to invasive plants found in the neighboring community, a wide net was cast in our selection of species, specifically looking at their relationship to other documented allelopathic plants, anecdotal suggestions, and field observations. Plants screened for potential allelopathy included a combination of native plants, non-natives, and invasive species.

3.3.1 Selection of Plants for Study

Acacia confuse, common name Formosan Koa, was observed to have suspected allelopathy after aqueous leaf extracts reduced plant growth inhibition in lettuce, alfalfa and Chinese cabbage. The suspected allelopathic chemicals include ferulic, vanillic, caffeic, gallic, *m*-hydroxybenzoic, and *m*-hydroxyphenylacetic acids among other non-identified chemicals, with no single chemical-plant interaction being the single factor for plant growth inhibition (Chou, 1998). This plant was chosen for comparison to a native Hawaiian Koa in the same genus, *Acacia koa*. The native Koa has been recommended for reforestation or lumber production on degraded Maui farmland and, like many native plants of Hawaii, does not display strong allelopathic characteristics upon environmental observation (Jenkins, 2020).

Atriplex semibaccata, common name Australian salt bush, is a plant that can be found growing along the sand dunes of the North shore of Oahu Island. It has a unique property of being able to grow in salinized soil conditions (Ibrahim, 1998). While not shown to be allelopathic, this plant is a part of a genus that also has other members with suspected allelopathic properties. Invasive *Atriplex canescens* was found to be able be able to inhibit the growth of native *Salsola rigida* with leaf extracts in a study of native vs invasive plant species (Dehdari, 2009). *Atriplex lentiformis* was found to contain the most inhibitory allelopathic chemicals in its leaves compared to other plant organs by ways of inhibiting the growth of *Medicago scutellate* with aqueous leaf extracts (Ebrahimi, 2016).

Of the 4 bauhinia species, *Bauhinia blakeana, Bauhinia galpinii, Bauhinia monandra,* and *Bauhinia purpurea*, only *Bauhinia purpurea* has been previously shown to be allelopathic. Studies have found that leaf and bark leachate application to crop plants *Triticum aestivum L,*

Brassica campestriss L. and *Hordeum vulgare* results in reduced germination rates (Chou, 1980; Singh et al., 2009). This provides a comparison with three unknown plants and 1known allelopathic plant within the same genus. There is also evidence that the genus holds more allelopathic plants, as *Bauhinia variegate* leaf leachates have been shown to inhibit the growth of *Vigna unguiculata* and *Zea mays* (Kaletha et al., 1996).

Although the two erythrina species are not native to Hawaii, they conduct the same comparison where *Erythrina abyssinica* has been previously shown to be allelopathic, while *Erythrina crista-galli* is an unknown factor. *Erythrina abyssinica* leaf litter had adverse effects on wheat seedlings due to a dual effect of suspected allelopathic interactions and nitrogen immobilizations (Anthofer et al., 1998). Within the same genus though, there exists other allelopathic plants. *Erythrina fusca* inhibited germination of lettuce with an ethanol water mixture of leaf material causing maximum reduction (Gris, 2019).

Jatropha integerrima has an unknown status concerning allelopathy, but within the same genus, *Jatropha curcas* has been greatly studied for its aqueous leaf extract inhibition of *Triticum aestivum L, Phaselous culgaris, Zea mays, Lacatuca sativa, Lycopersicon lycopersicum* and *Hibiscus esculentus*, thus inviting the study of this related species (Abugre and Sam, 2010; Mahmoud et al., 2016; Sanderson, 2013).

One of the other native species in the study, *Metrosideros polymorpha*, common name ohia lehua, has also not been greatly studied for allelopathy due to its role as an endemic keystone species in the Hawaiian Island watershed. Found across the island chain in a variety of morphologies, ohia lehua is often the first tree to colonize lava fields, yet can be found growing in symphony with at least 40 other native plant species in ecosystems ranging from swamps to high level elevations. With a long-fabled history in Native Hawaiian culture, the trees are prized for their ecological as well as cultural significance to the local community. Currently under assault from a variety of invasive plants and deadly fungus, the tree has declined in its omnipresence across the Hawaiian Islands and efforts are underway to preserve what few untouched groves remain (Friday and Herbert, 2006).

While not many studies have been done on *Pachira aquatica* in relation to allelopathy, there have been reports that this tree accumulates the fungitoxin isohemigossypolone in its bark, in addition to having seeds with antioxidant properties (Rodrigues, 2019; Shibatani et al., 1999).

Within the same genus, *Pachira quinate's* leaf litter was found to increase growth and improve survivability of *Saccharum spontaneum* when compared with control conditions (Cummings et al., 2012), perhaps providing a potentially non-allelopathic plant to balance out the many allelopathic or suspected allelopathic plants previously described.

While *Platymiscium stipulare* is a member of the Fabaceae family, home to other allelopathic containing genus groups described, such as Acacia, Prosopis, Bauhinia, Erythrina and Tamarind, this plant species specifically has not been examined for allelopathy. However, within the same genus, *Platymiscium yucatanum,* was found to be a tropical heartwood resistant to the fungi *Lenzites trabea* and *Coriolus versicolor* due to a variety of flavonoids and isolfavonoids found within its bark, including medicarpin, homopterocarpin, isoliquiritigenin, and liquiritigenin (Reyes-Chilpa, 1998).

Plectranthus amboinicus, common name Cuban oregano, is home to a spicy aromatic scent that has allelopathic potential. It was found that, through a liquid-liquid extraction with majority content of carvacrol, leaf extracts were able to reduce the germination and growth of *Sorghum bicolor* and lettuce (Pinheiro, 2015), making this plant a known allelopathic positive control. It was also found that aqueous leaf extracts of the *Plectranthus amboinicus* helped reduce the weed growth of *Phalaris minor* and *Anagalis arvensis* while boosting growth of crop plant *Pisum sativum* (El-Rokiek, 2018).

Prosopis pallida, commonly called kiawe in Hawaii, is known for its ability to grow in arid and dry regions, mainly beach fronts where few trees can grow. Under its canopy, biodiversity was found to be relatively limited compared to surrounding species, along with a plethora of leaf litter. An astute observation because it was found that aqueous leaf extracts inhibited the germination and growth of *Cenchrus ciliaris*, *Enteropogon rupestris, Zea mays*, and *Triticum aestivum*, potentially due to tryptophan levels. (Gallaher and Merlin, 2010; Getachew et al., 2012; Noor et al., 1995).

Pseuderanthermum carruthersii, common name Carruther's false face was included as a negative control as it is not thought to have allelopathic activity. Having a white flower to contrast its purple leaf coloration, it is considered a xeriscape plant and is highly recommended for groundcover in hotels and other business areas (Wong, 2008). In addition, a government study of the plant from a WRA specialist found no evidence that the plant forms dense thickets

in native or introduced range, no evidence of allelopathy, no toxicity to animals or humans and it is not a fire hazard ("PLANTS Profile for Pseuderanthemum carruthersii var. carruthersii () | USDA PLANTS," n.d.). However, its purple coloration has brought up additional questions, with the discovery of several flavonoids and their derivatives, including luteolin and apigenin (Vo, 2012).

The allelopathic potential of *Psidium cattleianum*, strawberry guava, was first brought to attention when the invasive common or American guava, *Psidium guajava*, was found to have allelopathic chemicals within its leaves. By grinding up the guava leaves and spraying a 10% extract concentration mixture on young lettuce plants and lettuce seeds, there was an almost 45% reduction of growth and germination rates as compared to non-affected plants and seeds (Chapla and Campos, 2010). Not many studies have been done on the related strawberry guava and, with strawberry guava being an entrenched and expensive problem for the State of Hawaii, much interest has been developed over the years as to why strawberry guava grows so prolifically and dominates the local ecosystem where it has become established.

The federally endangered endemic species *Sesbania tomentosa*, common name ohai, is a rare coastal shrubland plant that can be found growing from lava rocks to cliff sides and whose flowers were traditionally used in leis (Kim, 2020). While not many allelopathic studies have been done on this rare species, other species in the genus have been found to be potentially allelopathic. For example, *Sesbania virgata* seed leachate, containing sesbanimide, (+)-catechin, and other known phytotoxins, was found to strongly negatively affect *Leucaena leucocephala, Arabidopsis thaliana* and rice germination and seeding growth (Drewes et al., 1995; Simões, 2008). In another species, *Sesbania grandiflora* leaf extracts decreased seed germination and growth of *Cajanus cajan*, providing the groundwork to include this plant into the bioassay (Alagesaboopathi and Deivanai, 2011).

Tamarindus indica leaf extracts were found to be allelopathic to a large number of weeds and crop plants, including *Echinochloa crus-galli, Astragalus sinicus, Lolium perenne, Phleum pratense, Trifolium repens, Asarum canadense, Asparagus officinalis, Cucumber sativus, Lectuca sativus, Raphanus sativus, Sesamum indicum, Lycopersicum esculentum, and Allium fistulosum* for hypocotyl and radicle length, with potential allelopathic chemicals being oxalic

and tartaric acids. (Fujii, 2004; Parvez, 2003; Syed, 2014).

3.3.2 Analysis of Bioassay

All plants were tested for allelopathic potential initially by macerating the leaves and testing the resultant extract on the growth of green onion – 'Koba' (monocot) and Manoa lettuce (dicot). Chemical extraction was done in either water or acetone because the polarity difference (water being polar and acetone being nonpolar) between the two solvents would obtain different chemical profiles from the leaf. Analyzing the plant's respective effectiveness at a 14-15mg/mL extract solution concentration (chosen so because this dilution greatly separated those with allelopathic potential from those that did not), it was found that (of those plants not previously determined to be allelopathic by a previous study) if the growth of the lettuce and the green onion were affected by the test plant, the reduction of growth was generally greater for the monocot as compared to the dicot. Exceptions to this include strawberry guava, ohai and tamarind, which had the opposite affect and generally affected the dicots more. One reason for this differentiation between monocots and dicots could be because of the different modes of action of the allelopathic molecules. For example, if the mode of action affected auxin levels, monocots would respond differently than dicots, with monocots being less sensitive to auxin level changes compared to dicots (McSteen, 2010). A water and acetone screen (chosen for their different polarities and thus their ability to concentrate different chemical classes) of the selected plants was conducted and produced the results below. Additional data can be seen in Appendix A.

When looking at the leaf maceration data as an aggregate (Table 1), *Platymiscium stipulare* was the best candidate for whose both water and acetone macerated leaf extracts inhibited the germination and growth of both lettuce and green onion by 50% or more compared to the control. This warrants more study because, while *Platymiscium* is a genus of the Fabaceae family, not many allelopathy studies have been conducted with species in this genus. However, there exists potential allelopathic chemicals within the plant (Reyes-Chilpa, 1998). *Sesbania tomentosa*, while not having the same level of inhibition with the acetone extract for green onion, generally reduced both the water growth condition plants and acetone extract for lettuce by 50%. Much remains to be learned about *Sesbania tomentosa* as this plant species, despite being a highly versatile plant found growing in a multitude of environmental conditions, has not been

studied specifically for allelopathy. Common genus *Sesbania virgata* and *Sesbania grandiflora* have potential allelopathy (Alagesaboopathi and Deivanai, 2011; Drewes et al., 1995; Simões, 2008)

More generally, it seems that plants either excelled in inhibition of plant germination and growth with either water leaf extracts or acetone leaf extracts. For example, in water extracts, both *Prosopis pallida* and *Atriplex semibaccata*, greatly reduced both monocot and dicot growth. Perhaps not a surprising conclusion because of their classification as beach plants along with their ability to grow near the ocean and in salinated soil conditions (Getachew et al., 2012; Ibrahim, 1998; Noor et al., 1995). *Atriplex glabriuscula, Atriplex littoralis* and *Atriplex prostrata* were found to contain dry weight masses of over 30% sodium content, levels to which our lettuce and green onion would produce stunted plant growth (Ievinsh, 2021). Kiawe has previously been shown to be allelopathic but Ohai's potential may be related to relative species *Atriplex canescens* and *Atriplex lentiformis's* allelopathic abilities (Dehdari, 2009; Ebrahimi, 2016). Red and yellow *Psidium cattleianum* extracts also greatly affected both monocots and dicots, as was evident in its related species *Psidium guajava* (Chapla and Campos, 2010). *Pachira aquatic* was the single plant species that affected monocots and dicots with only its acetone leaf extract. Initially considered our control species due to a related species positive effects on plant growth, water chestnut leaves have a thick cuticle much like strawberry guava leaves and occasionally drop off sap from its leaves, potentially indicating a release mechanism for organics into the environment (Cummings et al., 2012). *Pseudoanthemum carruthersii* also surprised by showing germination and growth reduction of monocots with water extract, potentially due to the purple coloration of the leaves resulting from an accumulation of flavonoids (Vo, 2012). This was surprising but did not invalidate our results as the project had multiple negative controls moving forward, including *Metrosideros polymorpha*.

When comparing several species within the same genus, both Acacia plants produced inhibition of monocots, with *Acacia confuse* previously being illustrated as potentially allelopathic (Chou, 1998). While this is contradictory to the field observation for *Acacia koa*, due to the variety of weeds found in the field observation and the limited selection of test plants within our own study, perhaps plant specific interactions are being illustrated (Jenkins, 2020). Within the Bauhinias and the Erythrinias, each had a representative suspected allelopathic plant and several that were unknow. The non-studied trees performed relatively similar to the

representative species, with *Bauhinia purpurea* and *Erythrina abyssinica* producing results that were similar to the other Bauhinias and Erythrinias found in this study, indicating that perhaps at the genus level these plants contain something within their leaves that negatively affect external plant growth (Anthofer et al., 1998; Chou, 1980; Singh et al., 2009).

Metrosideros polymorpha was one of the plant species that did not produce a large amount of plant germination and growth inhibition for both monocots and dicots, indicative perhaps because of its role as a keystone species within native Hawaiian forests. It would be highly unusual for this keystone species, who is known to grow in tandem with many other species, to exhibit strong allelopathic properties, and as such, the results of this species' bioassay are consistent (Friday and Herbert, 2006).

Table 1: Species tested for potential allelopathic effects by leaf maceration with water or acetone chemical extraction. All plant species were identified on Oahu. Green onion – 'Koba' (monocot) and Manoa lettuce (dicot) were used as test species. Native Hawaiian species are indicated by an "*****". N.D. indicates no data currently available in the literature. More "+" Indicate higher allelopathic activity (greater plant growth inhibition and lower resultant fresh mass). "-" indicates no data available in the bioassay. Each "+" represents 20% growth inhibition compared to its respective category control.

Species	Common Name	Previously Identified Allelopathy	Allelopathic Activity at 4% Extract Concentration (14 mg/mL) Water		Allelopathic Activity at 6% Extract Concentration (15mg/mL) Acetone	
			Monocot	Dicot	Monocot	Dicot
Acacia confuse	**Formosan Koa**	**Chou, CH et al.**	++++	++	+	+
Acacia koa	**Koa***	**N.D.**	+++++	++	+++	++
Atriplex semibaccata	**Australia Salt Bush**	**N.D.**	+++++	+++++	+	+
Bauhinia blakeana	**Hong Kong Orchid Tree**	**N.D.**	+++	+	++	+
Bauhinia galpinii	**Pride of De Kaap**	**N.D.**	++++	+	+	+
Bauhinia monandra	**Napoleon's Plume**	**N.D.**	+++	+	-	-
Bauhinia purpurea	**Butterfly Tree**	**Singh, B, Jhaldiyal, V, and Kumar, M.**	+++	++	++	+
Erythrina abyssinica	**Red Hot Poker Tree**	**Anthofer, J, Hanson J, and Jutzi SC.**	++++	++	++	++
Erythrina crista-galli	**Corkspur Coral Tree**	**N.D.**	++++	++	+	+
Jatropha integerrima	**Peregrina/Spicy Jatropha**	**N.D.**	+++	+	++	++
Metrosideros polymorpha	**Ohia Lehua***	**N.D.**	++	++	+	+

Pachira aquatica	**Water Chestnut**	**N.D.**	+	+	+++++	++++
Platymiscium stipulare	**N.D.**	**N.D.**	+++++	+++	+++++	+++++
Plectranthus amboinicus	**Cuban Oregano**	**Pinheiro, PF et al.**	+++	+	+	+
Prosopis pallida	**Kiawe**	**Getachew, S, Demissew, S, and Woldemariam, T.**	+++++	+++	++	++
Pseuder-anthemum carruthersii	**Carruthers' Falseface**	**No, USDA**	++++	+	+	+
Psidium cattleianum (Yellow)	**Strawberry Guava**	**N.D.**	++++	+++++	+	+
Psidium cattleianum (Red)	**Strawberry Guava**	**N.D.**	++++	+++++	+	+
Sesbania tomentosa	**Ohai***	**N.D.**	+++++	+++	++	+++++
Tamarindus indica	**Tamarind**	**Parvez, SS et al**	+	+++++	-	-

With the large amount of data presented, trends can be hard to distinguish. As such, a subset of data utilizing *Pachira aquatica, Psidium cattleianum* Y*,* and *Pseuderanthemum carruthesii* is provided in Figure 16. The sensitivity of the bioassay can be shown in multiple ways. When looking at a comparison between *Pachira aquatica* and *Psidium cattleianum* Y, we see that *Pachira aquatica* has significant plant growth reduction in only the acetone extracts while *Psidium cattleianum* Y has significant plant growth reduction in only the water extracts. This shows the polarity difference of the extracts and that neither condition results in background plant growth inhibition. There are chemicals being extracted from the plant leaves that result in this plant growth reduction, not from a factor of the bioassay itself. These two plants either affect both lettuce and green onion growth or neither depending on what polarity range of chemicals extract is utilized. When comparing *Psidium cattleianum* Y*,* and *Pseuderanthemum carruthesii*, we see that in the water extract, *Psidium cattleianum* Y results in significant plant growth inhibition of both lettuce and green onion, while *Pseuderanthemum carruthesii* has inhibition only in the green onion. This shows that even within the same condition, the test plants can respond either similarly or differently based upon the chemicals being extracted from the leaves.

These initial screen procedures produced promising data, with water extracts from *Atriplex semibaccata,* both varieties of *Psidium cattleianum*, and *Tamarindus indica* and acetone extracts from *Patricia aquatica* and *Platymiscium stipulare* exhibiting early initial indications of allelopathic potential and carried to the next step of analysis.

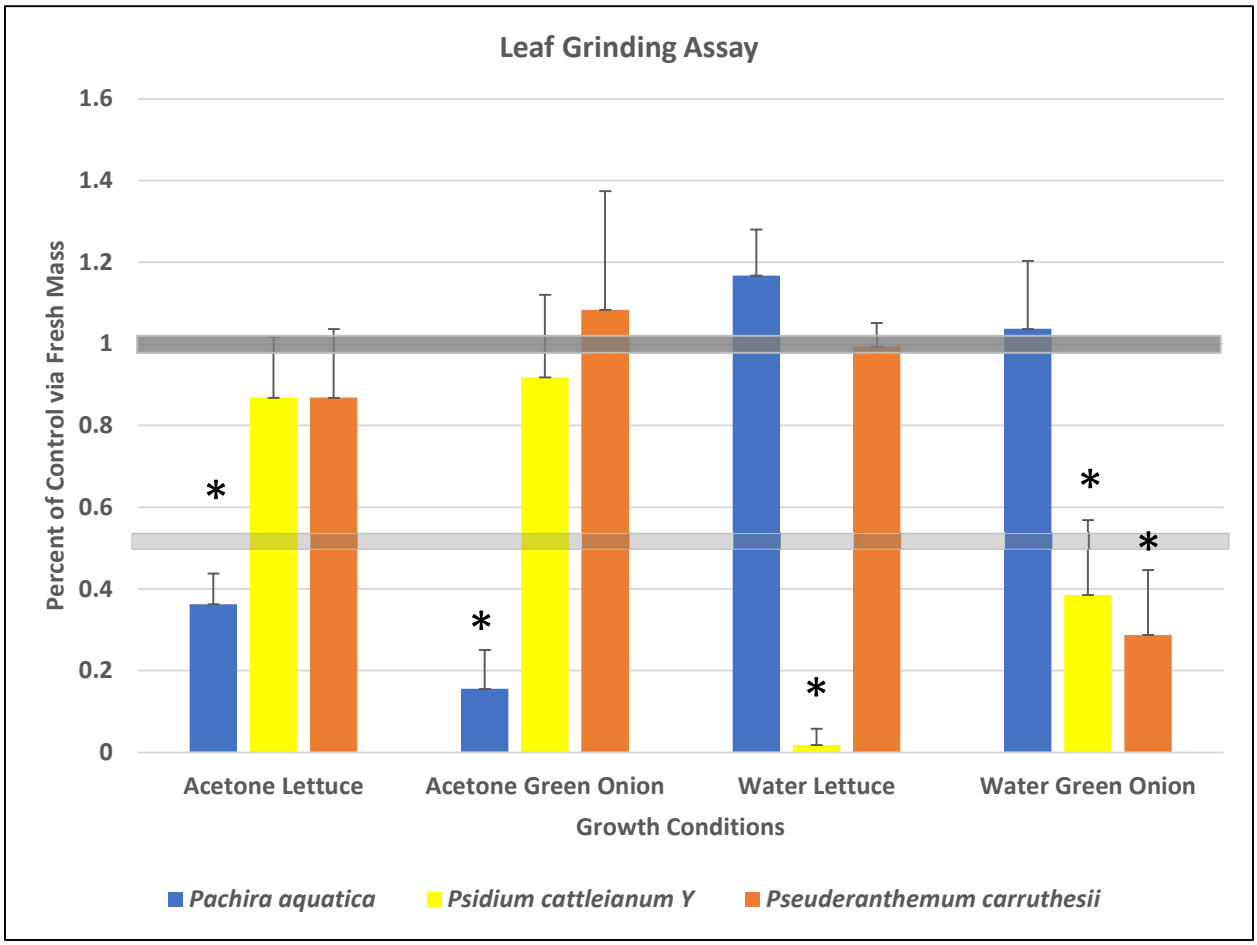


Figure 16. A subset of data showing sensitivity between water and acetone extracts along with sensitivity between lettuce and green onion responses to different experimental condition. The "*" denote a significance between the control and the experimental with $P < 0.01$ utilizing Anova: single factor. The dark horizontal line indicates 100% control growth while the smaller gray line indicates 50% control growth. N = 18

3.3.3 Analysis of Leaf Soak

Literature has shown that grinding of the leaf may produce false positives for illustration of allelopathy due to cellular contents (Harper, 1975). As such, the next step to validate the bioassay as being non-biased was to conduct a leaf soak of plant material in water, to replicate natural leaf degradation and expulsion of chemicals into the surrounding environment. This would also have other environmental implications including the possible buildup of chemicals in

surrounding soil to toxic levels along with potentially changing soil microbial composition, pH and other macro and micronutrient content.

Results generally fell into 4 categories (Initial high activity, Terminal low activity (1), Initial and Terminal high activity, Median low activity (2), Initial low activity, Terminal high activity (3), and Consistent high activity (4)), with green onion growth trends similar to those seen with lettuce. Additional data can be seen in Appendix B. Category 1 (Initial high activity, Terminal low activity) is characterized by having earlier days such as day 1 and day 2 fresh mass being generally lower than later days day 16 and day 32 and can be exhibited by having an easy or intentional release of plant growth inhibiting chemicals. The leaves themselves maintain their shape and rigor throughout the time course due to the thick glossy cuticle. These leaf characteristics are exhibited with both varieties of strawberry guava as well as ohia lehua. For ohia lehua, a member of the myrtle family, its ability to consistently reduce the growth of our test plants lettuce and green onion could be related to its nature as a first responder, being able to colonize lava fields and other hazardous environments. While this is conversely related to its role as a keystone species, the plasticity in terms of shrub vs tree forms, leaf colors and shapes, and floral colors speaks to the varying physiological and thus resultant chemical aspects of this plant contained and exhibited within its leaves (Friday and Herbert, 2006).

For strawberry guava (Figure 17), the greatest days of growth inhibition are in day 1 and day 2, after which the remaining days are markedly less affected. This can be attributed to the suspected allelopathic nature of strawberry guava, of which, after dispersion of its plant growth inhibiting molecules in question via soaking into the surrounding water, the plant no longer has the ability to negatively affect the growth of the test crops. This can also be attributed to the glossy cuticle of the strawberry guava leaves, which helped the leaves maintain rigor and overall structural integrity through day 32, allowing only what was designed to be released to leech into the water (Chapla and Campos, 2010). The red variety of strawberry guava also holds a stronger growth inhibition on day 2 compared to the yellow variety, an interesting result as the red fruit also holds a higher content of polyphenolic compounds including the flavonoid, hyperoside, and more of the anthocyanidin, cyanidin (Biegelmeyer, 2011).

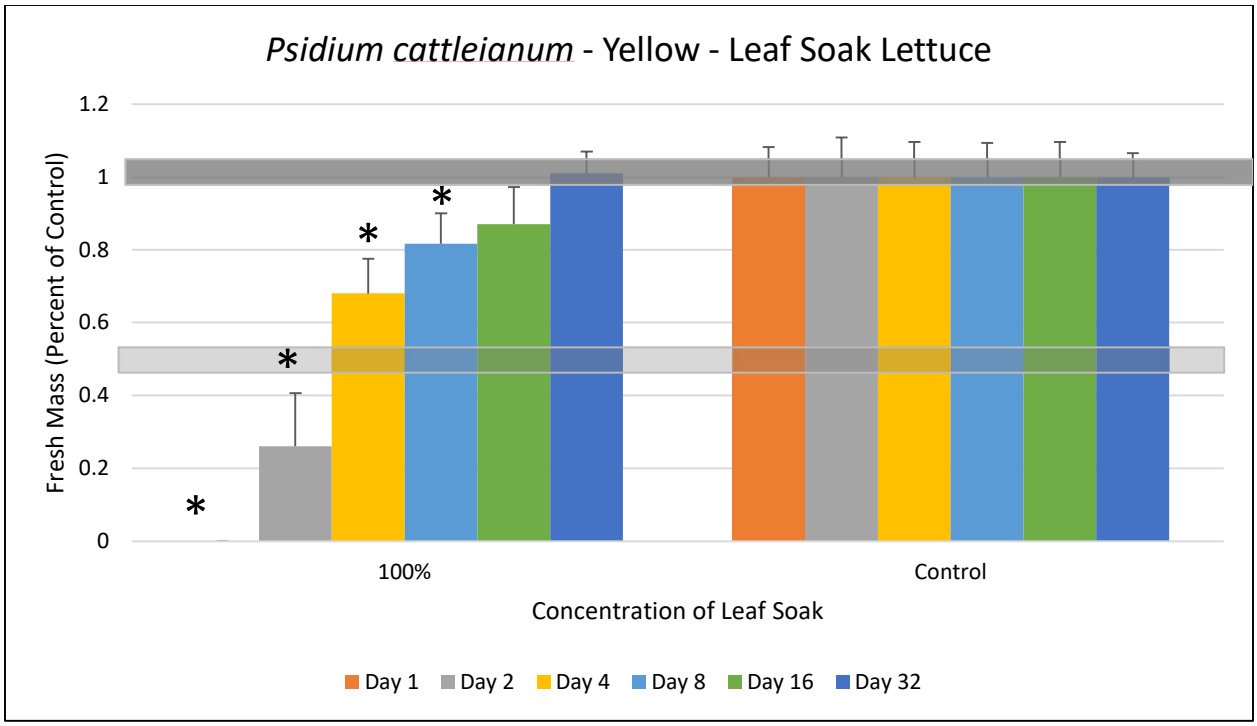


Figure 17. A time course of continuous soaking of *Psidium cattleianum* yellow variety leaves and their effect on the growth of lettuce. This highlights the trend seen in Type 1: Initial high activity, Terminal low activity. The dark horizontal line indicates 100% control growth while the light grey line indicates 50% control growth. Values are fresh mass displayed as a percent of the control. Inhibition of growth was greatest in day 1, and 2, with the least plant growth inhibition on day 32. The "*" denote a significance between the control and the experimental with $P < 0.01$ utilizing Anova: single factor. $N = 18$

The next category of interest (Initial and terminal high activity, Median low activity (2)), which is characterized by having early days, such as day 1, and later days, such as day 32, closely aligned in similarly high levels of growth inhibition. The day with the least inhibition is usually at the end of the first quarter of the month, or day 8. This category includes the plants *Sesbania tomentosa* and *Pseuderanthemum carruthersii*. While both plants possibly resulted in low growth of the test plants at the later sampling dates due to pronounced leaf decomposition, their early inhibition reasoning may differ from each other. *Sesbania tomentosa* has fragrant leaves with a plentiful number of hairs on each leaf, indicating the potential presence of chemical volatiles and leaf structures designed for release of materials (Alagesaboopathi and Deivanai, 2011). *Pseuderanthemum carruthersii*'s (Figure 18) leaves are large and required breaking up into chunks before being placed within the containers. As such, their purple coloration, which can be attributed to the leaves containing and subsequently releasing a variety of chemicals

(including luteolin and apigenin) that may have inhibited the growth of our test plants (Vo, 2012).

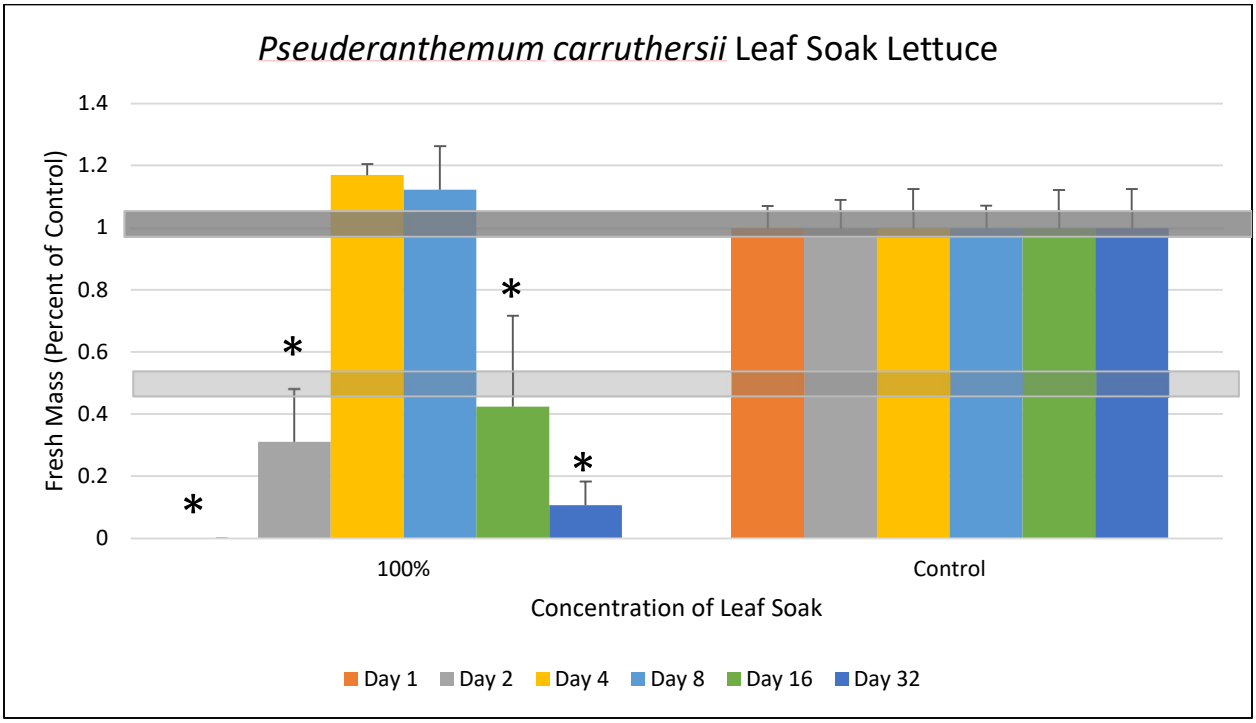


Figure 18. A time course of continuous soaking of *Pseuderanthemum carruthersii* leaves and their effect on the growth of lettuce. This highlights the trend seen in Type 2: Initial and terminal high activity, Median low activity. The dark horizontal line indicates 100% control growth while the light grey line indicates 50% control growth. Values are fresh mass displayed as a percent of the control. Inhibition of growth was greatest in day 1, and 32, with the least plant growth inhibition on days 4 and 8. The "*" denote a significance between the control and the experimental with $P < 0.01$ utilizing Anova: single factor. $N = 18$

The third category (Initial low activity, Terminal high activity (3)), characterized by having early days with high levels of growth compared to later days which have a lower level of growth, includes the plant *Pachira aquatica* (Figure 19). This pattern follows previous bioassay, which found that the easily water soluble components of the *Pachira aquatica* leaf was non-allelopathic. The resultant decline in growth as time progresses may be the release of the non-polar constituents of the leaf through decomposition and resultant growth inhibition. While a few other species have exhibited growth slightly greater than the control, this stands out as being the largest positive growth difference. While potentially contributing more nitrogen and other essential nutrients and minerals due to leaching from the leaf, it is a variation that requires more study, yet is similar to the previously mentioned boost to the growth of *Saccharum spontaneum* seen with *Pachira quinate's* leaf litter (Cummings et al., 2012).

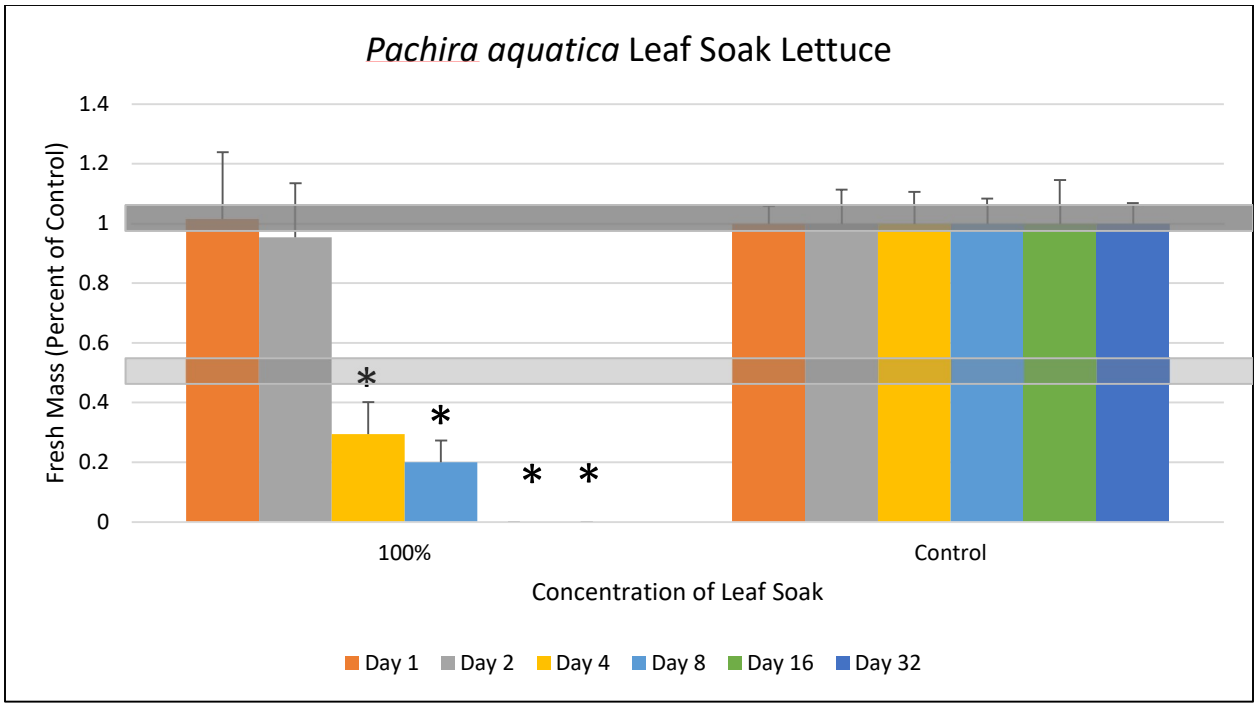


Figure 19. A time course of continuous soaking of *Pachira aquatica* leaves and their effect on the growth of lettuce. This highlights the trend seen in Type 3: Initial low activity, Terminal high activity. The dark horizontal line indicates 100% control growth while the light grey line indicates 50% control growth. Values are fresh mass displayed as a percent of the control. Inhibition of growth was greatest in day 16 and 32, with the least plant growth inhibition on days 1 and 2. The "*" denote a significance between the control and the experimental with $P < 0.01$ utilizing Anova: single factor. $N = 18$

Pachira aquatic and strawberry guava have inverse levels of pH as seen below. This is reflected in their day course percent of fresh mass data, where low pH is correlated with low fresh mass and vice versa.

Table 2: pH measurements of *Pachira aquatica* and strawberry guava taken from their leaf soak time course. *Pachira aquatica* saw a pH decrease as time elapsed while strawberry guava saw a pH increase.

Plant Species	Day 1 Leaf Soak pH	Day 2 Leaf Soak pH	Day 4 Leaf Soak pH	Day 8 Leaf Soak pH	Day 16 Leaf Soak pH	Day 32 Leaf Soak pH
Pachira aquatica	6.13	5.1	5.17	5.27	4.94	4.72
Psidium cattleianum Red	4.23	4.51	5.01	5.32	5.55	6.16
Psidium cattleianum Yellow	4.49	4.62	4.82	4.8	5.55	6.35

Serial dilution of a pH 4 (water + HCl) solution had no statistical significance in the growth of both lettuce and green onion. This is because the addition of water to an unbuffered pH solution should not change pH greatly because H+ ion levels are staying relatively the same. Thus dilutions shouldn't affect pH greatly and should not affect the growth of the plants. In addition, growing lettuce and green onion in a range of pH solutions (4-6.5) produced no significant growth differences within the pH range. While there have not been much seeding growth studies quantifying pH affects in our two cultivars (UH Manoa lettuce and green onion – 'Koba'), our study only grew the test plants for 5 days, a time when the seedlings are utilizing endosperm nutrients. Additional data can be seen in Appendix C. pH is not a great factor within our study as long as there is water for seed germination because pH is a greater factor upon pulling nutrients from soil/hydroponics as low or high pH reduces the availability of nutrients to the plant and can cause nutrient deficiency in mature plants (Anderson, 2017; Kane, 2006; Samarakoon, 2020).

The last category (Consistently high activity (4)) is characterized by having continuous plant growth inhibition throughout the entire 32 day time frame and contains the plant *Prosopis pallida*, a known allelopathic plant that can grow in the harshest of conditions and can currently be found across the Hawaiian island chain (Figure 20) (Noor et al., 1995).

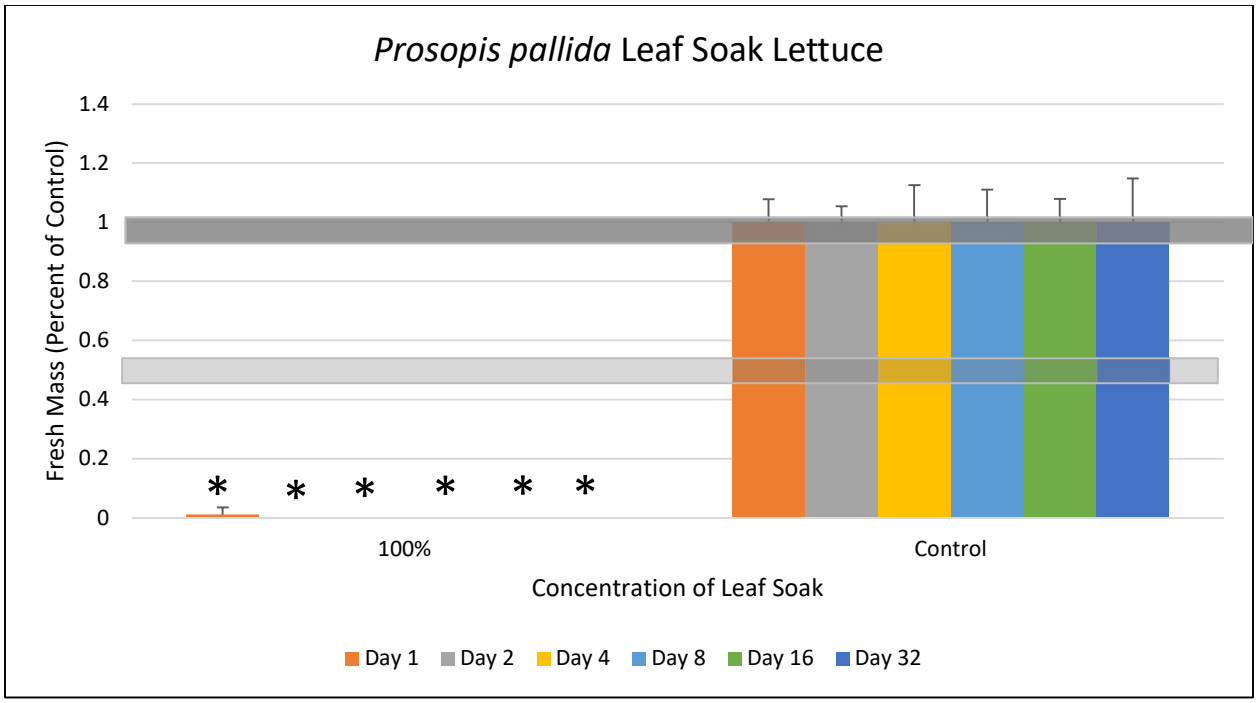


Figure 20. A time course of continuous soaking of *Prosopis pallida* leaves and their effect on the growth of lettuce. This highlights the trend seen in Type 4: Consistently high activity. The dark horizontal line indicates 100% control growth while the light grey line indicates 50% control growth. Values are fresh mass displayed as a percent of the control. Inhibition of growth was greatest in day 16 and 32, with the least plant growth inhibition on days 1 and 2. The "*" denote a significance between the control and the experimental with $P < 0.01$ utilizing Anova: single factor. N = 18

This has shown the creation of a non-biased bioassay for detection of potential allelopathic properties from a solution containing suspected allelopathic properties. While the method of extraction may vary and produce a more complete picture of a plant's potential allelopathy, the bioassay portrayed here itself does not discriminate and cause allelopathy due to systemic bias. Strawberry guava has garnered attention for inhibiting test plant growth in both the maceration extraction and the leaf soak analysis and garners further investigation.

Chapter 4: Illustration of Strawberry Guava Allelopathy

4.1 Introduction

A bioassay with minimum bias has been produced to test for allelopathy. Now, the investigation continues by focusing on an invasive species in the surrounding community, strawberry guava. As detailed previously, the allelopathic potential of *Psidium cattleianum* was first brought to attention when the invasive common or American guava, *Psidium guajava*, was found to have allelopathic chemicals within its leaves. By grinding up the guava leaves and spraying a 10% extract concentration mixture on young lettuce plants and lettuce seeds, there was an almost 45% reduction of growth and germination rates as compared to non-affected plants and seeds (Chapla and Campos, 2010). However, the researchers did not test if there were differential effects of the extract on monocots vs dicots, a distinguishing test because monocots and dicots are different classes of plants that are affected by herbicides differently depending upon the herbicide's mode of action. For example, synthetic auxin herbicides such as 2-4 D, affect dicots more greatly than monocots and are often utilized to selectively control broadleaf (dicot) weeds in cereal (monocot) crops (McSteen, 2010). On the other hand, there exist grass selective herbicides such as clethodim, which will more greatly harm monocots compared to dicots due to the monocot's more sensitive acetyl-coenzyme A carboxylase inhibition by this class of chemical (Rendina and Felts, 1988). Within the same genus as *Psidium guajava* exists strawberry guava, *Psidium cattleianum*, an invasive species that has also colonized the Hawaiian Islands in large groves, linking local knowledge with the possibility that the strawberry guava plant might also have allelopathic properties (U.S. Forest Service, 2016; Wikler, 2000). More is known about the common guava as it has been well studied for its various uses.

The common guava (*Psidium guajava)* is native to Central and South America. Its fruit is edible and it is cultivated around the tropics for agricultural and medicinal applications, building material, and firewood, with wild groves quickly becoming established with help from their allelopathic activity. Aqueous extracts of its leaves inhibited the germination and growth of the model plant lettuce, the invasive weeds *Parthenium hysterophorus* (Santa Maria feverfew) and *Cassia occidentalis L* (‘au‘auko‘i in the Hawaiian language) and the tree *Croton megalocarpus*

(Croton) (Chapla and Campos, 2010; Kapoor, 2019; Kawawa, 2016). However, due to the conditional and species-specific response to allelochemicals, it was found that *Helianthus annuu)* (sunflowers) and the trees *Markhamia lutea* (Nile tulip) and *Diospyros mespiliformis* (African ebony) were not affected with aqueous extracts up to 20% concentration (El-Rokiek et al., 2012; Kawawa, 2016). Sunflowers (*Helianthus annuus*) are important crop plants for oil production but is limited by competition with weeds such as *Portulaca oleracea* and nematodes (specifically *Meloidogyne incognita*) costing farmers yield losses of up to almost 50% (El-Rokiek et al., 2012). *Portulaca oleracea* (purslane), is a host plant to a variety of nematodes, especially the sunflower damaging nematode *M. incognita*. To combat these threats, farmers often utilize synthetic pesticides and herbicides in an attempt to control this damaging weed-nematode relationship, a solution that is not considered environmentally friendly. It was found that, when utilizing guava leaves, either as an aqueous extract or as leaf litter, sunflower yield was increased while the number of nematode galls and egg masses and purslane growth and germination rates were reduced (El-Rokiek et al., 2012). In addition, guava leaves as a mulch were found to decrease weed density in fields of *Solanum lycopersicum* (Mabele and Ndong'a, 2019). This provides evidence of the potential for guava as an alternative method to combat various weeds and nematodes in sustainable agriculture (Jabran et al., 2015).

With the current literature, it was decided to look further into the application potential of a purportedly allelopathic close cousin of the guava, *Psidium cattleianum* (strawberry guava). Strawberry guava is an ornamental fruiting tree native to Brazil that was brought to Hawaii in 1825 by traders for firewood, fruit, and aesthetic purposes. The tree grows up to 16ft tall in elevations ranging from sea level to 4000ft and produces small edible red or yellow fruit year-round. The trees also have economic traits that are utilized by small scale farmers around the tropics including for firewood, fruits and animal feed (U.S. Forest Service, 2016). The wood and leaves of the trees have a glossy texture that is often used in landscaping and furniture and can be burned to smoke meat. The fruit has tart skin and sweet insides, which can be harvested and made into jam, pies, or eaten raw. Current literature suggests that there is a chemical as well as physical difference between the red and yellow fruit (Biegelmeyer, 2011). The seeds can be roasted and eaten as a snack and the leaves can be made into tea. In addition to these traditional uses, there are indications that the strawberry guava tree may harbor a natural herbicide

(Motooka, 2003). Regardless of its potential, the tree is considered to be one of the worst invasive species to have impacted the state of Hawaii.

Strawberry guava trees spread rapidly and are extremely resistant to drought conditions, currently covering hundreds of acres of once native tropical rainforest. The trees crowd out native plants, disrupt animal communities, alter water flow and provide refuge for other introduced species such as fruit flies. During their study of the tree, the US Forest Service found that forests overrun with strawberry guava lose 25% more water to the atmosphere as compared to native forests, reducing overall groundwater retention and reservoir recharge. After a decade of research, the US Forest Service is in the initial stages of implementing the introduction of a natural predator, the beetle *Tectococcus ovatus*, to stem the expansion of the tree (U.S. Forest Service, 2016)

While the state implements the last resort of introducing another non-native species to combat the growing threat, our lab is looking to repurpose the tree into something that may one day assist organic farms and home gardeners in their battle with weeds. While much research remains to be done to commercialize the product, there is hope that this may one day provide the source for a local, organic, human and pet friendly herbicide harvested, refined and sold all in same state.

The research continues into strawberry guava's leaf chemicals, specifically how they spread and what parts of the test plants (lettuce and green onion) they effect. While no two allelopathic chemicals are the same, the symptoms of their affects often overlap and include stunted growth, burned roots and lack of germination to name a few. In order to bridge the gap between lab-based bioassays and outdoor soil trials, we conducted a modified agar bioassay utilizing leaf tissue to see if the allelopathic affects remain and helps to validate soil tests (Dornbos and Spencer, 1990; Nakano, 2001). Lastly, soil trials were conducted as both pre and post germination greenhouse assays were conducted for barnyard grass (monocot) and pigweed (dicot), two agricultural weeds that have significant effects on total crop yields and harvests.

4.2 Materials and Methods

4.2.1 Plant Material

Barnyard grass (*Echinochloa sp.*) and red root pigweed (*Amaranthus retroflexus*), both noxious weeds, were purchased from Azlin Seed Services in Mississippi, USA.

4.2.2 Soil Analog Experiment

To determine the effect of the potential allelochemical(s) on root and shoot length, 200mg of strawberry guava leaf (either fresh or freeze dried) was placed (or ground with liquid nitrogen then placed) into the center of a dish (9 x 4.5 cm) containing 200mL of cooled autoclaved 0.4% agar culture medium (Figure 21). A central cylinder core had been excavated (2cm diameter x 1.75cm deep), into which the material was inserted and covered with remaining agar culture medium cooled to 40° C. Lettuce (*Lactuca sativa* L.) seeds, as the test species, were placed on the agar culture medium in three concentric circles at increasing 10mm intervals from the core. The dishes were covered in foil and placed into climate-controlled darkness for 3 days at 25°C within a New Brunswick Scientific Innova 4230, after which the radicals and hypocotyls of each lettuce seedling was measured. Protocol was adopted from (Nakano, 2001).

Figure 21. An example of the soil analog experiment, where 200mg of strawberry guava leaf (shown here as fresh or freeze dried) was ground with liquid nitrogen then inserted into the center of a dish (9 x 4.5 cm) containing 200mL of cooled autoclaved 0.4% agar culture medium. A central cylinder core had been excavated (2cm diameter x 1.75cm deep), into which the material was inserted and covered with remaining agar culture medium cooled to 40°C. Lettuce (*Lactuca sativa* L.) seeds, as the test species, were placed on the agar culture medium in three concentric circles at increasing 10mm intervals from the core.

4.2.3 Greenhouse Experiment

To determine the effect of the potential allelochemicals when application is simulating a "natural" environment, 7cm x 7cm x 7cm pots were filled with Sungro Horticulture: Professional Growing Mix sown with 9 seeds of either pigweed or barnyard grass grown in a grid formation

within each pot. Pots were grown in flats of 21 members. Application of 14mL of 100% strawberry guava day 2 leaf soak extract (either yellow or red variety) was done with a hand sprayer held 4.5cm above the soil to each pot. A single application was done to each pot either at the time of sowing (pre-germination condition) or after 2 weeks of growth (post-germination condition). Pots were shielded from spraying drift by physical barriers between pots and a water control was utilized for each condition. Root and shoot length of the pigweed and barnyard grass were measured 2 weeks after application of extracts. Total growth time for pre-germination assay was 2 weeks while total growth time for post-germination was 4 weeks.

4.3 Results and Discussion

The next step was to proceed with the initial allelopathic screening by macerating strawberry guava leaves to extract potent chemicals utilizing the same extraction and bioassay materials and procedures as described in Chapter 3. Water extracts of macerated strawberry guava leaves inhibited both the germination and growth of lettuce and green onion, a dicot and monocot respectively (Figure 22 and 23). This leaf maceration inhibition result has been postulated to perhaps have been caused by the levels of macro or micronutrients contained within the leaf. In a comparison of micro and macro minerals of regular guava to strawberry guava, it was found that strawberry guava had slightly higher levels of Mg (0.46% dry leaf weight) and moderately higher levels of Na (0.68% dry leaf weight), levels that are not different enough to cause plant growth differences (Adrian, 2015). This shows that the allelopathic chemical is water soluble, and likely charged, with it affecting both major groups of weeds.

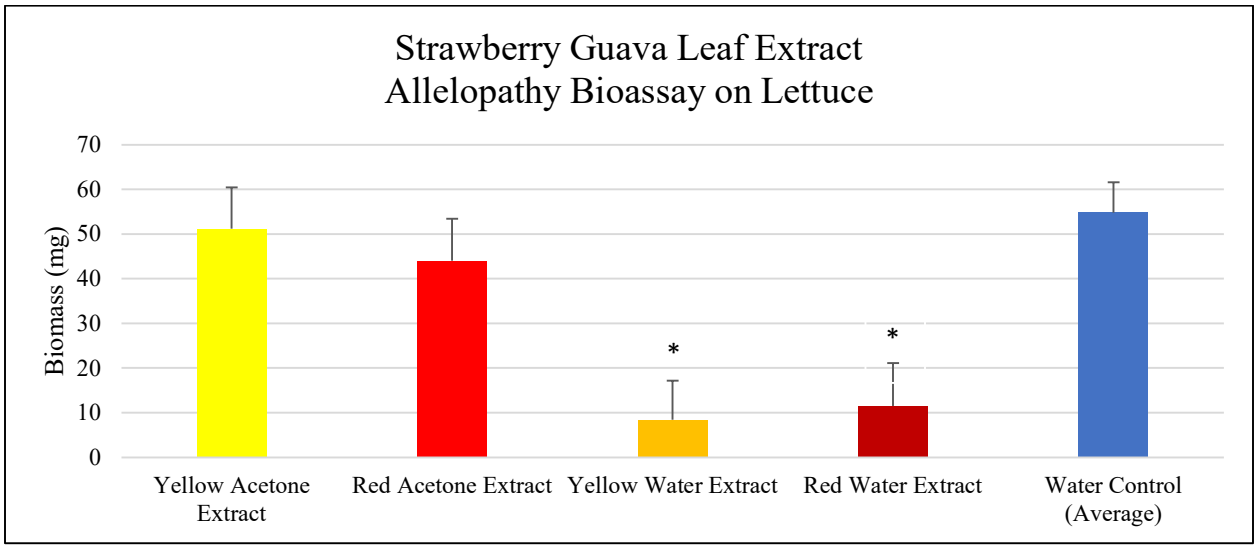


Figure 22. A comparison of lettuce growth in macerated strawberry guava water leaf extract (10.5 mg/mL) vs acetone leaf extract (11.23 mg/mL) utilizing the cell-well allelopathy bioassay. The "*" denotes a significance between the extract and the control with $P < 0.05$ utilizing ANOVA: Single factor. N = 18

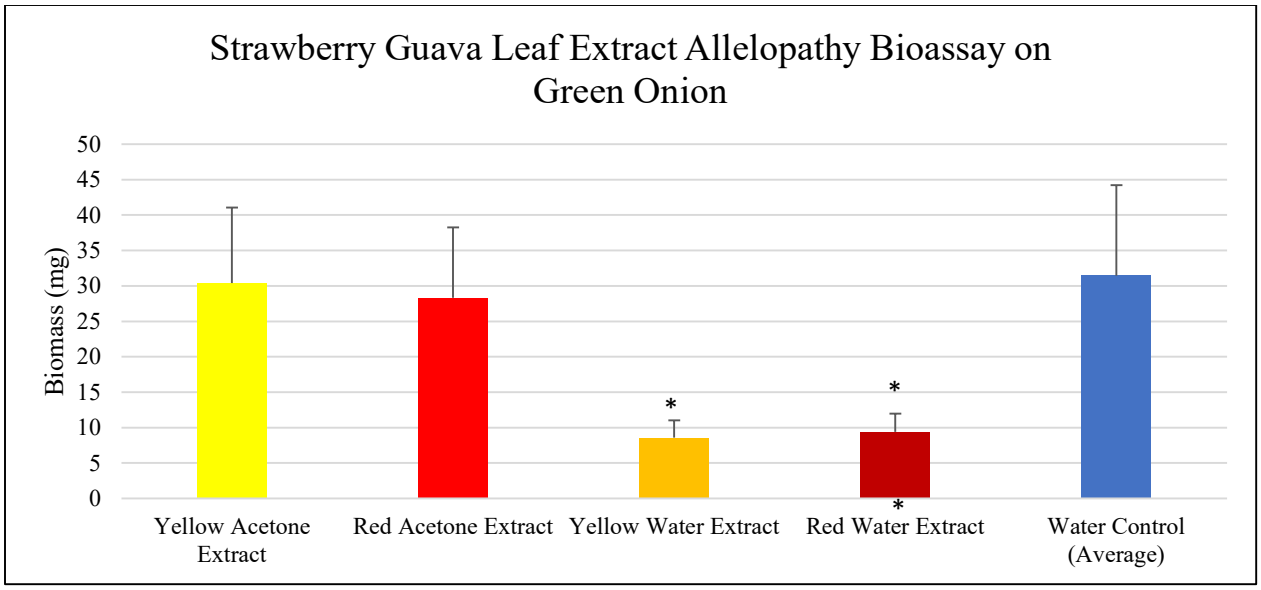


Figure 23. A comparison of green onion growth in macerated strawberry guava water leaf extract (10.5mg/mL) vs acetone leaf extract (11.23mg/mL) utilizing the Cell-Well Allelopathy Bioassay. The "*" denote a significance between the extract and the control with $P < 0.05$ utilizing Anova: Single factor. N = 18

Next, a dilution series was created to determine at what concentration of water soluble material was necessary for plant growth inhibition (Figure 24). The water-soluble allelopathic activity could be diluted to zero, allowing us to calculate an IC_{50} (Inhibition concentration 50%) value utilizing the Instituto Nacional de Enfermedades Respiratorias's Mario Vargas's April 2000 IC_{50}plus v1.0 worksheet. For the lettuce, the yellow strawberry guava water leaf extract completely inhibits germination at 17.5 mg/mL while the red strawberry guava water leaf extract completely inhibits germination at 13.2 mg/mL. The IC_{50} value, the chemical concentration at which to achieve 50% plant growth inhibition, for the yellow strawberry guava water leaf extract was 10.74 mg/mL, and the IC_{50} for the red strawberry guava water leaf extract was 9.57 mg/mL. For the green onion dilution series, the yellow strawberry guava water leaf extract and the red strawberry guava water leaf extract never caused a complete inhibition of growth. The IC_{50} value for the yellow strawberry guava water leaf extract was 12.2 mg/mL, while the red strawberry guava water leaf extract was 10.67 mg/mL. In addition, the red raw leaf extract tended to be more potent than the yellow raw leaf extract, reflected in the IC_{50} values. While these may be relatively high compared to other IC_{50} values for their respective target sites, including natural product herbicides such as 0.00405mg/mL for Sarmentine, 0.015mg/mL for Manuka oil,

0.00314mg/mL for Leptospermone, and 0.00022mg/mL Grandiflorone, the dose response curve produced by these raw extracts is similar to that of pure compounds (Dayan, 2015, 2007).

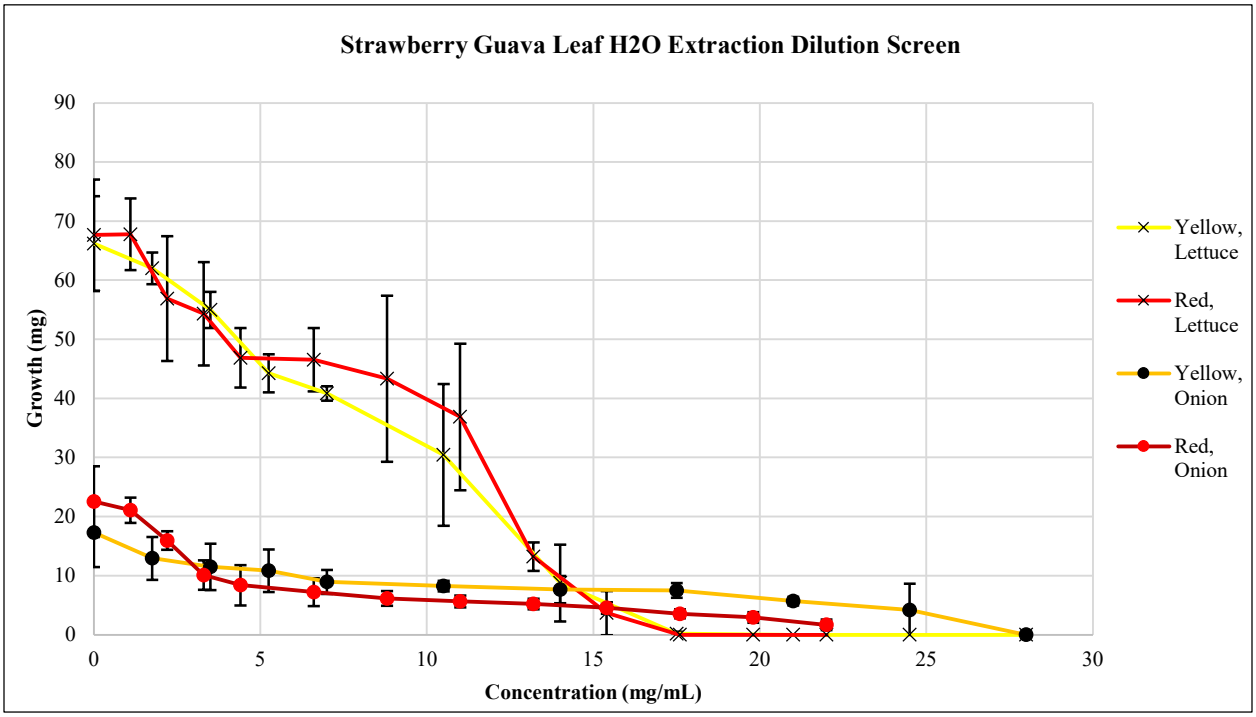


Figure 24. A comparison of the growth of lettuce and green onion in red strawberry guava water leaf extract vs yellow strawberry guava water leaf extract. The IC_{50} value for the yellow strawberry guava water leaf extract was calculated to be at 10.74 mg/mL for lettuce and 12.2 mg/mL for green onion, while the red strawberry guava water leaf extract was determined to be 9.57 mg/mL for lettuce and 10.67 mg/mL for green onion, indicating that the red strawberry guava water leaf extract is more potent than the yellow strawberry guava water leaf extract. N = 18

Strawberry guava leaves have a dry matter percentage of 34.51% (water content 65.49%) (Adrian, 2012) and a Na dry matter percentage of 0.68% (Adrian, 2015). This allows estimation of the salt content of 50g of fresh mass strawberry guava leaves as roughly 0.117g of Na (equivalent to 0.298g NaCl). Assuming full extraction of the salt content in our extraction (50g leaf in 50mL water = 0.298g NaCl in 50mL water, 0.102M). The salt concentration of strawberry guava leaves statistically affected the growth of both the test plants lettuce and green onion as seen in previous studies, with green onion having the exhibited mild tolerance to salt (Garrido, 2014; Shimose and Hayashi, 1983). This provides evidence that salt may contribute to the reduction in growth as seen in previous and subsequent bioassays but is not the only factor contributing as the effect on lettuce and green onion that was seen in the salt trials does not equal the effect observed in the bioassay. Additional data presented in Appendix D.

To test the viability that strawberry guava leaves were releasing potential allelopathic chemicals as they were breaking down, a leaf soak time course experiment was conducted, but this time the leaf soak extracts were concentrated by lyophilisation and resuspended in water (Figure 25-28). It was previously found that some leaves released their allelopathic chemicals into the water (Nakano, 2002), and this was also the case in strawberry guava, as the 2, 4 and 8 day time lapse had similar levels of allelopathic affects. However, as time went on, inhibition of growth was weaker even though the leaves were continuing to break down, as seen in the majority of the day 16 vs day 32 reduction in inhibition trends, which were often less effective than their early day counterparts. While we previously reported that strawberry guava leaves retained their structural integrity throughout the entire experiment, that comparison was made in relation to the other plant's leaves conditions. Here, the leaf analysis was made based upon visual observation, including discoloration and olfactory differences of later day leaves compared to freshly harvested leaves. This might indicate that the leaves have released all of their allelopathic chemials, and now the growth inhibiton being seen is being caused by cellular breakdown byproducts and fermentation. The red variety has only a weakening of activity in day 32, as compared to the yellow which had a weakening in day 16 and day 32. This may have been caused by the different level of allelopathic chemicals in its leaves, with the red variety being slightly more detramental to the test plants possibly due to its higher concentration of chemicals.

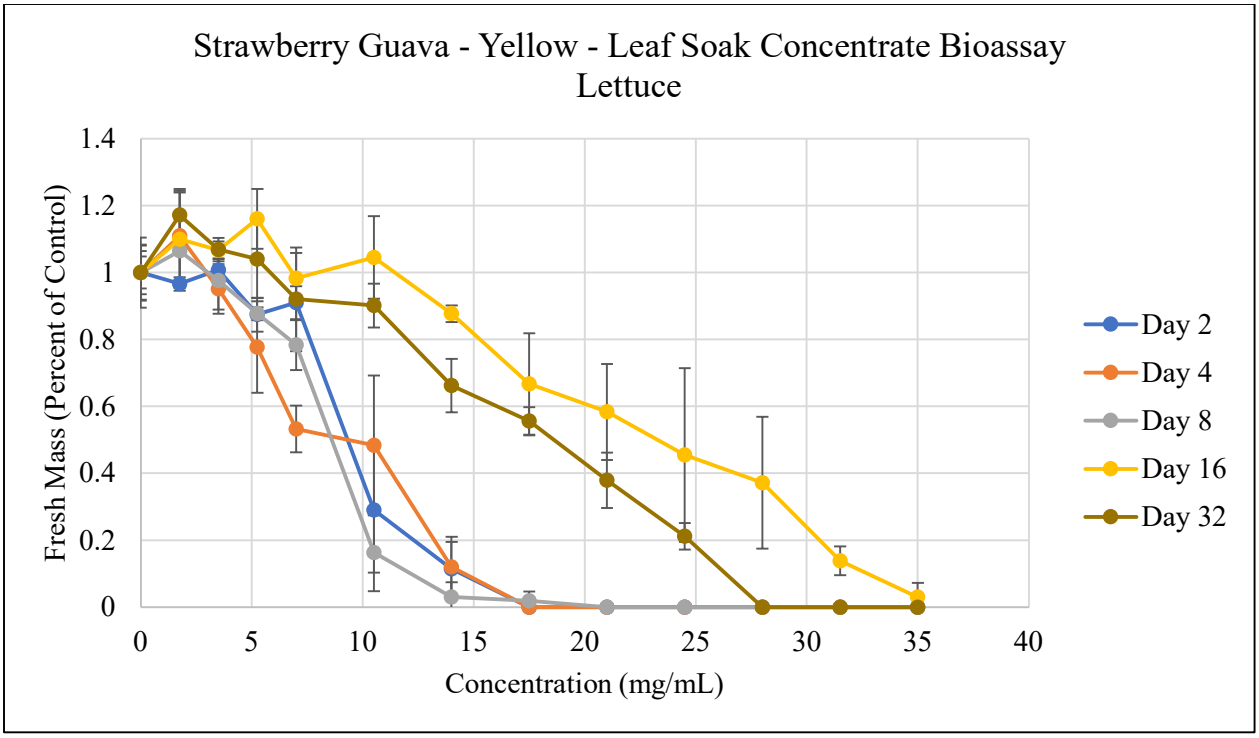


Figure 25. A time course of continuous soaking of yellow strawberry guava leaves and its effects on the growth of lettuce As time passed, less plant gro25wth inhibition was observed, as seen with the difference between the 8 day and the 16 day and 32 day inhibition trends. However, this time course produced interesting data because, contrary to expected results, day 16 was the weakest instead of day 32 at growth inhibition. N = 18

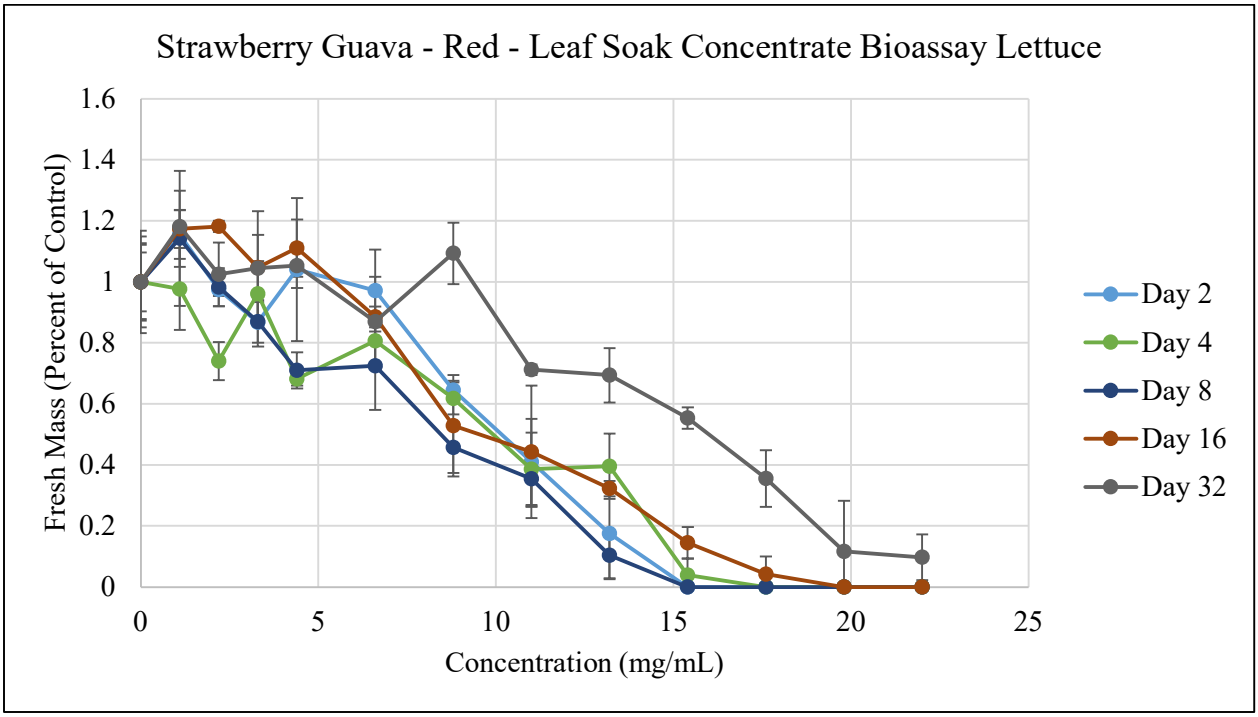


Figure 26. A time course of continuous soaking of red strawberry guava leaves and its effects on the growth of lettuce. As time passed, less inhibition of growth was observed, as seen with the difference between the 16 day and 32 day inhibition trends. N = 18

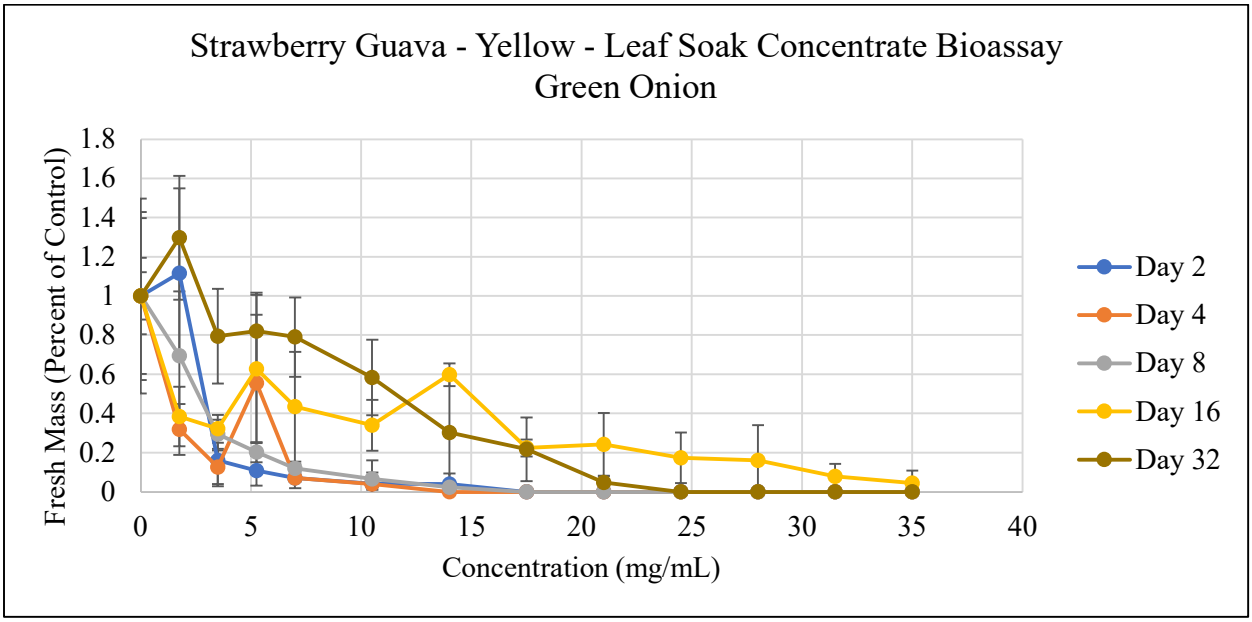


Figure 27. A time course of continuous soaking of yellow strawberry guava leaves and its effects on the growth of green onion. As time passed, there was an increase in growth, as seen with the difference between the 8 day and the 16 day and 32 day inhibition trends. However, this time course produced interesting data because, contrary to expected results, day 16 was the weakest instead of day 32 at growth inhibition at the higher concentrations. N = 18

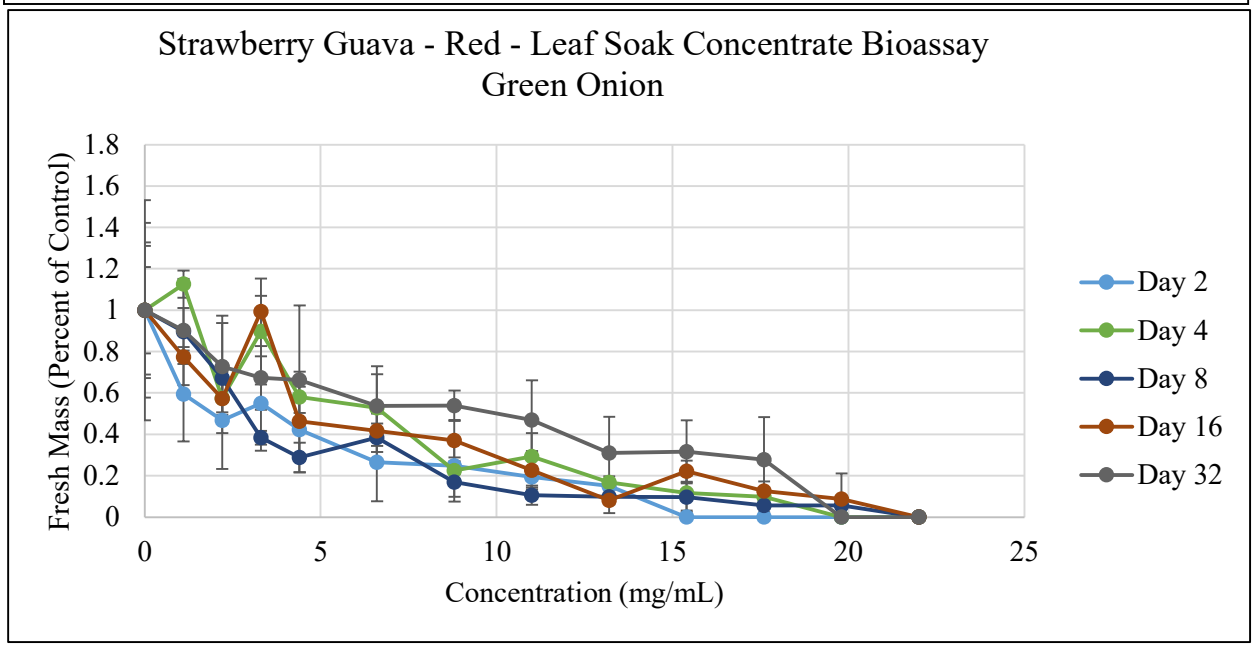


Figure 28. A time course of continuous soaking of red strawberry guava leaves and its effects on the growth of green onion. As time passed, there was a reduction in the inhibition of growth, as seen with the difference between the 16 day and 32 day inhibition trends. N = 18

How the chemicals were released into the environment and to what degree germination rates and root and shoot lengths were affected was also investigated. Strawberry guava's exudates of fresh or freeze-dried whole leaf or macerated leaf were tested in an agar gel soil analog experiment and it was found that freeze dried macerated leaves were the most effective at reducing the growth of lettuce (Figure 29-31). Fresh leaves were less effective than freeze dried leaves, while whole leaves were less effective than macerated leaves. The former affect seen can be contributed to moisture content. As the leaves have their moisture removed, their chemical composition is concentrated, and, when revitalized by the moisture in the agar gel, is released in a more concentrated manner compared to leaves that have not been freeze dried. As for the leaf condition, breaking up the leaf allows for more direct and increased rate of release of chemicals as compared to being released through the thick cuticle. Roots were more greatly affected compared to shoots. This is indicative of the chemicals being taken up through the roots and can be seen visually in Figure 31 with the displayed root burn. As the allelopathic chemicals are polar in nature, it's a high probability that it is being taken up with water to have its affect. As the distance from the core increased there was generally a reduced inhibitory growth effect on roots and shoots, with all negative effect being lost after 10mm for all leaf conditions. These trends were seen in previous experiments utilizing a similar false soil analog but with mesquite leaves (Nakano, 2001).

Figure 29. A comparison of the effects of yellow and red strawberry guava leaves on the growth of lettuce roots. The "*" denote a significance between the control and the experimental with $P < 0.05$ utilizing Anova: single factor. The dark horizontal line indicates 100% control growth. Freeze dried leaves are generally more effective at inducing plant growth inhibition, with ground freeze dried leaves producing the largest negative effect on root elongation. Red strawberry guava leaves generally inhibited the elon4gation greater than yellow strawberry guava leaves. N = 20

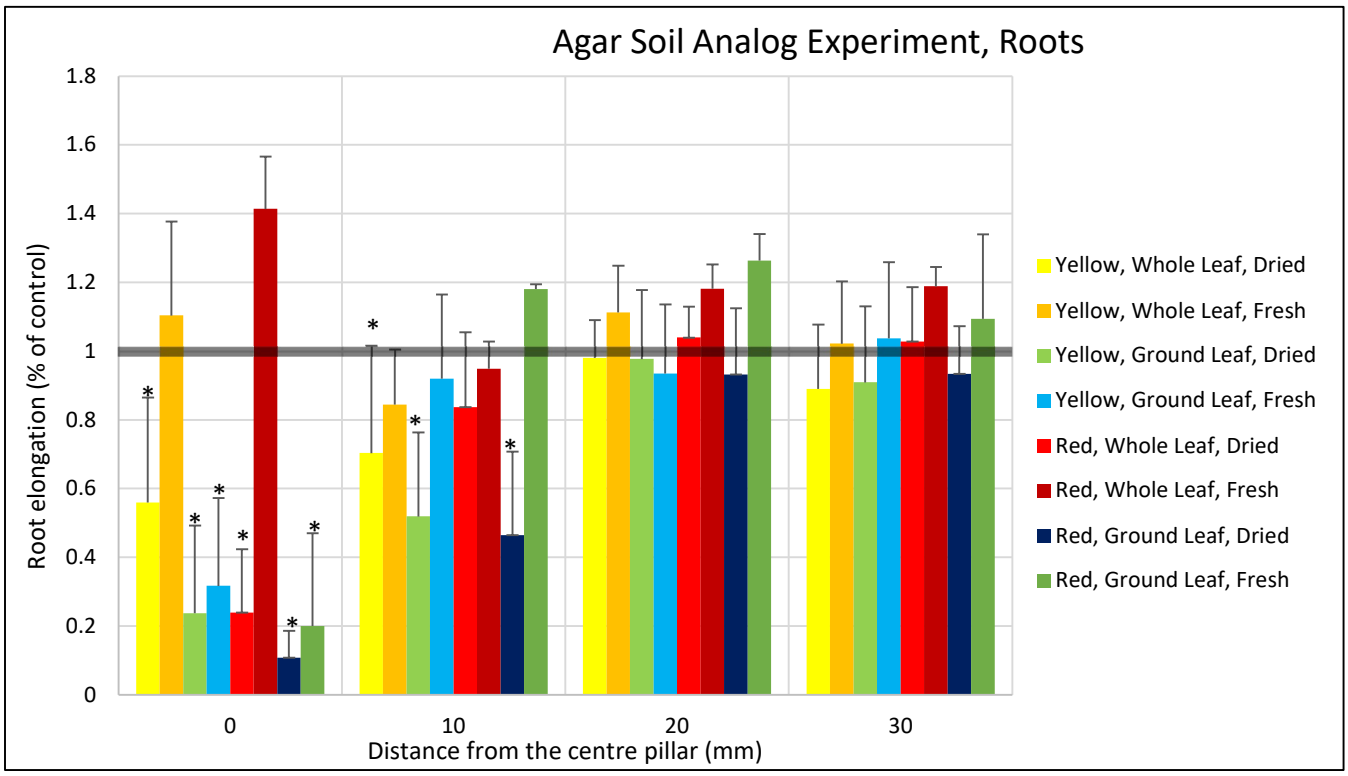


Figure 30. A comparison of the effects of yellow and red strawberry guava leaves on the growth of lettuce shoots. The "*" denote a significance between the control and the experimental with $P < 0.05$ utilizing Anova: single factor. The dark horizontal line indicates 100% control growth. Freeze dried leaves are generally more effective at inducing plant growth inhibition, with ground freeze dried leaves producing the largest negative effect on shoot elongation. Red strawberry guava leaves generally inhibited the elongation greater than yellow strawberry guava leaves. N = 20

Figure 31. A comparison of spread of the allelopathic chemicals, from the central column to 30 mm out, with data points taken at every 10mm. Note that roots under distress have stunted growth and are crooked in appearance.

In addition to affected lettuce and green onion (our indicator species), strawberry guava's allelopathy was also tested upon two common weeds found in agricultural fields, barnyard grass (*Echinochloa sp.*) and red root pigweed (*Amaranthus retroflexus*), within our costar-cell well

bioassay (Figure 32). It was found that a leaf soak of the red variety of strawberry guava had a greater affect upon both barnyard grass and pigweed, with complete inhibition of germination of both species at 100% concentration and a greater reduction in growth at the 50% concentration. These trends follow the previous experiment's results and showcase how strawberry guava's allelopathy affect a wide range of plant life.

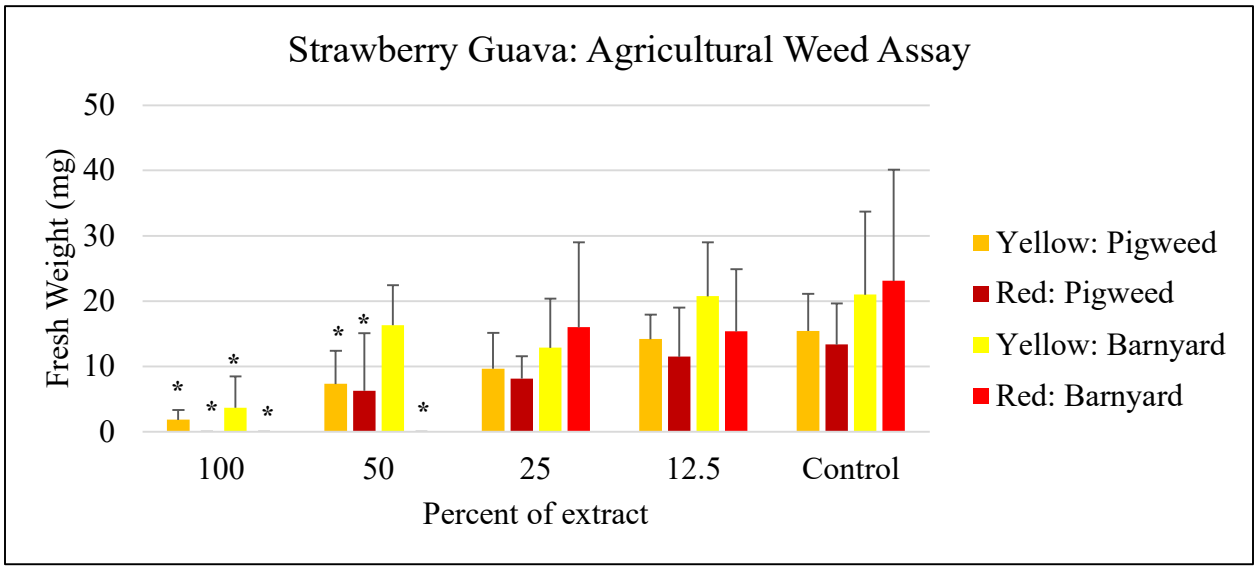


Figure 32. A comparison of the affects of different concentrations of red or yellow strawberry guava leaf soak on the growth of pigweed and barnyard grass. A "*" denote a <0.05p value between the treatment and the control. Statistical tests were done with ANOVA: single factor. N = 18

In order to best test strawberry guava's allelopathic potential in a more agriculturally significant scenario, strawberry guava leaf soak was applied to pigweed and barnyard grass in a pre- and post- germination assay conducted with soil trials in a greenhouse setting. In the pre-germination assay, it was found that the leaf extract had a greater affect upon the monocot barnyard grass germination rates, as compared to the dicot, the pigweed (Figure 33). This trend, which follows the previous experiment's results, may have been because of the nature of the seed's themselves. Pigweed seeds are very small, while barnyard grass seed are larger and have a chaff on them. This chaff allows for the strawberry guava liquid to soak in and penetrate the barnyard grass through a larger surface area than is exposed for the pigweed seeds.

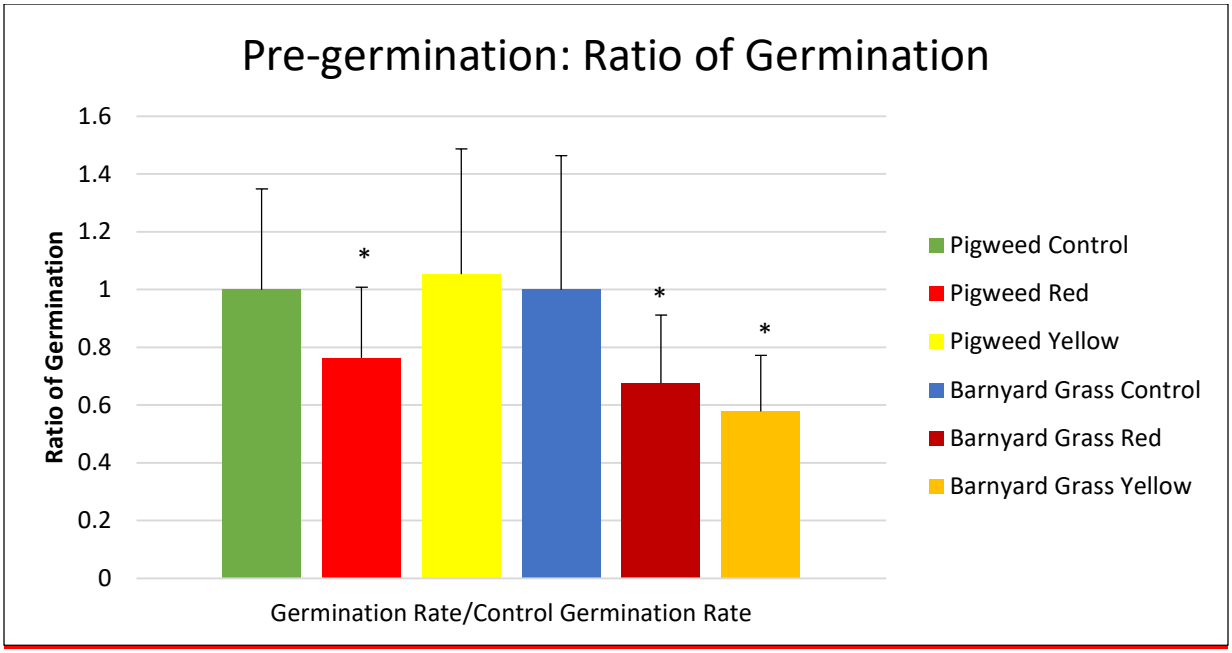


Figure 33. A comparison of the ratio of germination of seeds with the application of strawberry guava leaf extract (red or yellow variety). A "*" denote a <0.05p value between the treatment and the control. Statistical tests were done with ANOVA: single factor. N = 18

It was also found that, when comparing the pre-germination ratio of root to shoot, that strawberry guava extracts reduced the growth of barnyard roots as compared to the control for the reasons previously described (Appendix E). In comparison to post-germination assays, which found no statistical difference within any of the categories previously described, strawberry guava extract application has shown pre-germination activity, with the chemicals in question affecting roots greater than shoots. This may have been caused due to a variety of factors, including but not limited to that the mode of action of the allelopathic chemical in question may only affect young growing tissue or that older more mature plants may have a way to detoxify the allelopathic chemical. These types of inquiries require more detailed investigations, as discussed in the following chapter.

Chapter 5: Allelopathic Compound – Gallic Acid

5.1 Introduction

Until now, leaf extracts of strawberry guava and their allelopathic affects as a raw mixture have been described. Next, the chemistry and properties of its allelochemical(s) will be explored.

Gallic acid, otherwise known as 3,4,5-trihydroxybenzoic acid, has the molecular formula of $C_7H_6O_5$, the exact mass of 170.02152329 m/z, a melting point of 251°C, is very polar and water soluble (12g/L) (McCall, 1980), and is the starting point of hydrolysable tannin synthesis ("National Center for Biotechnology Information. "PubChem Compound Summary for CID 370, Gallic acid," n.d.; Ossipov et al., 2003). It has a K_{oc} value (organic carbon-water partition co-efficient, a higher number indicates stronger attraction of chemical to the soil, indicating lower mobility) of 57 L/kg, which indicates very high mobility with water ("National Center for Biotechnology Information. "PubChem Compound Summary for CID 370, Gallic acid," n.d.). This value is within a similar range for atrazine (38-288L/kg), while it contrasts with glyphosate's K_{oc} of 9-60,000L/kg (Battaglin, 2005). During water runoff events from farms, atrazine was more commonly found in higher concentrations and in more sample sets than glyphosate (Battaglin, 2005), indicating that gallic acid would be more likely to follow atrazine's route to flow and concentrate in water than stay attached to soil and spread little. Salts and esters of gallic acid are called "gallates" with the origin of the name coming from their original harvesting location of oak galls. Gallic acid has been isolated from peroxisomes and vacuoles in the leaf tissue of tea plants (*Camellia sinensis*) (Zhou, 2020). It is biosynthesized via the shikimate pathway, where shikimic acid (a compound before EPSP) undergoes oxidation to dehydroshikimic acid via shikimate: $NADP^+$-oxidoreductase (shikimate dehydrogenase). This intermediate compound is found only in trace amounts in a living leaf and is quickly dehydrated to gallic acid by dehydroshikimate dehydrogenase and $NADP^+$ (Ossipov et al., 2003, p. 4). Gallic acid can then be utilized to make ellagic acid, along with hydrolysable tannins such as gallotannins and elagitannins which are important for their antimicrobial, antifungal, antioxidant, and anti-inflammatory properties ("National Center for Biotechnology Information. "PubChem Compound Summary for CID 370, Gallic acid," n.d.). It is broken down by several bacterial

species in the genus *Pseudomonas* (including *Pseudomonas putida*) having the enzyme gallate dioxygenase to utilize this acid as a carbon and energy source (Beveridge and Hugo, 1964). Gallic acid is considered nontoxic to humans, with the average intake from food being 1g/day in the US, while it has a no-observed-adverse-effect-level for rats of 120mg/kg/day (Lu, 2006).

Gallic acid's antioxidant activity can be contributed to the multiple hydroxyl groups and the carboxylic acid attached to its aromatic ring. Part of the effect has shown to be caused by intramolecular hydrogen bonding between hydroxyl groups while free radical scavenging, allowing for stability of antioxidant radical formation (Wright et al., 2001). The carboxylic acid aids in the captodative effect, where the hydroxyl group is an electron-donating group and the opposite carboxylic acid is an electron-withdrawing group, helping to stabilize the radical via a number of resonance forms (Leopoldini, 2004). Gallic acid in biological systems help provide protection against the radical's hydroxyl ($HO^{\cdot}$) and peroxyl ($ROO^{\cdot}$) and the non-radicals, hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) groups (Badhani et al., 2015). Gallic acid, given to rats at 15mg/kg daily for 10 days and induced with treatment by isoproterenol 100mg/kg for 2 days, was found to help elevate activity levels of antioxidant enzymes superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and glutathione-S-transferase, helping the body naturally relieve oxidative stress (Priscilla and Prince, 2009). It can also help reduce lipid peroxidation, the reaction of oxygen and lipids to form hydroperoxides and peroxide radicals, caused by H_2O_2 (Abdelwahed, 2007). While gallic acid performs well in antioxidant studies *in-vivo*, *in-vitro* its effects are less pronounced and it has a low toxicity due to limited absorption after ingestion, difficulty in reaching the desired target areas of the body, and limited ability to enter the cell through the cell membrane (Teixeira, 2013). While the exact mechanism of plant transportation of gallic acid is not well understood, gallic acid in human cells was found to be transported across the intestinal epithelial cell layer via the paracellular pathway, the transfer of substances between the intercellular space between cells, as in tight junctions. This transfer is pH gradient independent and linearly increases with concentration. However, the rate of transfer across the cell layer is very small, being maximum 0.22 nmol/min. Gallic acid was taken up more readily by the cells themselves at a lower pH 6 compared to pH 7.4 by a factor of 3, with a maximum rate of 0.23 nmol/min (Konishi et al., 2003).

Gallic acid, due to its weak metal chelating ability and strong reducing property (electron-donating effect) (Halliwell et al., 1995), can also be a prooxidant and cause production

of free radicals when in the presence of transition metals, such as iron, and H_2O_2, where it helps catalyze the reaction of Fe(III) with H_2O_2 to Fe(II) with the gallic acid being oxidized to a hydroxyquinone (Hynes and Coinceanainn, 2001). At pH ranging from 3-10 in the presence of H_2O_2 and peroxidases, if the ratio of gallic acid to Fe(III) is smaller than 2 (low gallic acid concentration; 0.2-0.6mM – human cell study), gallic acid is a prooxidant, slightly increasing H_2O_2, and resulting in DNA damage and potential human cell apoptosis(Yen et al., 2002). If the ratio is larger than 2 (high gallic acid concentration; 4.17mM – human cell study), it is an antioxidant as previously described (Strlic, 2002). However, even in the absence of H_2O_2 but in the presence of peroxidase, gallic acid may act as an oxidizing compound, resulting in DNA damage, human cell toxicity, inhibited MAPK/ERK (mitogen-activated protein kinase)/(extracellular regulated kinase) and AKT (serine/threonine protein kinase) (normally inhibits apoptotic processes) cell survival signals, and high levels of oxidative stress biomarkers (MDA-lys (malondialdehyde-lysine adducts), CML (Nε-carboxymethyl lysine), and neuroketal-adducted proteins)), and high eIF2 α activation (α-subunit of eukaryotic initiation factor 2) (occurs under high endoplasmatic reticulum stress; may result in impairment of ubiquitin-proteasomal activity and accumulation of damaged proteins), due to its property of being easily oxidized which can result in the formation of hydrogen peroxide, quinones and semiquinones in human cancer cells (Eslami, 2010; Serrano, 2010). Gallic acid, at 0.01mM, may also help reduce gastric adenocarcinoma cell metastasis by resulting in inhibition or downregulation of MMP-2/9 (matrix metalloproteinases), NF-KB (transcription factor activator of MMP-2/9), and cytoskeleton reorganization signal pathway Ras (GTPase), Cdc42 (cell division control protein 42 homolog), Rac1 (Ras-related C3 botulinum toxin substrate 1), RhoA (Ras homolog family member A; GTPase), RhoB (Ras homolog family member B; GTPase), PI3K (Phosphoinositide 3-kinase) (activator of NF-KB), and p38MAPK (p38 mitogen-activated protein kinase) (cell signaling) and cytoskeletal F-actin (reduced activity and organization) causing cell death (Ho, 2010).

5.2 Materials and Methods

5.2.1 Chromatography and Mass Spectroscopy

Psidium cattleianum leaf extracts that were found to potentially contain allelopathic molecule(s) (showing statistically significant growth reduction compared to a control) were separated into fractions of 0, 20, 40, 60, 80 and 100% methanol/water (v/v) (2mL) by C18 cartridge chromatography (Sep-Pak Vac 35 cc (10 g), Waters). Inhibitory activity was found in the 0% methanol fraction, and was subsequently separated into 0, 20, 40, 60, 80 and 100% methanol/water (v/v) (1.4mL) by polar silica cartridge chromatography (Sep-Pak Silica Classic cartridge (690 mg), Waters). Inhibitory activity was found to be concentrated in the 100% methanol phase. This sample was then analyzed for components by HPLC. HPLC was done utilizing a Waters Nova-Pak C-18 (4um 3.9mm I.D. x 150mm) with a 22 minute gradient of Acetonitrile and Water + Acetic Acid (15%) (5-65% Acetonitrile) on a Waters Alliance 2695 Separations Module with a Waters 996 Photodiode Array Detector. Isolation of peaks was done by hand and sampled in the previously described bioassay. Peaks showing bioactivity were taken to Hawaii Pacific University Hawaii Loa Campus under the expertise of Dr. David Horgen, Jessie Nguyen, and Olivia Honaker, where LC/MS was conducted.

Gallic acid was measured in extracts by LC-MS/MS using reversed-phased chromatography (Waters, Acquity HSS T3, 1.8 μm, 2.1 mm x 150 mm) on an Agilent 6530 QTOF mass spectrometer with an Agilent 1200 HPLC system. The mobile phase was a linear gradient of acetonitrile/water with 0.1% formic acid (5% acetonitrile over 0-5 min; 5-100 % acetonitrile over 5-10 min); flow rate was 0.20 mL/min; injection volume was 2 μL. The QTOF parameters were as follows: positive-mode, gas temp 300 ºC, VCap 3500 V, fragmentor 160, *m/z* 70-1700, MS/MS collision energies 15 and 20 V. Calibration curves for standard gallic acid was prepared from semi-log gallic acid dilutions (0.1-32 ng/μL), integrating extracted ion chromatograms (50 ppm width) for *m/z* 171.0288 (calc for MH^+), and generating a linear regression with excellent fit (R_2= 0.9). Calculations for amount of gallic acid were done in replicates of six based upon the standard curve. Identification of the gallic acid peak was accomplished by high resolution mass of MH^+ (δ < 1 mmu), MS/MS base peak at *m/z* 125, retention time, and co-injection of gallic acid with the extract. Analysis and integrations were performed using MassHunter Analysis software (Agilent).

5.3 Results and Discussion

Separation with Waters C18 Sep Pak columns was first conducted. The allelopathic chemicals were highly concentrated in the 100% water fraction, but were lost as the percentage of methanol was increased to each wash and resultant fraction, affecting both lettuce and green onion similarly (Figure 34-35). Thus, it was determined that the allelopathic chemicals are likely polar in nature.

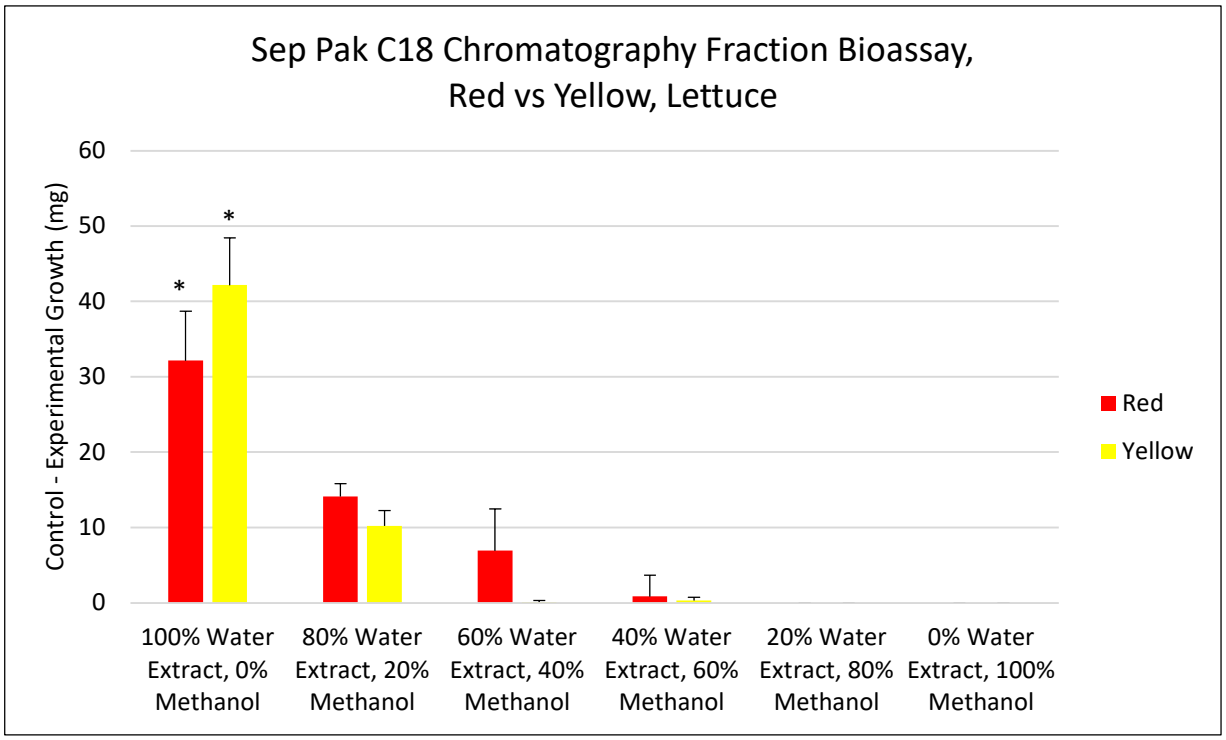


Figure 34. A comparison of the difference between the growth of lettuce in control solution or with a treatment (10% fraction solution, either red or yellow). A "*" denote a <0.05p value between the treatment and the control. Statistical tests were done with ANOVA: single factor. N = 18

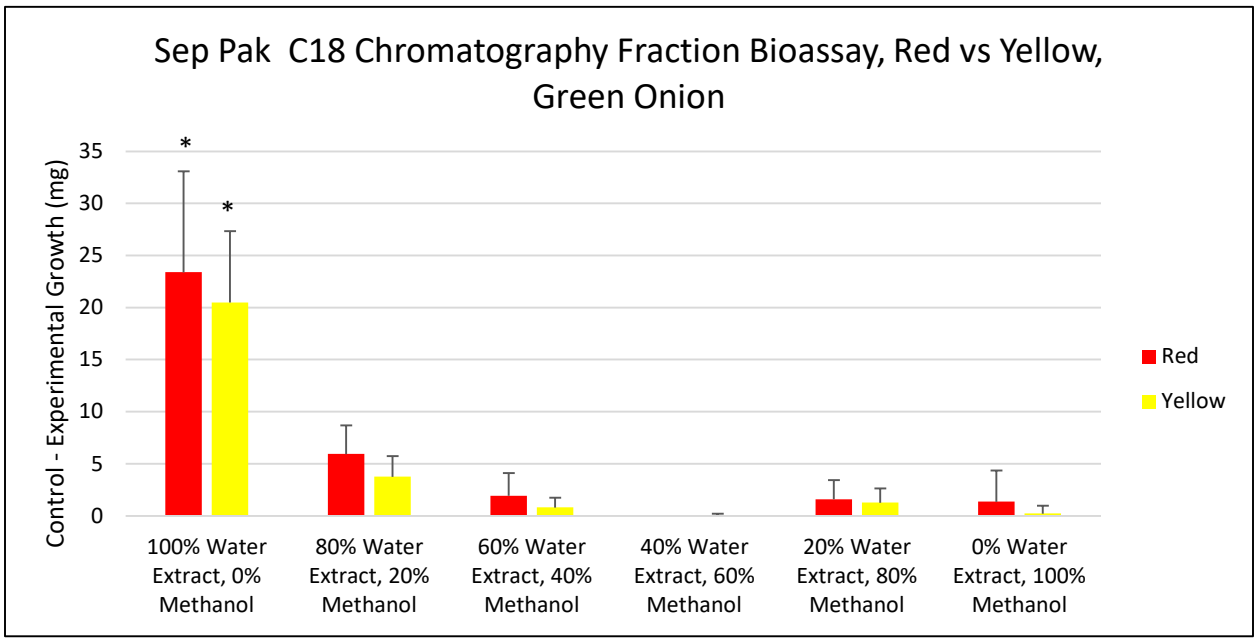


Figure 35. A comparison of the difference between the growth of green onion in control solution or with a treatment (10% fraction solution, either red or yellow). A "*" denote a <0.05p value between the treatment and the control. Statistical tests were done with ANOVA: single factor. N = 18

The 100% water fraction was then taken and underwent Waters Sep Pak Polar Cartridge Separation, with allelopathic activity being found in the 0% water, 100% methanol fraction for lettuce and green onion (Appendix F).

This bioactive fraction was then run on a Waters Nova-Pak C-18 (4um 3.9mm I.D. x 150mm) with a 22 minute gradient of Acetonitrile and Water + Acetic Acid (15%) (5-65% Acetonitrile) on a Waters Alliance 2695 Separations Module with a Waters 996 Photodiode Array Detector, where 3 peaks of interest were discovered.

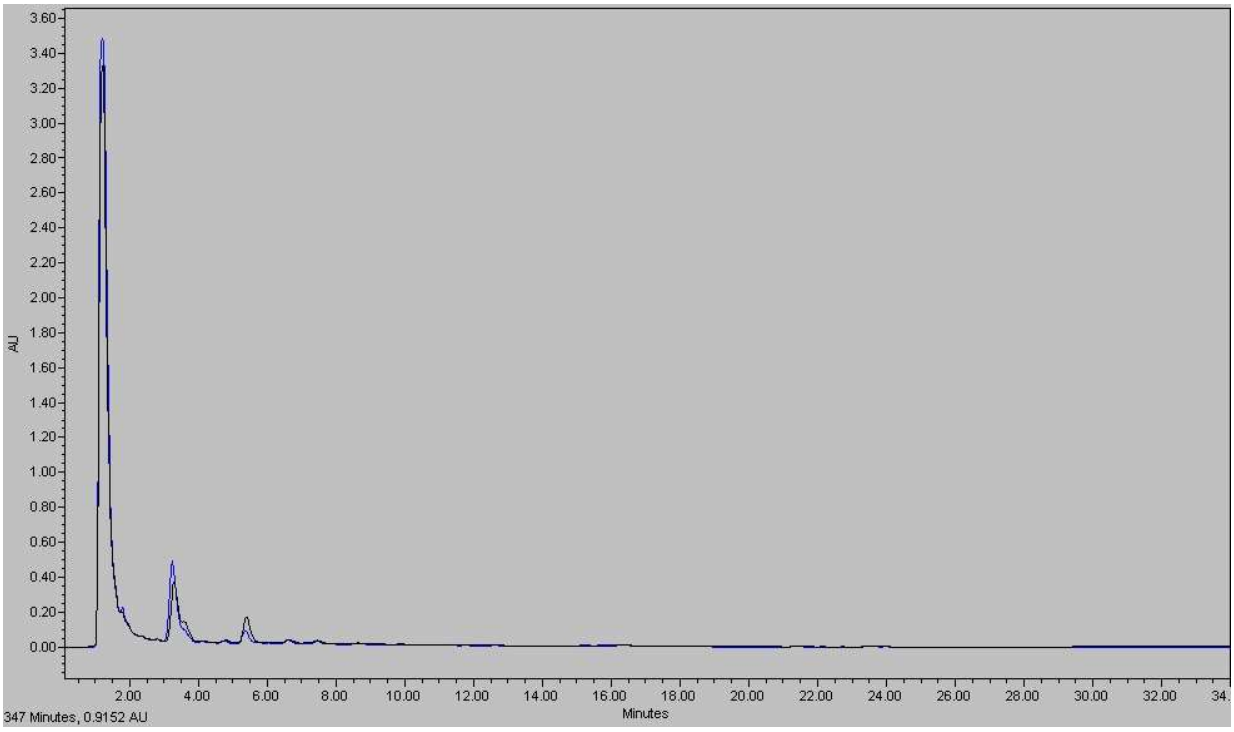


Figure 36. Waters Nova-Pak C18 4um 3.9mm I.D. x 150mm with a 22 minute gradient of Acetonitrile and Water + Acetic Acid (15%) (5-65% Acetonitrile) at 254nm of *Psidium cattleianum* red variety leaf soak overlapped with the *Psidium cattleianum* yellow variety 10 uL inject. Elution volumes overlapped at around the solvent front, 3.346 minute and the 5.347 minute mark, indicating that the chemical(s) causing *Psidium cattleianum* allelopathy are quite possibly the same between the two varieties.

As shown in figure 36, three distinct peaks appeared, at the solvent front, 3.346 and 5.347 minute runtime are shown in both the red variety and yellow variety of *Psidium cattleianum,* constituting that perhaps both varieties carry the same allelopathic chemical(s) in question (Appendix G).

Bioassay mediated fractionation produced Figure 37 and 38, showing that the solvent front had the most inhibitory effect on plant growth out of the 3 peaks of interest shown previously and affected both lettuce and green onion. Mixing the solvent front with equal parts of either isolated peak A or peak B resulted in a roughly 50% reduction in growth (equivalent to the addition of the solvent front), while mixing of peak A and B did not result in a statistical difference in growth compared to the control, indicating that the solvent front contains the primary component producing the observed reduction in growth.

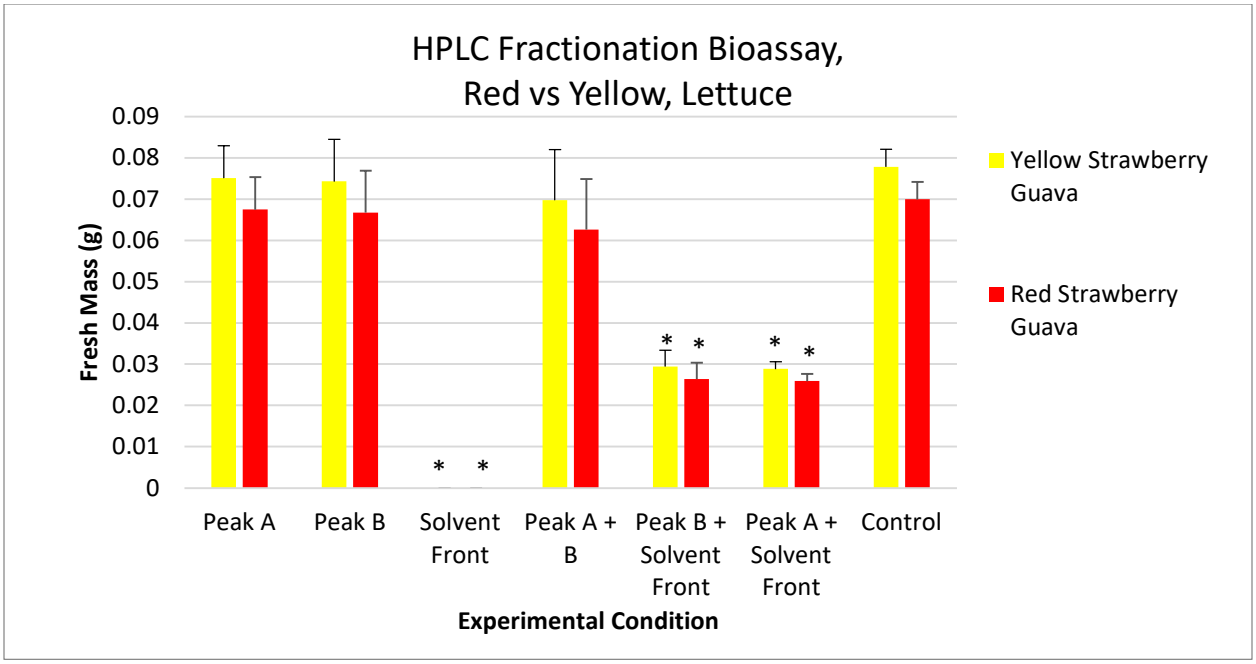


Figure 37. Peaks isolated from a Waters Nova-Pak C18 4um 3.9mm I.D. x 150mm with a 22 minute gradient of Acetonitrile and Water + Acetic Acid (15%) (5-65% Acetonitrile) and tested in a bioassay of lettuce. The Solvent Front has a retention time of 1.25 minutes, Peak A has a retention time of 3.346 minute, while Peak B has a retention time of 5.347 minute. The Solvent Front had greatest reduction in growth. A "*" denote a <0.05p value between the treatment and the control. Statistical tests were done with ANOVA: single factor. N = 18

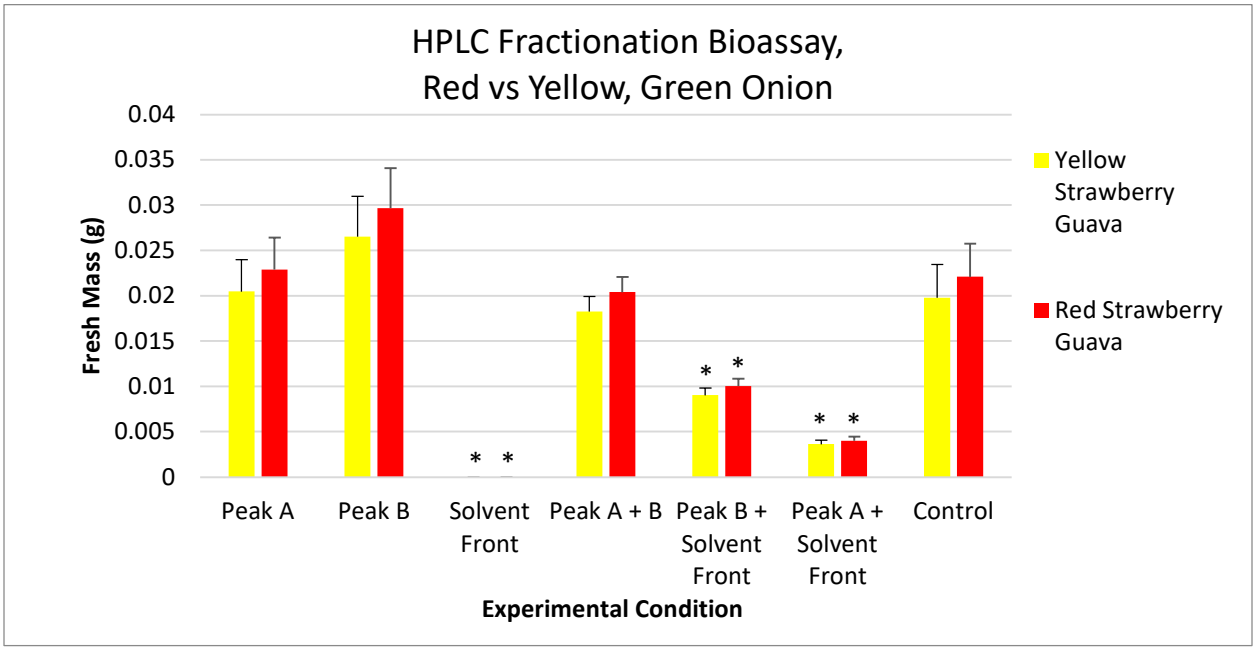


Figure 38. Peaks isolated from a Waters Nova-Pak C18 4um 3.9mm I.D. x 150mm with a 22 minute gradient of Acetonitrile and Water + Acetic Acid (15%) (5-65% Acetonitrile) and tested in a bioassay of green onion. The Solvent Front has a retention time of 1.25 minutes, Peak A has a retention time of 3.346 minute, while Peak B has a retention time of 5.347 minute. The Solvent Front had greatest reduction in growth. A "*" denote a <0.05p value between the treatment and the control. Statistical tests were done with ANOVA: single factor. N = 18

Collaboration with Dr. David Horgen, Jessie Nguyen and Olivia Honaker at Hawaii Pacific University, Hawaii Loa Campus, was conducted with the isolated solvent front of both red and yellow variety strawberry guava on LC/MS as described previously, with similar results produced for both varieties. Searching within the mass spectroscopy data for suspected allelopathic compounds found in regular guava, it was discovered that gallic acid (171.0300 m/z for H+ ionization) may be contained within both samples, with an estimated retention time of 2.69 minutes for yellow (171.0291 m/z; 0.9mmu; 5.26ppm) and 2.68 minutes for red (171.0296 m/z; 0.4mmu; 2.34ppm), with the standard for gallic acid at 2.71 minutes (171.0289 m/z; 1.1mmu; 6.43ppm) as seen in figure 39, 40 and Appendix H.

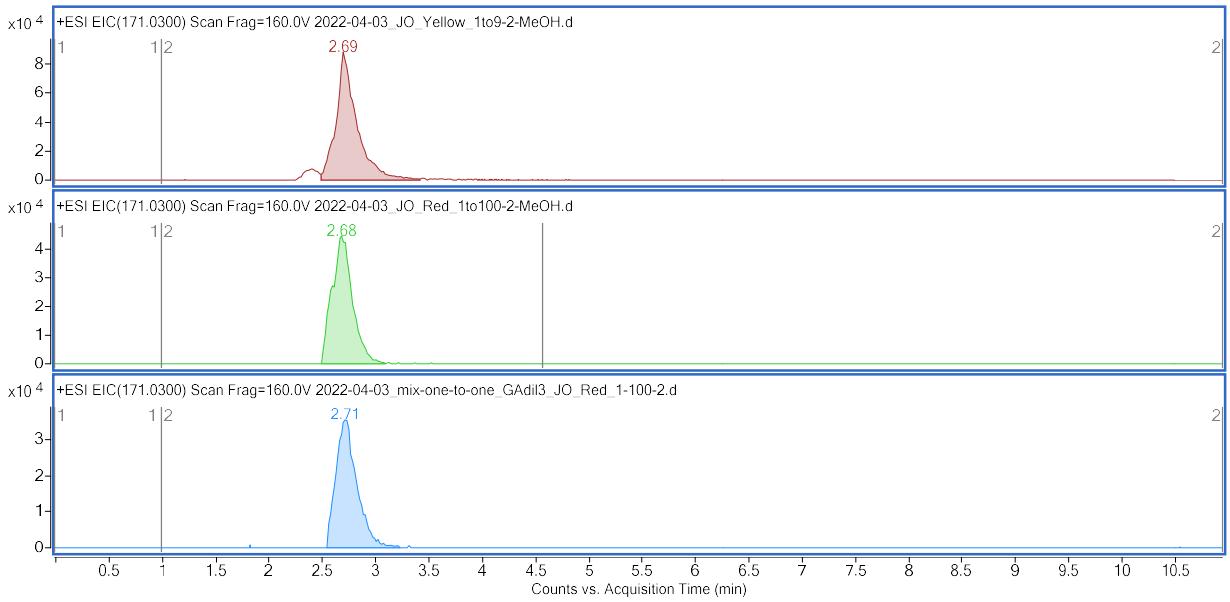


Figure 39. Potential hit of gallic acid to mass spectroscopy data in positive mode when searching for H+ ionization 171.0300 (m/z). Retention time of 2.69 minutes (171.0291 m/z; 0.9mmu; 5.26ppm) for yellow strawberry guava, 2.68 minutes red (171.0296 m/z; 0.4mmu; 2.34ppm) for red strawberry guava, and 2.71 minutes (171.0289 m/z; 1.1mmu; 6.43ppm)for the standard gallic acid. Mmu = milli mass error ppm = parts per million for the molecular formula C7H6O5.

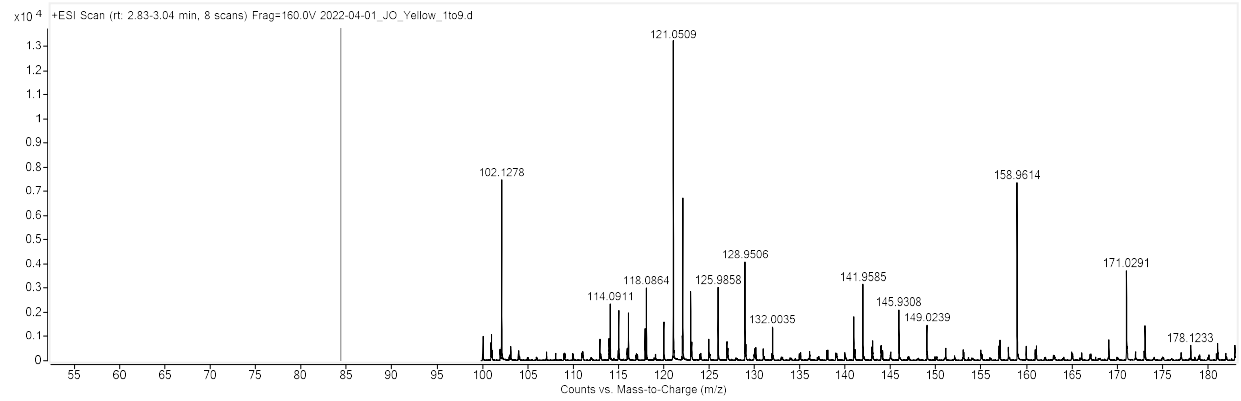


Figure 40. Potential hit of gallic acid to mass spectroscopy data in positive mode when searching for H+ ion 171.0300 (m/z). Retention time of 2.68 minutes (171.0291 m/z; 0.9mmu; 5.26ppm) for yellow strawberry guava. Mmu = milli mass error and ppm = parts per million for the molecular formula C7H6O5.

MS/MS fractionation was conducted in order to determine the fragmentation pattern of the suspected gallic acid compound and compared to the gallic acid standard fragmentation pattern and relevant literature as seen in Figure 41, 42 and appendix H. Gallic acid mass fragmentation patterns in positive mode at 15V collision energy was predicted and shown to have fragments of 153.018m/z (loss of water) and 125.023m/z (loss of CO_2 and formation of a dione) based upon multiple databases (RIKEN PlaSMA, NIH-NLM-ChemID*plus*, and the MassBank of North America), predictive software CFM-ID ("National Center for Biotechnology Information. "PubChem Compound Summary for CID 370, Gallic acid," n.d.; Tsugawa, 2019; Wang et al., 2021) and previously shown work (Fernandes and Salgado, 2016; Mahfoudhi, 2014). Identification of gallic acid was proposed by comparison of overlapping retention times and similar fragmentation patterns, specifically the presence of constitutive fragments 153.016m/z and 125.022m/z as seen in our standard, but also to fragments found in the literature such as 139.003m/z, 135.0049m/z, 115.039m/z, and 111.007m/z (Hossen, 2020; Rudrappa, 2007). In yellow strawberry guava, fragments were found to be 153.0151m/z (0.9mmu, 5.88ppm), 139.0026m/z (0.4mmu, 2.88ppm) 125.0239m/z (1.9mmu, 15.2ppm), 115.0393m/z (0.3mmu, 2.61ppm) and 111.0062m/z (0.8mmu, 7.21ppm). In red strawberry guava, fragments were found to be 153.0145m/z (1.4mmu, 9.15ppm), 134.8954m/z (109.5amu, 811ppm), 125.0228m/z (0.8mmu, 6.4ppm) and 115.0399m/z (0.9mmu, 7.82ppm). Negative mode on MS/MS was also conducted, with the majority peak being from the standard being 125.0247m/z (loss of CO_2) (Mahfoudhi, 2014; Okba, 2021), with yellow having the fragment of 125.0246m/z

(0.1mmu, 0.8ppm) and red having the fragment 125.0241m/z (0.6mmu, 4.8ppm) as seen in Appendix H. These results strongly suggest that gallic acid is the constituent of interest.

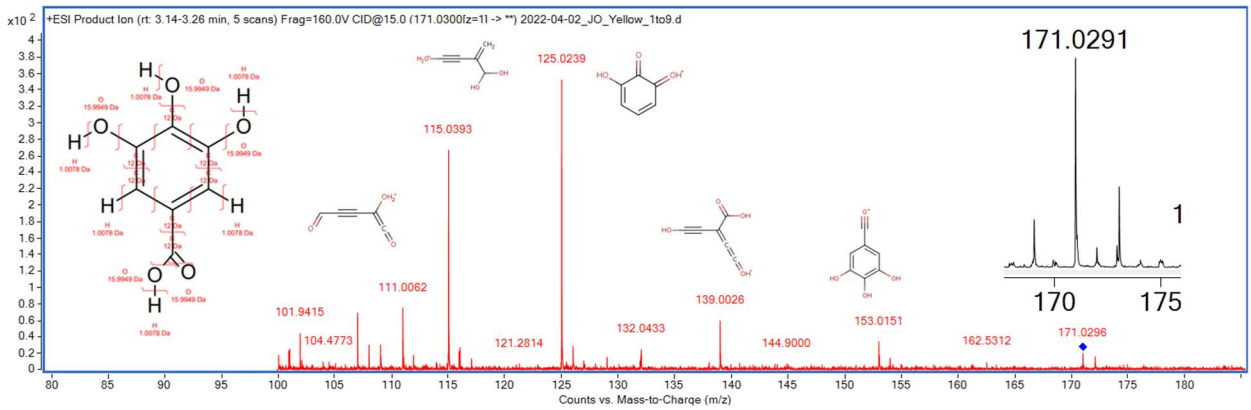


Figure 41. MS/MS analysis at 15V collision energy of suspected gallic acid peak for yellow strawberry guava in positive mode. Fragmentation pattern of gallic acid, predicted fragments, and close up of LCMS ionization state, are noted on the chromatogram, with special focus given to fragment 153.0151m/z (0.9mmu, 5.88ppm) and 125.0239m/z (1.9mmu, 15.2ppm), commonly found in both our fragment, the standard and relevant literature for gallic acid.

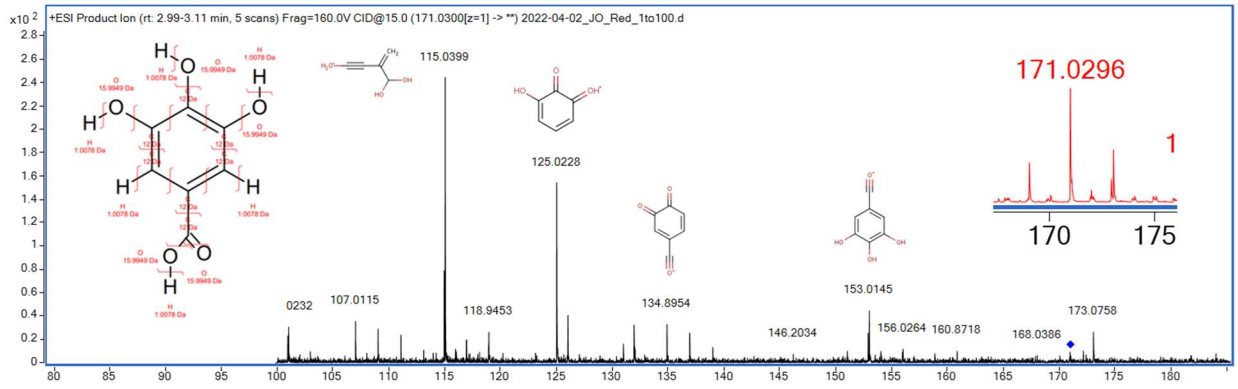


Figure 42. MS/MS analysis at 15V collision energy of suspected gallic acid peak for red strawberry guava in positive mode. Fragmentation pattern of gallic acid, predicted fragments, and close up of LCMS ionization state, are noted on the chromatogram, with special focus given to fragment 153.0145m/z (1.4mmu, 9.15ppm) and 125.0228m/z (0.8mmu, 6.4ppm), commonly found in both our fragment, the standard and relevant literature for gallic acid.

Gallic acid has been previously reported in regular guava (*Psidium guajava*) leaves and its health benefits have been noted as having antioxidant effects (Gutiérrez et al., 2008; Wang, 2021). In strawberry guava (*Psidium cattleianum*) fruits, gallic acid has been found to be one of the primary phenolic compounds (Pereira and Elisa, 2018). A study of strawberry guava leaf extract addition to chicken feed was found to have an antimicrobial and antioxidant capacity on

eggs, with lower *Escherichia coli* count in shells as well as greater antioxidant capacity in the yolks (Santos and FA, 2020). However, there has not been much work done on the leaves of strawberry guava in terms of allelopathy. This is, to the best of our knowledge, the first time we present evidence of gallic acid in strawberry guava leaves and propose it contributes to observed allelopathic activity.

Gallic acid was identified in both monocot and dicot plants *Aegiceras corniculatum* (river mangrove), *Ageratum conyzoides* L. (billy goat weed), *Delonix regia* (royal poinciana), various eucalyptus including *Eucalyptus tereticornis*, *E. camaldulensis*, *E. polycarpa* and *E. microtheca*, *Fagopyrum tataricum* (buckwheat), *Picea schrenkiana* Fisch. et Mey. (Schrenk spruce), *Prosopis juliflora* (Sw.) (mesquite), *Wedelia chinensis* (Wedelia) or their surrounding soil and their extracts were found to be allelopathic to both monocots and dicots *Brassica chinensis* (Chinese cabbage)*, Brassica napus L.* (rapeseed), *Echinochloa crus-galli* (L.) P. Beauv. (barnyard grass), *Lactuca sativa* (lettuce), *Lepidium sativum L* (cress), *Lolium multiflorum* Lam. (Italian ryegrass), *Medicago sativa L.* (alfalfa), *Phaseolus mungo L* (blackgram), *Phleum pratense* L. (timothy), *Vulpia myuros* (L.) C.C. Gmel. (foxail fescue), and even alge *Cyclotella caspia* (Hossen, 2020; Li, 2010; Liu et al., 2013). Gallic acid was found to affect both roots and shoots of both monocot Italian ryegrass and dicot cress, with 80% reduction of growth of both cress and Italian ryegrass at 30mM. IC_{50} values for Italian ryegrass was 6.6mM for shoots and 2.3mM for roots while cress was 15.4mM for shots and 13.8mM roots, with Italian ryegrass being more sensitive (lower IC_{50} value) but cress showing a steeper decline in health with higher concentration (Hossen, 2020). Blackgram germination was also inhibited by 1mM gallic acid (Li, 2010). *Arabidopsis thaliana*, *Nicotiana tabacum* (tobacco), *Brassica juncea* (Chinese mustard), *Lactuca sativa* (lettuce), and *Spartina alterniflora* (saltmarsh cordgrass) saw a significant reduction in root length and in growth with an estimated IC_{50} of 0.1mM gallic acid. No affect was observed on rice (*Oryza sativa*) or wheat (*Triticum aestivum*), both monocots, while *Brassica rapa* (bok choy, dicot) showed resilience to gallic acid's effects, thus indicating that gallic acid's affects are species specific and vary with effectiveness against both monocots and dicots.

In our own studies, it was found that lettuce and green onion were affected quite similarly by gallic acid, as seen in Figure 43, with IC_{50} values for lettuce calculated to be 10.23mM while

green onion was slightly higher at 12.69mM. These values are within the range of those previously described and are very close to our own strawberry guava leaf raw extract values for yellow strawberry guava lettuce 10.74mg/mL, green onion 12.2mg/mL and red strawberry guava lettuce 9.57mg/mL, green onion 10.67mg/mL, providing more evidence for gallic acid the allelopathic molecule. Our data follows similar trends where affected plants have a reduction in growth generally around 80% reduction when approaching 30mM. The slight hormesis affect (hormetic effect; at very low rates, a traditional plant growth inhibitor can stimulate plant growth as the plant puts all its energy into detoxification and hopeful subsequent survival; this is not uncommon with herbicidal compounds and has been previously observed and studied with the common herbicide glyphosate and with gallic acid) (Brito, 2018). Gallic acid, as one of multiple constituents from the tree *Moringa oleifera*, was shown to have a positive growth effect on *Lepidium sativa* at low concentrations (Perveen, 2021). Gallic acid as a single constituent was found to have a slight positive growth effect on cress in concentrations lower than 3mM (Hossen, 2020). While very similar to the values found previously in our own experiments, the variation shown in the literature indicates that there is no consensus on whether gallic acid affects monocots or dicots more or less. This chemical can be found in both monocots and dicots and as such, its effects are species specific and more study is recommended on a case by case basis (Rudrappa, 2007).

1g samples of fresh yellow strawberry guava leaves, taken from the same grove but from multiple trees and presented as an aggregate, were found to contain 0.080533mg ± 0.017874mg of gallic acid, with 1g fresh red strawberry guava leaves found to contain 0.023914mg ± 0.0071mg of gallic acid. This difference is significant, with a Anova: single factor test returning a p-value less than 0.01, but alone is not enough to explain the relatively close relationship in the bioassays of red vs yellow strawberry guava. Indeed, by previous observations, red strawberry guava, which has a slightly greater plant growth inhibitory ability, should contain more gallic acid, yet we see here that it actually contains less. Estimations of 1g of dry leaves showed yellow strawberry guava leaves containing 0.2211667mg ± 0.051779mg of gallic acid while 1g of dried red strawberry guava leaves contained 0.07267mg ± 0.022955mg. This dry mass estimation of gallic acid content within strawberry guava leaves is less than was found for regular guava, which contained 0.608mg of gallic acid / 1g of dried leaf tissue, indicating that perhaps the allelopathic properties of both have a similar gallic acid component (Du et al., 2009). One

potential explanation for the difference in allelopathic ability and gallic acid content could be that strawberry guava's allelopathic activity is the result of more than one chemical. For example, *Wedelia chinensis*'s allelopathy has been attributed to the presence of both gallic acid and vanillic acid (Hossen, 2020). Multiple studies have shown the diversity of regular guava's phenolic and metabolic profile, with strawberry guava's profile sure to rival regular guava's in diversity, indicating that, while gallic acid may contribute to its allelopathy, there are likely other chemicals contributing to its detrimental effects on surrounding plant life (Gutiérrez et al., 2008; Wang, 2021).

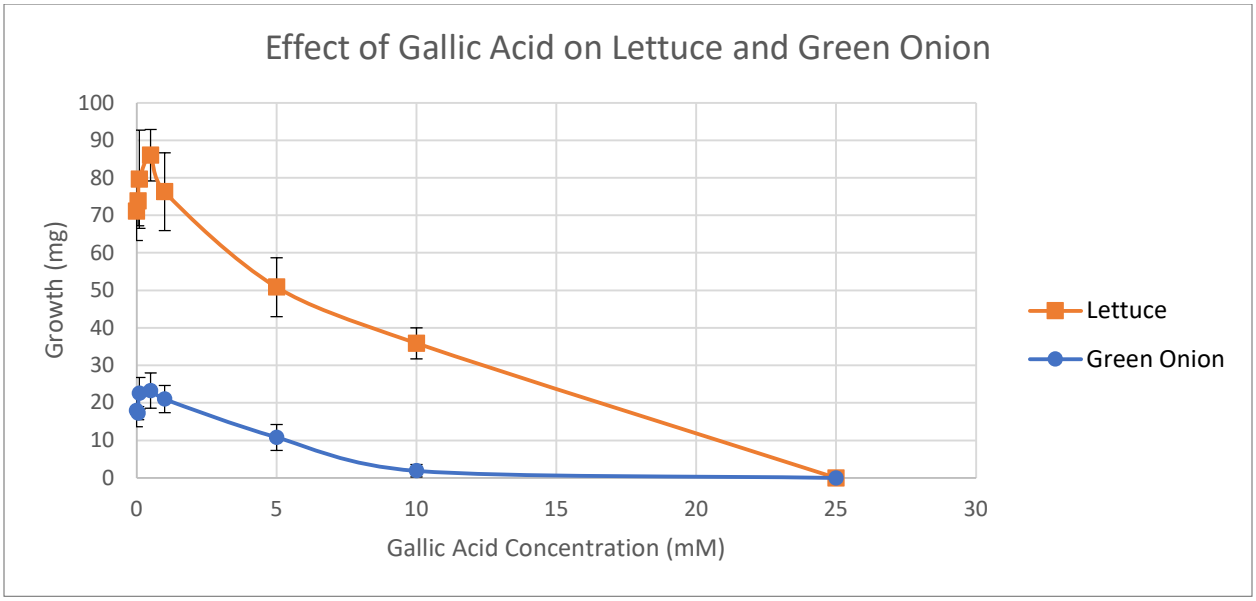


Figure 43. A comparison of the growth of lettuce and green onion in varying concentrations of gallic acid. The IC_{50} value for gallic acid for lettuce was calculated to be at 10.23 mM and for green onion 12.69 mM, indicating that gallic acid is slightly more effective on the green onion N = 12

In regards to its mode of action, gallic acid was found not to inhibit germination, but to render plants non-viable after gemination with eventual death. It has also been shown to create elevated levels of reactive oxygen species in treated plant roots by visulization of fluorescent H_2DCFDA (cell-permeant 2',7'-dichlorodihydrofluorescein diacetate), which activates in response to reactive oxygen species. The reactive oxygen species have then been shown to disrupt root architecture by damaging root microtubule assembly, with gallic acid affected *Arabidopsis thaliana* roots showing GFP-tagged microtubles (*Arabidopsis thaliana*-CTD-PAPK1-GFP) in dismantled localized aggregates, resulting in suppression of plant growth by the death of the primary root. This was reversed with ascorbic acid, a reactive oxygen species

quencher and antioxidant. *Arabidopsis thaliana* roots treated with 0.1mM gallic acid showed loss of fluorescence and thus loss of viability (lack of enzymatic acitvity) with fluorescein diacetate staining compared to control roots (Rudrappa, 2007). This root death by reactive oxygen species can be seen visually (Figure 44), and supports strawberry guava leaves having gallic acid, as the affects shown as root burn and browning in addition to stunted growth, is similar in both strawberry guava leaf extract and gallic acid solutions.

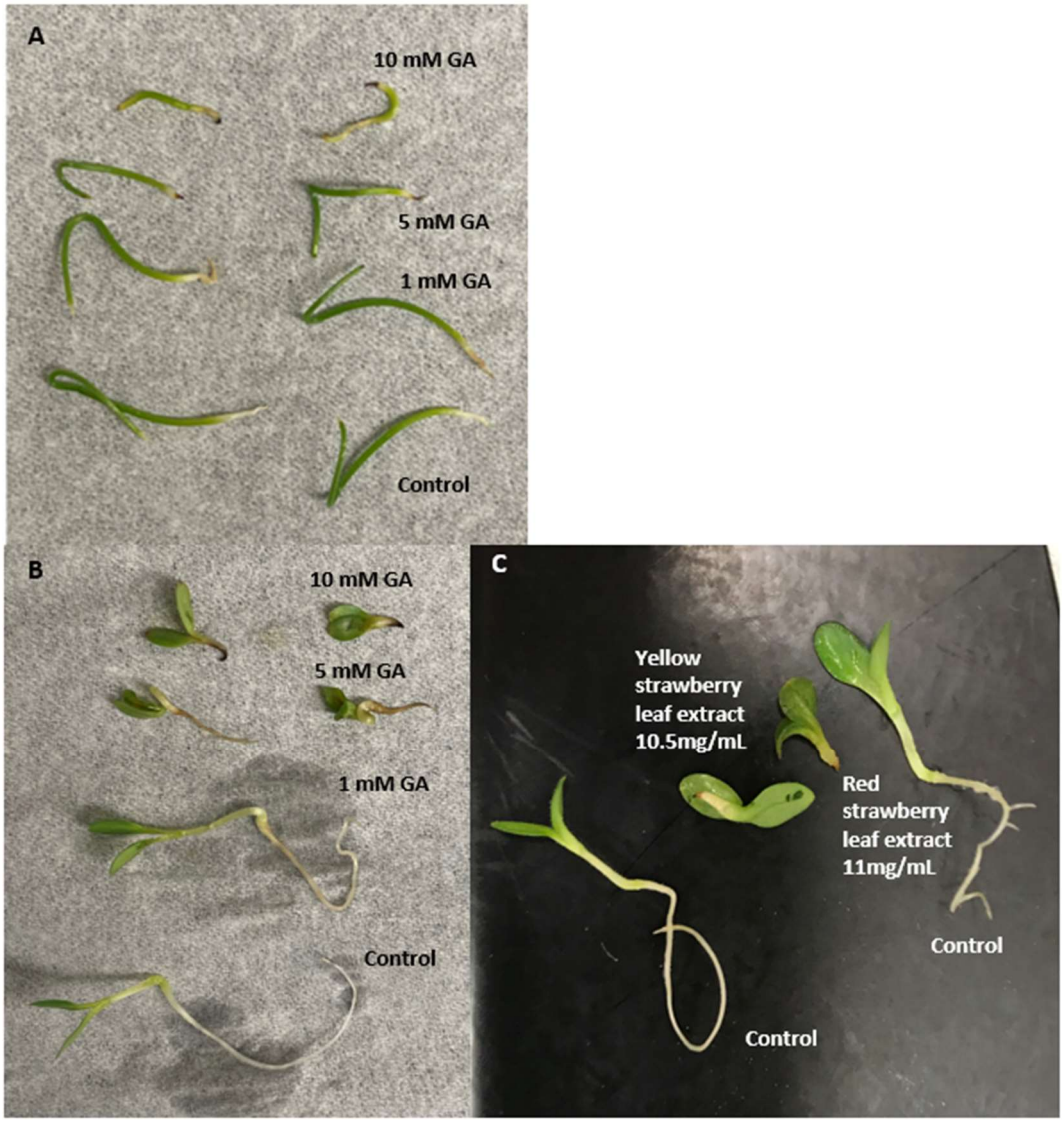


Figure 44. A composition of figures showing gallic acid effect or strawberry guava leaf extract on various test plants. All plants affected have visible root burn and stunted growth compared to the control. **A**. Gallic acid effect on monocot green onion, with concentrations at 10 mM, 5 mM, 1 mM and a control reference. **B**. Gallic acid effect on dicot lettuce, with concentrations at 10 mM, 5 mM, 1 mM and a control reference. **C.** The effect of raw strawberry guava leaf extract on dicot lettuce (for monocot green onion see Figure 31).

Arabidopsis grown in media culture with gallic acid (20mg/kg of water) were found to have upregulation of certain genes related to oxidative stress, including ubiquinol–cytochrome *c* reductase complex (binding factor for iron-sulfur protein), calmodulin kinase (signal transduction in Ca^{2+} signaling pathway), Cu–Zn superoxide dismutase (converts superoxide to hydrogen peroxide and molecular oxygen), peroxidase (catalyzes oxidation by hydrogen peroxide), glutathione S-transferases (herbicide detoxification by peroxidases with hydrogen peroxide), heat-shock proteins/chaperones (protein folding under stress), stress response transcription factors (WRKY and myb) and cytochrome P450s (Golisz et al., 2008). This is also similar to the previously mentioned antioxidant enzymes superoxide dismutase, glutathione peroxidase, glutathione reductase, and glutathione-S-transferase, that were upregulated in the human body in response to oxidative stress, providing evidence that gallic acid produces these types of oxidative stress response resulting from reactive oxygen species production. This reactive oxygen species could have been a result of the young seedlings being iron deficient and interactions with gallic acid (Yen et al., 2002). While that may occur, one hypothesis in the literature is that increases in intracellular hydrogen peroxides and hydroxyl radicals after gallic acid treatment (50 μM) could have resulted from the influx of hydrogen peroxide resultant from superoxides created outside of the cell by the auto-oxidation of gallic acid, leading to apoptosis as was seen in human cells (Inoue et al., 2000). However, this cellular apoptosis was inhibited by pre-treatment with Ca^{2+} chelator Bapta-AM, indicating, potentially, reactive oxygen species generation is followed by Ca2+ elevation resulting in arrest of the cell cycle and apoptosis as was seen in humans (Inoue et al., 2000). Newer studies have found gallic acid's partition coefficient in an octanol-water system to be about 0.29 (log value -0.53), indicating that this molecule is hydrophilic and may potentially have trouble diffusing through the hydrophobic lipid bilayer of a membrane. However, gallic acid showed no diffusion bottleneck across a liposomal membrane (20-100 μM solution concentrations) and was able to conduct itself as an antioxidant in the presence of lipophilic free radical DPPH, although human cell Ca^{2+} levels without the free radical was increased by 10% in the presence of gallic acid (100 μM) (Lu, 2006). In soybean, external application of H_2O_2 resulted in an increase of cytosolic Ca^{2+} sufficient to cause cell death exhibiting collapse of cellular structure, convulsions at the cell surface though infoldings of the plasma membrane, cell shrinkage by disengagement of protoplast from cell wall, condensation of cytoplasm, condensation of nucleus and pyknosis,

including DNA damage of chromatin collapse and large fragmentation, indicative of programed nuclear deconstruction and characteristics of apoptosis in animals (Levine, 1996). Elevated calcium levels (greater than 1 μM) have been found to result in calmodulin destabilizing and inhibiting the formation of microtubules in cultured carrot (*Daucus carota L.*) and tobacco (*Nicotiana tabacum L.*) cells (Fisher and Cyr, 1993; Hepler, 2016). Elevated calcium levels have also been found to enhance the microtubule-depolymerizing activity of *Arabidopsis thaliana* microtubule-destabilizing protein 25, in which increasing levels of calcium (2-6 μM) resulted in greater microtubule bundle disruption and fewer microtubule filaments (Qin, 2012). This would help explain what Rudrappa et al saw previously where GFP-tagged microtubles (*Arabidopsis thaliana*-CTD-PAPK1-GFP) were found to be in dismantled localized aggregates after gallic acid treatment. In addition to calmodulin and microtubule-destabilizing protein 25, kinesin-like clamodulin binding protein (a minus end directed microtubule motor protein unique to plants that is upregulated during mitosis and cell divsion) in the presence of calcium and calmodulin loses microtuble binding affinity as well as reduced ATPase activity, imparing the cell cycle (Hepler, 2016; Vos, 2000).

Gallic acid is a benzoic acid derivative and, while the two molecules share many differences, there is a known herbicide classified as a benzoic acid, albeit chlorinated. Dimethyl tetrachloroterephthalate (chlorthal-dimethyl or DCPA) is an active herbicide ingredient which controls both monocots and dicots as a pre-emergent herbicide. It is classified as an inhibitor of microtubule assembly, whose mode of action has been described as disrupting microtubule formation, cell wall formation, chromosomal replication and division, and overall cellular division (Cox, 1991). In wheat (*Triticum aestivum)*, bermuda grass (*Cynodon dactylon* L., var 328), corn (*Zea may* L., var. Pioneer 310) and onion *(Allium cepa* L., var. Yellow Globe Danvers) roots, DCPA resulted in complete stoppage of root growth. Affected cells showed abnormal cell wall orientation, including partially completed walls, walls with abnormal thickness or projections, or even walls in the wrong orientation. Cell division was also arrested in most often prometaphase, with unorganized condensed chromosomes, or produced non-viable cells with multiple nuclei or missing cell walls. All of these conditions may be attributed to the disruption of microtubules, potentially due to the release of intercellular calcium as was noted in caffeine treatments (Paul and Goff, 1973; Vaughan and Vaughn, 1990).

We propose that gallic acid treatment, due to its auto-oxidation, results in H_2O_2 and other radical production, that causing oxidative stress, including raising cytosolic Ca^{2+} levels. This results in a cascade of sustained elevated Ca^{2+} dependent cell responses including microtubule disruption, arrest of the cell cycle and resultant necrosis of affected cells. While this may not be the entire cell signaling and response map, as previously mentioned there are many genes that are upregulated in response to gallic acid treatment, including those responding to oxidative damage and elevated Ca^{2+} levels, this suggests combining multiple studies into the proposed mode of action of gallic acid beyond "death by reactive oxygen species" and explaining the root architecture collapse previously observed. However, while gallic acid is most likely an allelopathic chemical within strawberry guava leaves of both varieties, it may not be the only allelopathic chemical, as the metabolomics of regular guava leaves reveal quite a diversity of phenolics and other potentially allelopathic chemicals that remain to be studied in strawberry guava (Gutiérrez et al., 2008; Wang, 2021).

Chapter 6: 4-Hydroxyphenylpyruvate dioxygenase (HPPD)

6.1 Introduction

While further analysis by subsequent fractionation and purification, chemical identification with mass spectrometry, nuclear magnetic resonance, and additional mode of action determination were planned as the next steps with strawberry guava, due to the unprecedented situation of the global severe acute respiratory syndrome coronavirus 2 (SAR-CoV-2 or Covid-19) pandemic, an adaptation of the project was undertaken. Due to the limitations on personnel spacing and ventilation requirements as determined by the CDC for the health and wellbeing of faculty, staff and students over a 2-year period, a decision was made to bring a portion of this dissertation to an *in-silico* environment to protect the health of all personnel involved with this project.

This transition is a standard and obvious progression in the weed science field and is a critical contribution in the overall understanding of natural product discovery and development. This method of discovery and characterization is epitomized by the natural product leptospermone. Work with leptospermone took longer than a decade even with dedicated commercial funding, equipment and personnel for its discovery, characterization of herbicidal activity, and subsequent development into the Syngenta herbicide Mesotrione (Beaudegnies, 2009; Cornes, 2005). This integrated approach was utilized to work not only on the discovery aspect of this research, where a bioassay was created and found potential allelopathy, but also within the development aspect as well. Utilizing a known molecular target site, HPPD, and known competitive inhibitor ligands, a working in-silica system was developed. With these processes happening in parallel, akin to a candle burning from both ends, we move forward in progressive development and technological establishment as described below.

In collaboration with partners at Colorado State University and The University of Brasilia, we investigated the molecular target site HPPD to further the knowledge base of how synthetic derivatives of the natural product herbicide leptospermone interacted with HPPD and resulted in plant health decline and further used this information to develop an *in silico* model to aid future herbicidal development against this molecular target site. Much of the analog synthesis

and protein assays were handled by collaborators with our focus on the *in silico* computational and final analysis. This work establishes the process to undertake future allelopathic molecule analysis. Utilized in this analysis are two computer programs, Avogadro and Autodock. Avogadro is an open-source software designed by Marcus Hanwell in 2012 and can be utilized as a molecule editor and visualizer to produce molecular models. These models can then be utilized in other computational software which are promoted for use in computational chemistry, bioinformatics, and material sciences (Hanwell et al., 2012). Autodock is an open-source software developed by the Scripps Research Institute specializing in predicting how small molecules bind to a receptor of known 3D structure. It has become well known in the molecular modeling field as an accessible software that allows for differentiating between ligands with micromolar and nanomolar binding constants compared to those with millimolar binding constants (Rizvi et al., 2013). While there are many different computational docking software besides Autodock, such as FlexX, GLIDE, and DOCK, each with their own particular method of calculating and reporting ligand-target interaction, Autodock has been shown to be the most reliable in identifying actual inhibitors of various target enzymes from a database set, including the targets CDK2 (cyclin-dependent kinase 2), PTP1B (protein tyrosine phosphatase 1B), PDE4 (phosphodiesterase 4) and COX-2 (cyclooxygenase-2) (Park et al., 2006). Autodock has even been suggested to be incorporated into chemistry laboratories in an educational strategy to show early screening and visualiztion of known target site crystal structure and known inhibitory activities (Helgren and Hagen, 2017).

Screening natural products, which have been the subject of much attention in medicine and herbicide development, via molecular docking screens, helps reduce the high costs, long production and assay times and health risks required of physically synthesizing and screening a large number of candidates (Chen et al., 2017). This is currently being done in the medical field. During the Covid-19 pandemic, when time and information was in short supply, docking studies were one of the first indications of what existing medications could be utilized against the disease. The natural product quercetin, previously mentioned for its allelopathic properties but now highlighting it's antiviral properties, was calculated by Autodock to inhibit the SARS-CoV-2 3-chymotrypsin-like protease (3CLpro) and papain-like protease (PLpro) with docking of binding energies -6.25 and -4.62 kcal/mol, providing evidence for the usage of such supplements as a holistic guard against the diesase (Agrawal et al., 2020; Derosa, 2021). In terms of

repurposing medications, Autodock was utilized to test existing protease inhibitor drug binding potential against Covid-19's protease, including drugs remdesivir and ritonavir. It was found that the protease structure had multiple active sites for remdesivir compared to the other protease drugs, indicating that this drug would be a good potential condidate for an off-label perscription for Covid-19 (Mothay and Ramesh, 2020). Clinical studies proved that this molecular modeling study was correct in its findings, with hospitalized patients who were perscribed remdesivir recovering almost 5 days faster, along with a reduced rate of mortality and less serious adverse affect events compared to those patients who took a placebo (Group, 2020).

Herbicide development has also utilized this virtual screening stategy to help reduce the list of potential metabolite candidates to test via bioassay or to create a model to help design new more efficient ligands. As in the medical field, one of the processes to follow could be to search a database for potential ligands that have a favorable binding energy to a protein with a known 3D structure base on an established model. These are then synthesized and tested for activity. In addition, correlation between binding energy and the bioactivity can be utilized to create a model to highlight important motifs between ligand and protein for binding. For example, transketolase has an important role in plant photosynthesis, reversibly converting sedoheptulose 7-phosphate and glyceraldehyde 3-phosphate to ribose 5-phosphate and xylulose 5-phosphate, as well as fructose 6-phosphate and glyceraldehyde 3-phosphate to xylulose 5-phosphate and erythrose 4-phosphate in the dark reactions (Suzuki et al., 2017). This enzyme also has a critical role in phenylalanine and other aromatic amino acid biosynthesis (Ikeda, 1999), yet its role as a herbicide has been understudied. The College of Plant Protection in the Agricultural University of Hebei utilized molecular modeling and docking of a corn transketolase to pare down 1 million metabolites from the commercially available ZINC database to 6 candidate molecules, a reduction of 99.999%. The 6 identified compounds were tested via bioassay using rape seed and barnyard grass with one chemical, ZINC12007063 displaying significant herbicidal activity. Further benzofuran replacement or pyrimidine modification resulted in derivatives with increased herbicidial and/or fungicidal activity. Analysis of docking energy values resulted in the highlighting of important π-π bond interactions (benzene ring benzotetrahydrofuran and His103) hydrogen bonding witin the catalytic site (tetrahydofuran ring and oxygen with Leu194 and His340), along with important hydrophobic interaction (Gly234 and regional motif of ligand). Interestingly, changes in functional group substitutions did not affect the overall ligand-protein

binding schematic, but did impact biological activity, a detail highlighted when comparing both bioassay and docking data (Huo, 2018). This study showed how starting with a query into a known database with a known herbicidal target site can result in potentially undiscovered herbicidal compounds supported by bioassay results.

Another enzyme under analysis for herbicide discovery is protoporphyrinogen oxidase (PPO), an important enzyme in chlorophyll and heme biosynthesis where it catalyzes the oxidation of a protoporphyrinogen IX to protophorphyrin IX utilizing molecular oxygen (Dayan et al., 2018). Multiple herbicides have been developed for this target site, including Flumioxazin, a low-toxicity, broad-spectrum, N-phenyl phthalimide core strucuture herbicide. These herbicides have important key features including a N-subsititued phenyl group and a tetrahydrophthalimide, which allows for H-bonding (3) or π-π bond interactions with active site residues (Arg98, Gly175, Leu372, Phe392, and FAD600) (Chaudhari, 2017). In the Plant Protection College, Hebei Agricultural University, derivatives of Flumioxazin were tested for binding affinity via molecular docking on tobacco PPO. It was found that, while there was a reduction of hydrogen bonding (from 3 in Flumioxazin to 0 in the derivatives) those with the lowest binding energy had phthalimide rings with conserved π-π interactions with Phe392 and phenyl rings with hydrophobic interactions with Leu356 and Leu372. The derivatives were synthesized and tested upon *Brassica campestris* (field mustard), *Amaranthus retroflexus* (pigweed) and *Digitaria sanguinalis* (crabgrass), where herbicidal activity for those with the lowest binding energy was found to be comparable to the commercial herbicides Chlortoluron, Atrazine, and Flumioxazin. Enzyme assay of PPO with the most active derivative was found to be more effective than Flumioxazin by 10% (Gao, 2019). This study shows how adapting a known herbicidal compound, while retaining the key base structure, can be utilized via molecular modeling and bioassay to find herbicidal molecules that are potentially competetive with commerical herbicides. These two studies highlight the important integration of molecular modeling into herbicide development and how these new techniques can be utilized to find new herbicidal compounds. Here, the molecular target site HPPD is tested upon by a variety of derivatives of the natural product herbicide leptospermone.

6.2 Materials and Methods

6.2.1 HPPD Enzyme Assay

HPPD from model plant *Arabidopsis thaliana* was expressed in *E.coli* and assayed as a total soluble protein extract as described in (Dayan, 2009). Ligands were synthesized and enzyme inhibition activity was reported as IC_{50} (half maximal inhibitory concentration) by comparing control to experimental levels of HGA (the product of HPPD's conversion of HPP) identified by HPLC, with synthesis and enzyme assay measured as previously reported (Dayan, 2009). The smaller the IC_{50} value, the more effective the compound.

6.2.2 Model Creation

Each individual ligand was created atom by atom within Avogadro version 1.2.0. There were 5 classes of molecules, cyclohexane-1,3-diones with phenylene side chains, cyclohexane-1,3-diones with phenyl side chains, cyclohexane-1,3-diones with other side chains, non-diones with simple aliphatic side chains, and 2-acyl-cyclohexane-1,3-diones with simple aliphatic side chains (Figure 45 and 46). A selection of each, representative of their class of molecule, was created and further analyzed.

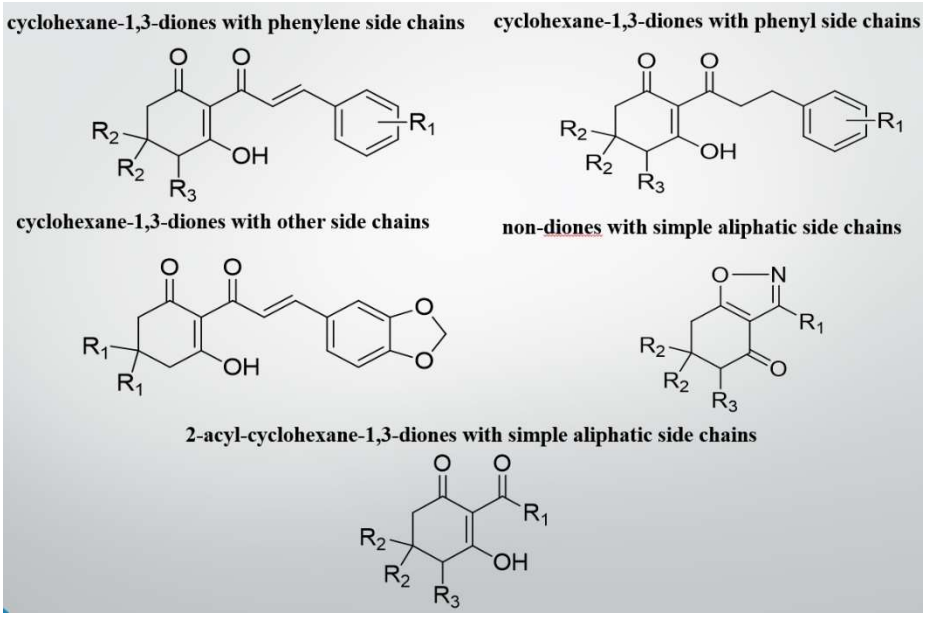


Figure 45. A display of the base structures utilized as HPPD inhibitors. The R groups differentiate different sub-groups that were added.

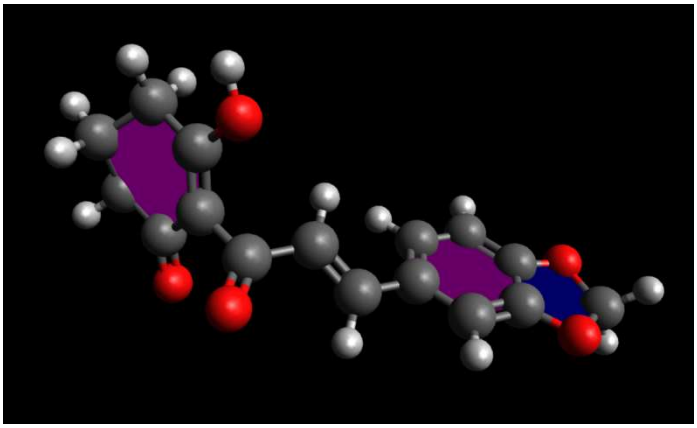


Figure 46. An example of a cyclohexane-1,3-diones with a benzodioxole side chain (13A) attached produced within Avogadro version 1.2.0.

6.2.3 Molecular Docking

Molecular docking was performed using Autodock version 4.2.6 (Figure 47). The HPPD protein itself was converted into a .pdbqt file based upon the crystal structure of the protein, with a 0.9 charge on the FE atom. Ligands were uploaded into Autodock from Avogadro as a .mol2 type file. Torsion of the ligand and conversion into a .pdbqt file was performed in Autodock. The grid utilized for docking had its covalent map set up around the coordinates x = 24.063, y = 9.506, z = -19.126 of the HPPD protein with an energy barrier of 1000, half-width to 5, and a gridbox being of dimensions x = 75, y = 75, z = 75, spacing = 0.2, centered over the binding domain/active site at coordinates x = 25, y = 5, z = -19. This box encompasses all amino acid residues known to be involved in inhibitor binding (Brownlee, 2004). Docking parameters with the macromolecule is the HPPD protein and the ligand is the "created ligands" from Avogadro, with default search parameters except for genetic algorithm which was changed to 25 runs. The parameters can be set to a variety of conditions, but in this analysis a Lamarckian genetic algorithm was chosen due to its ability to handle more degree of freedom in the ligand, along with its reliability and efficiency (Morris, 1998). A Lamarckian genetic algorithm entails the creation of a population of potential ligand confirmations. These then compete in a manner similar to biological evolution, where the final selected individual has confirmations that provide the lowest binding energy (Morris, 2009). These are the docking parameters utilized to create interactions and the resultant binding energies (kcal/mol), where the lower the binding energy, the more stable/favorable the molecular interactions between protein and ligand. Binding energy

is calculated as the sum of the intermolecular energy (hydrogen bonding, hydrophobic interactions, etc) and torsional free energy (free energy released due to the rotational bonds present in the ligand) (Iman et al., 2015). Analysis of the docking was done with *Arabidopsis thaliana* HPPD crystal structure (Yang, 2004).

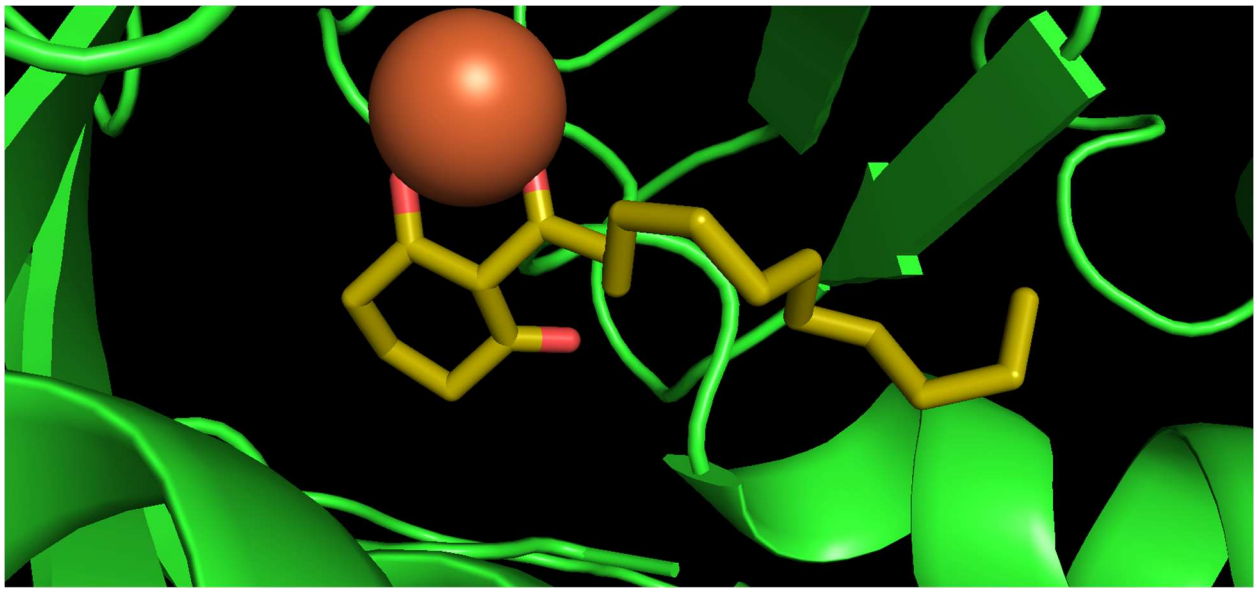


Figure 47. A screenshot of the docking between an HPPD inhibitor (4B: 2-acyl-cyclohexane-1,3-diones with a nonyl hydrocarbon side chain) and the *At*HPPD active site. You can see the iron interacting with the triketone feature (oxygen denoted as red).

6.3.1 Results and Discussion for Enzyme Analysis

Analysis of 5 different classes of ligands were undertaken as shown in Table 3 and Appendix I and summarized in graphic form in Figure 48. They are the cyclohexane-1, 3-diones with phenylene side chains, cyclohexane-1, 3-diones with phenyl side chains, cyclohexane-1, 3-diones with other side chains, 2-acyl-cyclohexane-1, 3-diones with simple aliphatic side chains and non-diones with simple aliphatic side chains. Comparing the selected compound's IC_{50} data points to known triketone natural product HPPD inhibitors leptospermone (IC_{50} value 3.14μM) and grandiflorone (IC_{50} value 0.22μM) and commercial HPPD inhibitor sulcotrione (IC_{50} value 0.25μM), half of the compounds have an IC_{50} value lower than leptospermone, while none of the compounds have values lower than either grandiflorone or sulcotrione (Table 3). These comparisons to known herbicidal compounds are important because all the ligands present in this study are derivatives of leptospermone. Those components with an IC_{50} value lower than leptospermone are of interest because they suggest greater activity than what can be most commonly found in nature for a β -triketone, but still represent a less effective molecule compared to the commercial inhibitor (Dayan, 2009). The most active compounds were those from the family 2-acyl-cyclohexane-1,3-diones with simple aliphatic side chains of 9 or 11 carbons (4B, 5B, 5F) or had a similar grandiflorone base structure containing a phenyl side chain (10B), possibly resulting from positive steric and hydrophobic interactions (Dayan, 2009). Comparatively, larger aliphatic side chains (greater than 11 carbons; 6A, 6B), smaller side chains (less than 9 carbons; 1B, 1F, 3B, 3E) resulted in a decrease of activity potentially due to steric hindrance (Dayan, 2009). It was also previously found that polar methyl groups or cyclohexyl groups in this position produced no inhibition and thus was not included in this study (Dayan, 2009). In cyclohexane-1, 3-diones with aromatic side chains, compounds with phenyl groups were more active than those with phenylene rings. Comparing compounds of similar base structure but with phenylene (8A, 8D, 10A, 10C) vs phenyl rings (8B, 8E, 10B, 10D), phenyl side chains had IC_{50} values 2-3 times more active (smaller) than those with phenylene side chains. The only difference, a double bond on the carbon chain leading out to the aromatic side group, may have resulted in a restriction of rotation that prevented a better fit in the binding pocket compared to what a single carbon-carbon bond could have provided. The addition of a 3-methoxy group or a methyl group to the base structure decreased activity and increased IC_{50} values by 2 to 5 times, indicating that these additional groups may interfere with the binding in

the active site. This decrease in activity may be due to the limited space around the Fe atom in the binding domain, which is unable to accommodate these extra side groups on the cyclohexane-1, 3-dione ring (Dayan, 2009). Non-diones with simple aliphatic side chains did not produce a notable IC_{50} value but were still included with this modeling assay. However, their very high IC_{50} values may be because they are missing the critical 1,3-dione structure to interact with the iron atom in the binding pocket (Dayan, 2009), but it
may still have affinity to the active site as shown by its binding energy.

Table 3. Structures and activities of leptospermone derivatives.

Molecule	Side chain R1	Side chain R2	Side chain R3	Lowest Binding Energy (kcal/mol)	Average Binding Energy (kcal/mol)	Standard Deviation (kcal/mol)	IC_{50} (uM)
1A	Methyl	H	H	-3.49	-3.48	0.00344	100
1B	Methyl	H	H	-3.87	-3.85	0.01406	23.09
1F	Methyl	Methyl	H	-3.03	-2.96	0.11696	53.12
3A	Pentyl	H	H	-4.62	-4.25	0.18279	100
3B	Pentyl	H	H	-4.23	-3.97	0.16871	2.93
3E	Pentyl	Methyl	H	-3.75	-3.45	0.16872	4.17
4A	Nonyl	H	H	-5.04	-4.71	0.14539	100
4B	Nonyl	H	H	-4.81	-4.39	0.25632	0.46
4E	Nonyl	Methyl	H	-4.14	-3.44	0.30581	2.66
5A	Undecyl	H	H	-4.65	-4.4	0.25264	100
5B	Undecyl	H	H	-5.44	-4.55	0.3429438	0.3
5F	Undecyl	Methyl	H	-4.19	-3.41	0.39712	0.77
6A	Hexadecyl	H	H	-4.67	-3.8	0.4536074	1.58
6B	Hexadecyl	Methyl	H	-3.41	-2.83	0.4273	1.7
8A	H	H	H	-5.83	-5.64	0.14745	5.43
8B	H	H	H	-5.06	-4.92	0.13667	1.27
8D	H	Methyl	H	-4.2	-4.11	0.07321	58.6
8E	H	Methyl	H	-4.17	-3.84	0.21501	2.17
10A	3-methoxy	H	H	-5.15	-4.97	0.08567	0.89
10B	3-methoxy	H	H	-4.97	-4.78	0.14771	0.31
10C	3-methoxy	Methyl	H	-4.17	-3.95	0.1303	6.4
10D	3-methoxy	Methyl	H	-3.45	-3.15	0.20144	1.5
13A	H	-	-	-5.91	-5.74	0.14843	9.51
13C	Methyl	-	-	-4.98	-4.76	0.19292	28.7

6.3.2 Results and Discussion for Molecular Modeling

In 2020, searching “Autodock” at PubMedCentral identified over 7,000 publications and a combined 30,000 citations as reported by Google Scholar for the three most-cited Autodock suite publications (Goodsell, 2021). The widening scale and scope of the publications, along with advances in computer technology, database management, open source software, and increasing public interaction, has brought molecular modeling into the mainstream of scientific literature. Autodock was designed to solve a specific problem; the docking of small, potentially drug-like or natural product-like molecules to biological macromolecules whose 3D structure is known (most likley from X-ray crystolography) (Goodsell, 2021). One major argument against such molecular modeling softwares is that the software doesn’t work very well with large ligands, specifically proteins 10 amino acids and larger. These ligands have too many degrees of freedom for the docking software (Forli, 2016). Another major argument is that the protein target may show a significant conformational flexbility or a induced fit upon binding. This is another drawback because, while Autodock may be able to model selective sidechain motion for limited conformational changes in the receptor, it generally employs a rigidity in the receptor during docking calculations (Forli, 2016). While advanced settings within autodock and molecular dynamics of the target protein can help facilitate utilizing Autodock for these particlar inquires (Forli, 2016), both of these concerns do not fall upon this study as the ligands in question are small with limited degrees of flexibility. Also, as described previously, the target protein *Arabidopsis thaliana* HPPD was found to have limited flexibility around the Fe atom in the binding domain (Dayan, 2009), making it a good fit for this type of molecular modeling.

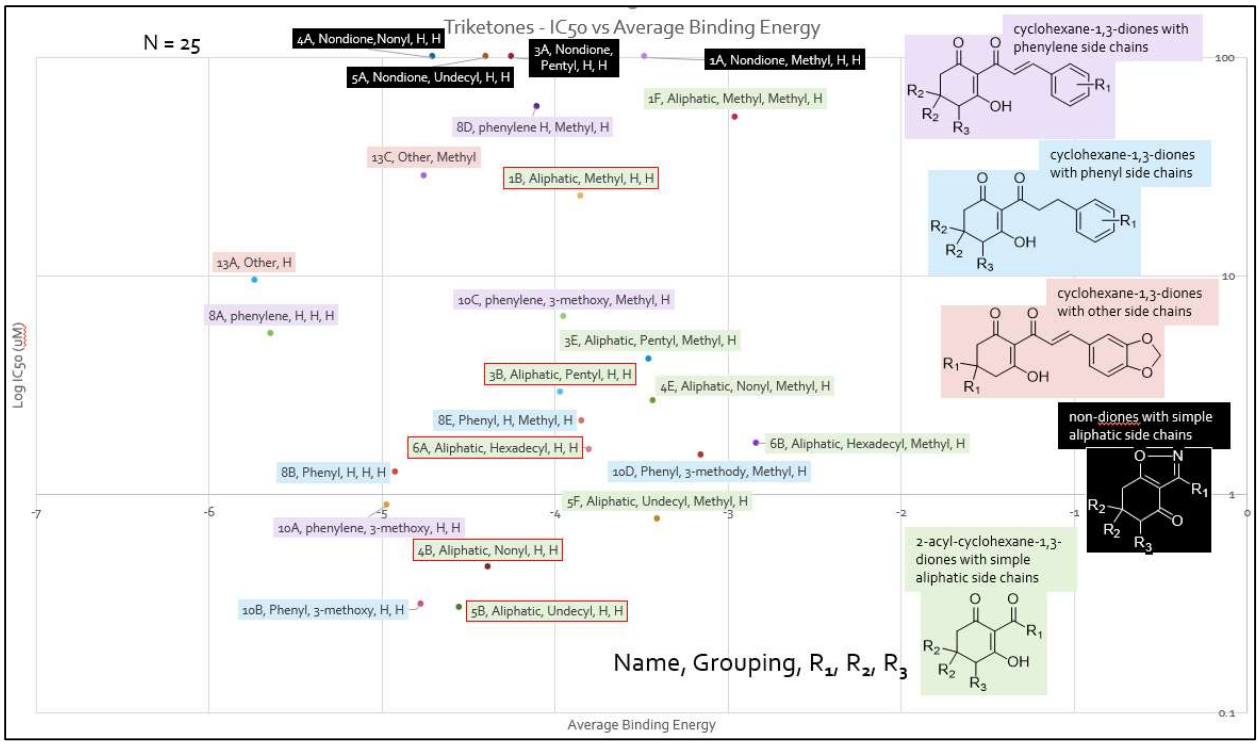


Figure 48. A display of all molecules, with predictive average binding energy correlated with Log IC_{50} values found *in-vivo*. Molecules are organized according to class of base structure (Grouping), seen on right. Each data label follows the pattern of "Name, Grouping, R_1, R_2, R_3", where "R_1, R_2, and R_3" refer to the functional group. The most optimal ligands are those that have the most negative binding energy and the lowest Log IC_{50}.

One trend can be seen when comparing 1B, 3B, 4B, 5B, and 6A (as shown in figure 49) the only difference in the structure of the molecules is the length of their hydrophobic tails (R_1 group). Previous studies have shown that the 1, 3-dione group is important to binding regardless of sidechains and extra motifs, and that this essential group interacts with the iron atom in the binding pocket (Fu, 2020; Li, 2018; Yang, 2004). When comparing the log of their IC_{50} values to their respective average binding energies, we see a positive correlation, where, as logIC_{50} values increase, binding energy approaches zero. A more positive binding energy indicates less binding affinity between substrate and active site and thus an interaction that is less thermodynamically favorable. The lowest binding energy (and smallest IC_{50} values) comes from 5B, with a R_1 of undecyl (11C) side group. With structures having a carbon tail of more carbons (4B, nonyl) or less carbons (1B methyl, 3B pentyl, 6A hexyl) having higher binding energy and a larger IC_{50} value. The longer than optimal carbon tail may result in not being able to fit sterically sound within the hydrophobic pocket of the active site, while too few carbons may result in not fully

utilizing the space and the potential stabilizing effect of the pocket. This trend was also supported by findings in similarly related triketone containing quinazoline-2,4 dione derivatives, which were also HPPD inhibitors, where, branching from the quinazline group, too small of a hydrophobic chain (CH_3), too long of a hydrophobic chain ($CH_2CH_2CH_2CH_3$), or addition of a polar group were detrimental for HPPD inhibition while intermediate non-polar tail lengths had better inhibition (Wang, 2015, p. 201). Both cases indicate that flexible, non-polar alkyl tail groups increase HPPD inhibition when accounted for the size of the binding pocket.

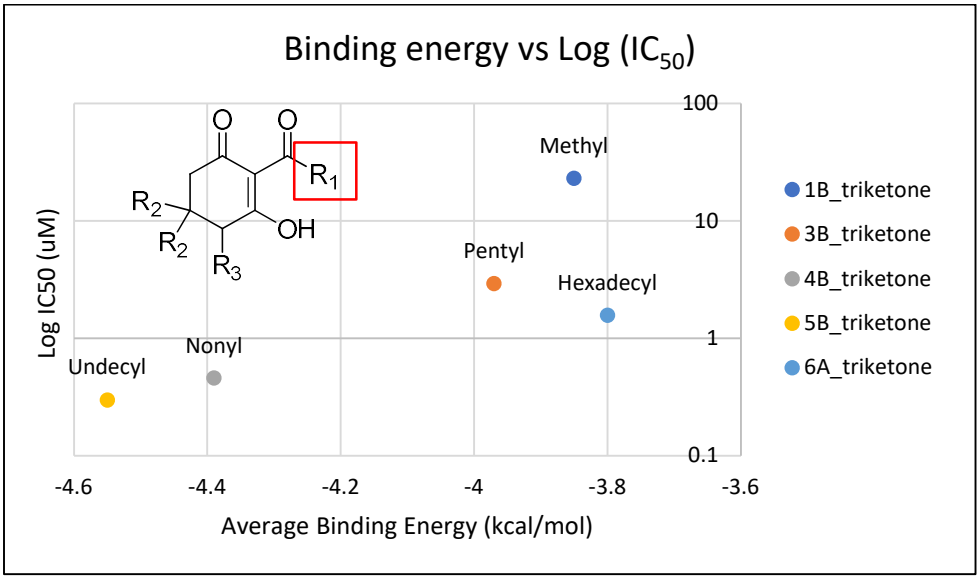


Figure 49. The relationship of binding energy and the logIC_{50} of a selection of 2-acyl-cyclohexane-1,3-diones with simple aliphatic side chains (R_1 subgroup). As the IC_{50} of the synthetic analog increases the model reflects a more positive binding energy, showing a correlation between the *in-vivo* and *in-vitro* models developed for the HPPD protein. The most effective molecule (5B) displayed in this graph is the triketone with the undecyl carbon chain length on the R_1 subunit location. This has both the lowest IC_{50} value (most effective) and most negative average binding energy (greatest affinity for the active site) compared to its peers who have either longer or shorter carbon length side chains.

Another trend involves the addition to a methyl group to the base ring structure as seen in 1F, 3E, 4E, 5F and 6B (as compared to their same carbon length counterparts 1B, 3B, 4B, 5B, and 6A in Figure 50) increases IC_{50} values and worsens binding energy, potentially due to sterics. This trend was also seen again in the similarly related triketone containing quinazoline-2,4 dione derivatives, which were also HPPD inhibitors, but this time with direct relation as these additional groups were on the similar triketone ring structure. Herbicidal activity decreased and K_i (inhibition constant, the concentration of inhibitor required to reach 50% enzyme saturation) increased as functional groups were added to the 1,3-cyclohexanedione ring (Wang, 2015).

These two trends, the perfect fit hydrocarbon tail and the reduction in binding ability with the addition of the methyl group to the head structure, can be seen in how the substrate is able to interact with the active site. While the active site does have a binding pocket with the stabilizing iron atom and a resultant hydrophobic region for the hydrocarbon tail, too large of a tail may cause the molecule to bend, resulting in increased steric hinderance, while the addition to the methyl group to the head structure may add steric hinderance, potentially decreasing its ability to interact with the HPPD active site.

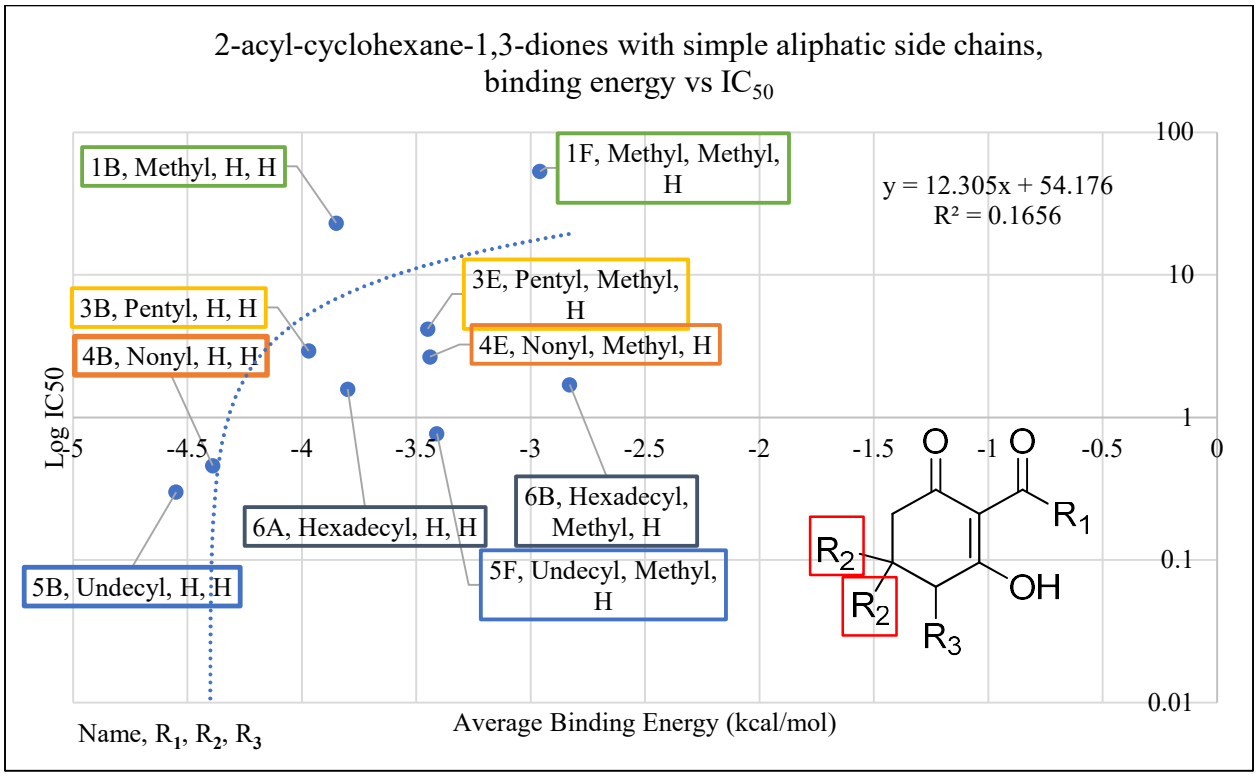


Figure 50. The relationship of binding energy and the logIC_{50} of a selection of 2-acyl-cyclohexane-1,3-diones with simple aliphatic side chains. Molecules are organized according to class of base structure (Grouping), seen on the bottom right. Each data label follows the pattern of "Name, Grouping, R_1, R_2, R_3", where "R_1, R_2, and R_3" refer to the functional group. Each grouping of similar tail length is shown by a colored box, with the only difference within the color selection being an addition of a head group at the R_2 location. The addition of a head group (methyl) results in a higher binding energy and a less favorable IC_{50} value.

This trend of the addition of a methyl group to the head structure resulting in an increase of IC_{50} values and a more positive average binding energy can also be seen when comparing cyclohexane-1,3-diones with phenylene side chains (8A vs 8D, 10A vs 10C), cyclohexane-1,3-diones with phenyl side chains (8B vs 8E, 10B vs 10D), and in cyclohexane-1,3-diones with

other side chains (13A vs 13C), for the reasons mentioned previously. However, an interesting comparison comes into play when factoring in a polar tail group (3-methoxy addition to R_1) in the phenylene side chains (8A vs 10A), the phenyl side chains (8B vs 10B), and the other side chain (benzodioxole group) (13A vs 13C). The 3-methoxy group is an electron-donating group by resonance (mesomeric effect), with the excess electron lone pairs being donating into the ring to increase its reactivity (Hagar et al., 2020). This can then cause the electrons to move around in conjunction with the tautomerization of the triketone structure (Gray et al., 1980), as we see a greater decrease in IC_{50} in the phenylene sidechain as compared to the phenyl sidechain. Both the 3-methoxy group and the benzodioxole group have the oxygens attached to a benzene ring that would allow them to assist in forming of pi bonds, with benzodioxole having the anomeric effect due to its carbon and oxygen confirmation, making its five member ring pucker (Laane, 2009). This would allow for the large bulky groups to be potentially stabilized with conserved amino acid resides Phe360 and Phe403 via $\pi - \pi$ stacking (Wang, 2015)(Wang et al 2015). This trend is also seen on the benzene ring from the quinazline group of the related quinazoline-2,4 dione derivatives, where substituting a Cl for a CH_3 slightly decreased K_i values, indicating slightly greater HPPD inhibition activity with the more reactive sidechain (Wang, 2015). This addition of a polar group may have increased the polarity of the dione group through the mesomeric effect and tautaumerization of the triketone and could be the reason for the larger IC_{50} value difference observed between phenylene side chain, which has an extra moveable double bond, and phenyl side chain, which does not have the electron flexibility. The addition of a methyl group to the triketone head group (R_2) with the methoxy group on R_1 resulted in the IC_{50} values returning close their original structures values (without the methyl head on R_2 or the methoxy on R_1), following the trend established previously whereby adding a methyl group to the base ring structure results in an increased binding energy and $IC_{50.}$ The same trend is seen with the quinazline group structures, where when adding methyl groups to the triketone group, Cl again outperforms CH_3 in terms of IC_{50} values (Wang, 2015). However, that trend of Cl outperforming CH_3, is reversed when a hydrophobic tail is brought into consideration, as the addition of a CH_2CH_3 tail to the quinazline group places CH_3 with a lower IC_{50} (but still higher than without the methyl group on the triketone head) compared to the Cl with the same hydrophobic tail addition and again emphasizes the importance of that hydrophobic interaction (Wang, 2015). Binding energy should follow the same trend as IC_{50} values, where addition of 3-methoxy should

have showed greater binding affinity followed by the addition of the head group, where binding affinity is worse, but Autodock is poor at evaluating and predicting largescale electron movements within a single molecule because its software is designed to analyze static interactions or small changes in charge, dipole movement or polarity rather than large dynamic shifts (Goodsell, 2021). This shows that a predictive model for the HPPD active site was created utilizing derivatives from the β -triketone leptospermone.

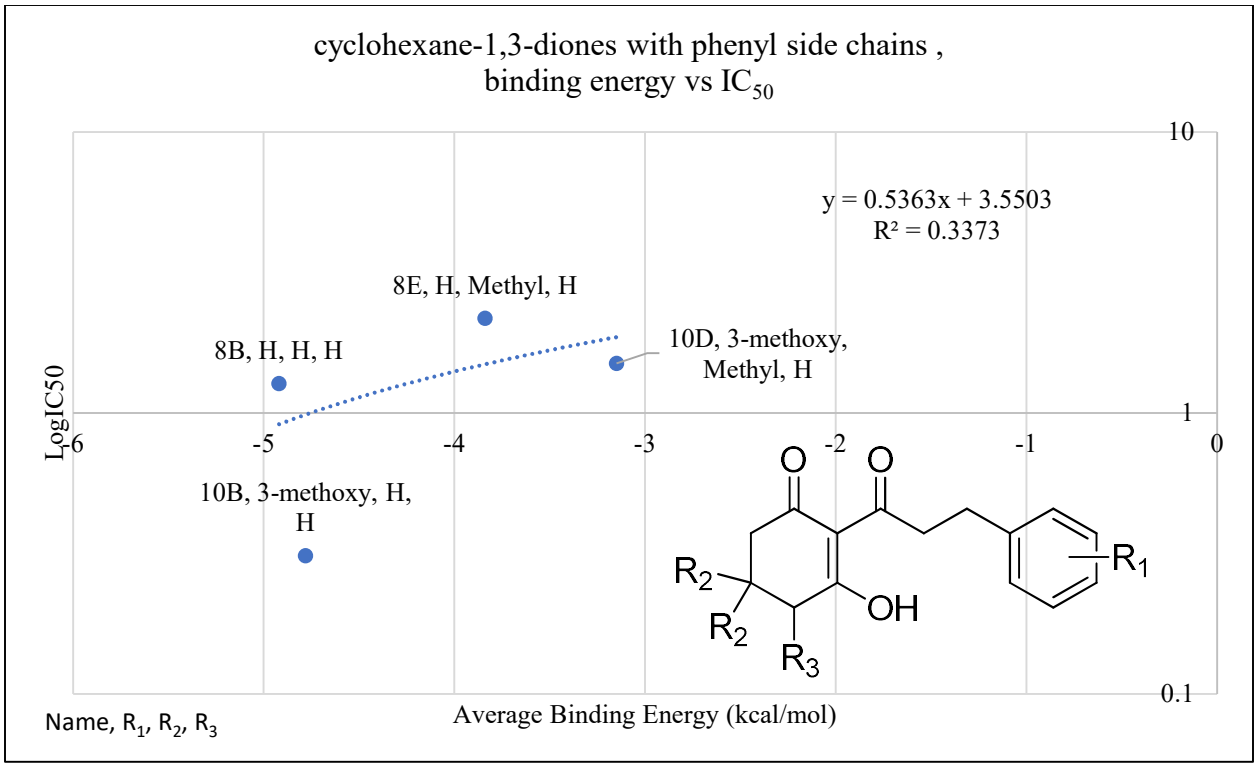


Figure 51. The relationship of binding energy and the $logIC_{50}$ of a selection of cyclohexane-1,3-diones with phenyl side chains. Molecules are organized according to class of base structure (Grouping), seen on the bottom right. Each data label follows the pattern of "Name, Grouping, R_1, R_2, R_3", where "R_1, R_2, and R_3" refer to the functional group. Addition of a head group to R_2 reduced binding energy and was found with a less effective IC_{50}. R_1 tail addition of 3-methoxy lowered IC_{50} compared to predicted with binding energy.

The work in this chapter stands shoulder-to-shoulder with other experiments of this nature done before with the HPPD enzyme, with the exception that most of the other work has been using modifications to the more complicated, synthetic mesotrione ligand rather than leptospermone, which was used in the original identification of the HPPD molecular target site. Although mesotrione is more biologically active than leptospermone, it is also more chemically complex. This leaves open the potential that a derivative of leptospermone that is simpler in

structure than mesotrione could be overlooked during screening. For example, the State Key Laboratory of Elemento-Organic Chemistry at Nankai University looked at derivatives of a hybrid base structure combining key elements of mesotrione and fischerellin A, a tetramic acid photosystem II electron inhibitor found to inhibit the growth of *Lemna minor* (Hagmann and Jüttner, 1996). Modifying the triketone into a diketone enamine and editing R groups while retaining the non-polarity of the left side of the molecule, they synthesized and evaluated for herbicidal activity derivatives. These assays resulted in injury symptoms (withered leaves, stunting and root inhibition on whole plants) on dicot *Brassica napus L* (rapeseed). and *Amaranthus retroflexusi* (pigweed) more than monocot *Echinochloa crusgalli (L.) Beauv* (barnyard grass). and *Digitaria adscendens* (crabgrass), with low IC_{50} values that were not indicative of traditional HPPD inhibition. A Hill reaction utilizing chloroplast from spinach leaves determined that the rate of oxygen evolution was correlated with the concentration of inhibitor added, indicating electron transport inhibition. To determine this, Autodock was utilized with their ligand and the target as Q_B binding site of protein D1 from photosystem II, for which the resulting binding energy supported their bioassay results, including indicating that there are hydrophobic interactions between the ligand's quinoline-2,4-dione and Phe255 and the *t*-Bu group with His215, Phe211, Phe274, Leu271, Leu275, and hydrogen bonding with the amide carbonyl of quinoline-2,4-dione and Phe265. In addition, the binding pocket has a large hydrophobic region that may help stabilize a large hydrophobic group such as a α-methylbenzyl or 2- tetrahydrofuranmethyl group (Liu et al., 2013). This structural modification of the base triketone changed the mode of action from a HPPD inhibitor to an electron transport inhibitor, with Autodock providing evidence of the nature of the active site affinity to the ligand. Such a change emphasizes the importance of maintaining the dione structure for a HPPD targeted herbicide and gives indications as to why the non-diones with simple aliphatic side chain showed potential to bind but did not have inhibitory affects in field trials, as was seen in our own data.

Chapter 7: Conclusion and Future Perspectives

The creation of a sensitive non-biased bioassay to show case allelopathy utilizing Manoa lettuce and 'Koba' green onion, two model plant species representative of dicot and monocots, has been achieved. Starting with 20 suspected allelopathic invasive and native plant species, courtesy of the large biodiversity found within the tropical and sub-tropical climate in Hawaii, aqueous and organic chemical extraction with an initial invasive leaf grinding and then a more natural leaf soak in water were conducted. Filtering via plant growth inhibition along with keeping in mind cultural sensitivity and local interests, the project was moved forward with the invasive species strawberry guava, *Psidium cattleianum*. Observations of its growth conditions in nature and in the green house found it to be characteristic of an allelopathic plant with reduced biodiversity, while leaf leaches were found to also have pre-germination effects on commercially relevant weeds pigweed and barnyard grass, representative dicot and monocot respectively. A closer look revealed that, while both roots and shoots of the test plants were affect, roots were more greatly affected than shoots. Putting numbers to the analysis, yellow variety of strawberry guava produced a IC_{50} of 10.74mg/mL for lettuce and 12.2 mg/mL for green onion while red variety produced a IC_{50} of 9.57mg/mL for lettuce and 10.67 mg/mL for green onion. Continuing with bioassay mediated fractionation, HPLC, and MS, it was proposed, for the first time to the best of our knowledge, that strawberry guava leaves contain gallic acid, with this chemical contributing to its allelopathic properties. Previously described were several reasons on how gallic acid interacts with a plant root system and causes oxidative damage, collapse of root architecture, and eventual death of the plant, yet many questions remain. For example, further confirmation of gallic acid in strawberry guava leaves would require NMR analysis. When looking at the mode of action, radiolabeling gallic acid and observing where in the plant roots and at what rate the gallic acid does or does not enter the plant root would help identify its exact role in the death of the plant. In addition, gallic acid may not be the only chemical in strawberry guava leaves to cause allelopathy and more metabolomic studies along with allelopathy studies are required to determine if gallic acid is the only allelochemical in the leaf or if there is a synergistic effect as was hinted at previously.

There are many other aspects of this project that can be expanded upon and adapted for additional plants. For example, in the initial allelopathy screening assays, it was found that the

native *Acacia koa* had a similar if not greater growth inhibition effect on the test plants compared to the known allelopathic *Acacia confuse*. This native vs non-native comparison would be of great interest as the biodiversity and isolation of Hawaii often leads to unique changes in native plant species compared to their continental counterparts. The *Bauhinia blakeana*, *Bauhinia galpinii* and *Bauhinia monandra*, all of unknown allelopathy, were slightly less allelopathic than their known allelopathic cousin the *Bauhinia purpurea*. This diverse collection of various related species would provide insight into why these plants showed allelopathic plant growth inhibition at all, perhaps through chemical means that remain to be discovered. *Erythrina abyssinica* and *Erythrina crista-galli* again provide that within genus analysis, where *Erythrina abyssinica* is known allelopathic while *Erythrina crista-galli* is suspected by our own bioassay. This similar relationship between known and unknown allelopathic plants parallels *Psidium cattleianum's* own relationship to the known allelopathic *Psidium guajava*, with the protocols utilized previously able to be applied to these other plant groupings for future study, utilizing the known allelopathic plant as a blueprint for the explanation of the potential allelopathy of the unknown related plant.

Two plants that did not get their fair share of analysis include *Pachira aquatica* and *Platymiscium stiuplare*, both plants whose acetone leaf extract greatly inhibited the growth of the test plants. This rarity in the selection of plants hinted at an allelopathy whose chemical nature is less water soluble. This provides a similar train of thought as to how a natural product may be discovered, invoking the history of manuka oil containing grandiflorone and eucalyptus oil containing citronellal and citronellol (to name a few). In addition, *Pachira aquatica's* water leaf extract had no inhibition of plant growth while *Platymiscium stiuplare* had inhibition of plant growth, indicating a various amount of potential situations that remain to be studied, including potential solubility of the chemical in question in both organic and aqueous solutions or perhaps multiple allelopathic chemicals.

While gallic acid may be one constituent of strawberry guava's allelopathy, it is most likely not the only allelopathic chemical within the leaves of the plant. For example, analysis of regular guava leaves provide a wide variety of chemicals that remain to be determined if they exist within strawberry guava and, if they are, their allelopathy remains to be tested. Within gallic acid however, many questions remain about its allelopathic ability. For example, how does

it get into the plant root when applied extracellularly? What exactly does it interact with within a cell to create the reactive oxygen species and resultant oxidative stress responses? While gallic acid may never become a ground-breaking herbicide, there is a lack of knowledge on how it affects the plant that remains to be discovered and can provide years of study for the next generation of students.

Working through an integrated approach for the progressive development and technology establishing of not only the discovery phase but also the development phase, an in-silica molecular docking was conducted with HPPD and known ligands to create a predictive model. The trends showed that, based upon 2-acyl-cyclohexane-1,3-diones with simple aliphatic side chains, the perfect R_1 tail length is an undecyl 11-C, with too short not fully utilizing the binding pocket and too long not fitting within the binding pocket properly, showcased by having a more positive binding energy and a less efficient IC_{50} value. This class along with cyclohexane-1,3-diones with phenyl side chains, phenylene side chains, or other side chains, also followed another trend, where the addition of a R_2 methyl group again made a more positive binding energy and a less efficient IC_{50} value possibly due to sterics. This showed proof of concept that we were able to correlate binding energy and measured IC_{50} values to create this predictive model that can be adapted to other molecular target sites and ligands.

The process and analysis outlined here, taking a known molecular target site and compare binding energies and wet lab IC_{50} values of ligands to create a molecular model for the advancement of herbicide development, can be applied to any number of other target-ligand pairings. While the creation of the model and verification of the data it produces will always involve a bioassay, the hope is that such a model will reduce the amount of unproductive and wasteful ligands being synthesized and tested. While many well known efficient herbicides currently exist, including glyphosate for EPSP synthase and grandiflorone for HPPD, there remains to be tested in the future newly discovered modes of action and, conversely, taking a look back upon old herbicides such as atrazine, whose herbicide resistance has grown in recent years. While the future of such a science is yet unknown and still in its infancy, with the development and implementation of artificial intelligence and machine learning, new developments in the molecular docking world are sure to be at the forefront of research and development teams in the coming decades.

While this work answers many questions, there always remains more to be discovered by future generations of scientists. This project has laid the groundwork for the next generation by highlighting the entire process of herbicide discovery and refinement, from determining suspected allelopathic ability to discovering a chemical constituent causing the activity to refining a natural product by molecular modeling backed up by bioassays. This process generally from start to finish takes more than a decade with commercial funding and a dedicated research team, yet here it has been shown that the majority of the steps can be completed within 6 years with publicly funded collaborations and analysis. It is with great pleasure that the achievements made within the compressed timeline could be considered monumental and was only possible due to the unwavering dedication and countless sleepless nights of multiple undergraduate students, graduate students and professors. Here we have laid the groundwork for future projects involving the plants of Hawaii or elsewhere in Dr. Daniel K Owen's laboratory and have opened the doors for new graduate and undergraduate cohorts to sharpen their skills and make new observations and conclusions in plant metabolite analysis.

Appendices

Appendix A. Range of Allelopathy

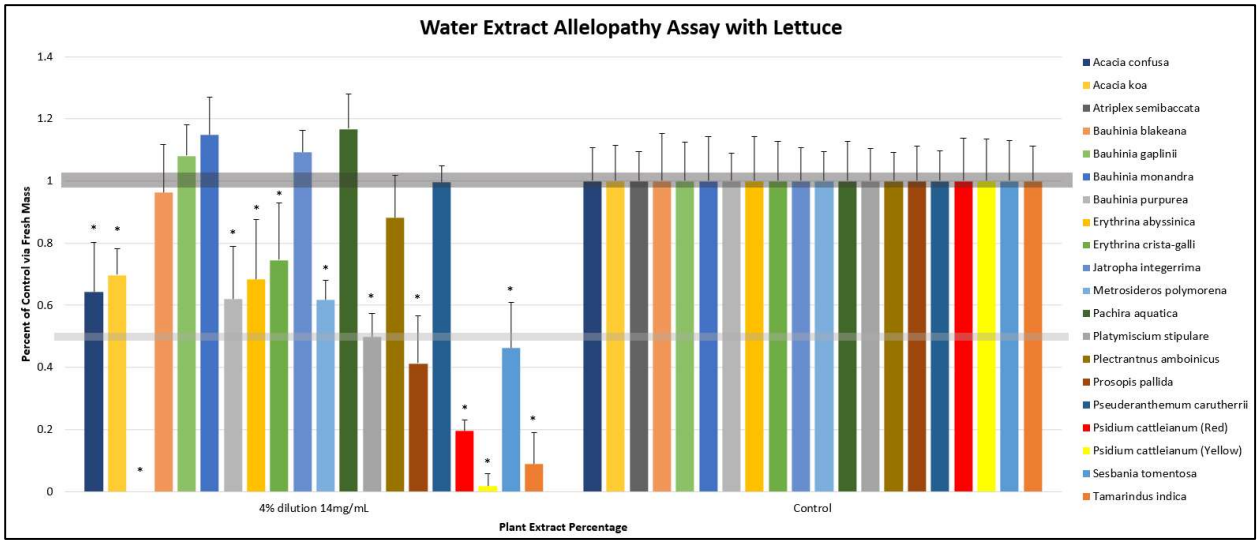


Figure 1. A comparison of the effects of various plant's macerated leaf water extracts at 4% concentration (14mg/mL) on the growth of lettuce, a dicot. The "*" denote a significance between the control and the experimental with $P < 0.01$ utilizing Anova: single factor. The dark horizontal line indicates 100% control growth while the smaller gray line indicates 50% control growth. N = 18

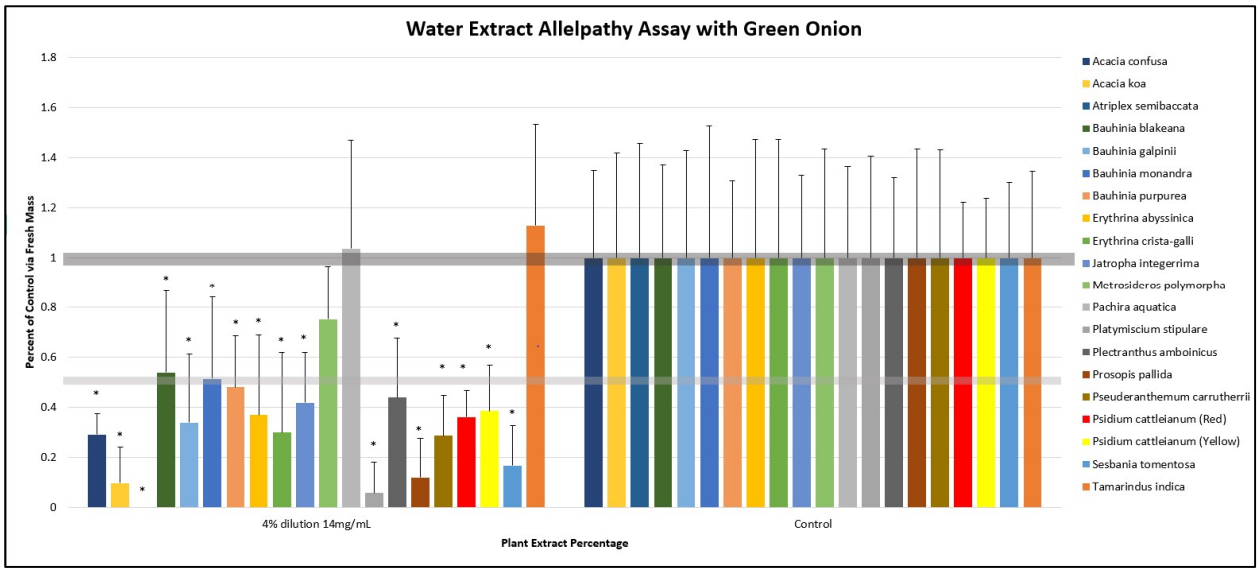


Figure 2. A comparison of the effects of various plant's macerated leaf water extracts at a 4% concentration (14mg/mL) on the growth of green onion, a monocot. The "*" denote a significance between the control and the experimental with $P < 0.01$ utilizing Anova: single factor. The dark horizontal line indicates 100% control growth while the smaller gray line indicates 50% control growth. N = 18

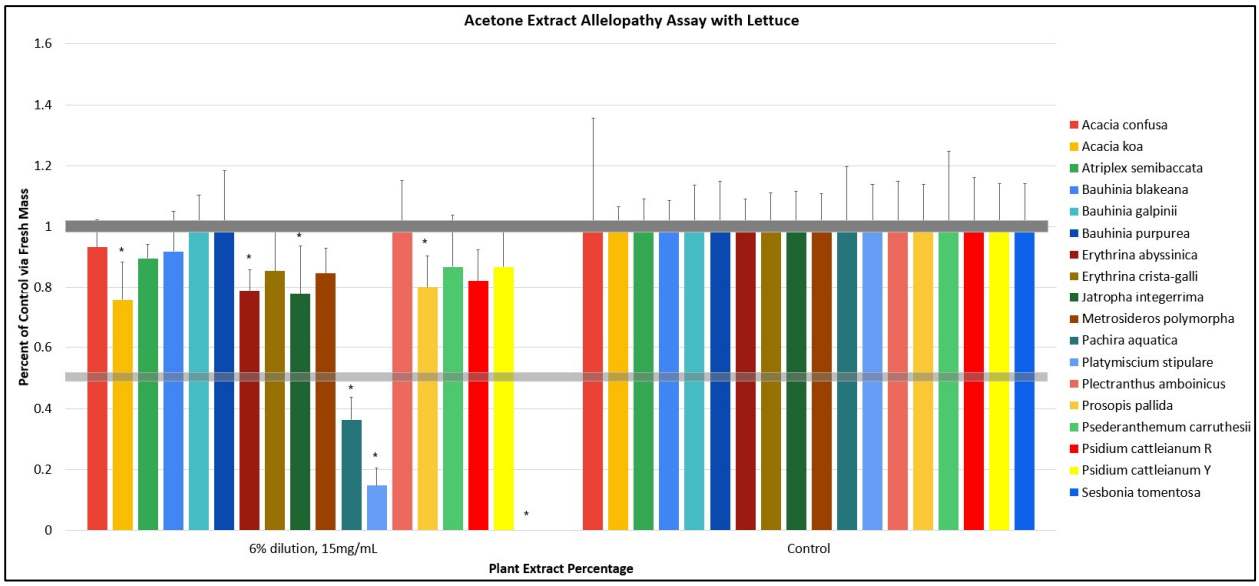


Figure 3. A comparison of the effects of various plant's macerated leaf actone extracts at a 6% concentration (15mg/mL) on the growth lettuce, a dicot. The "*" denote a significance between the control and the experimental with $P < 0.01$ utilizing Anova: single factor. The dark horizontal line indicates 100% control growth while the smaller gray line indicates 50% control growth. N = 18

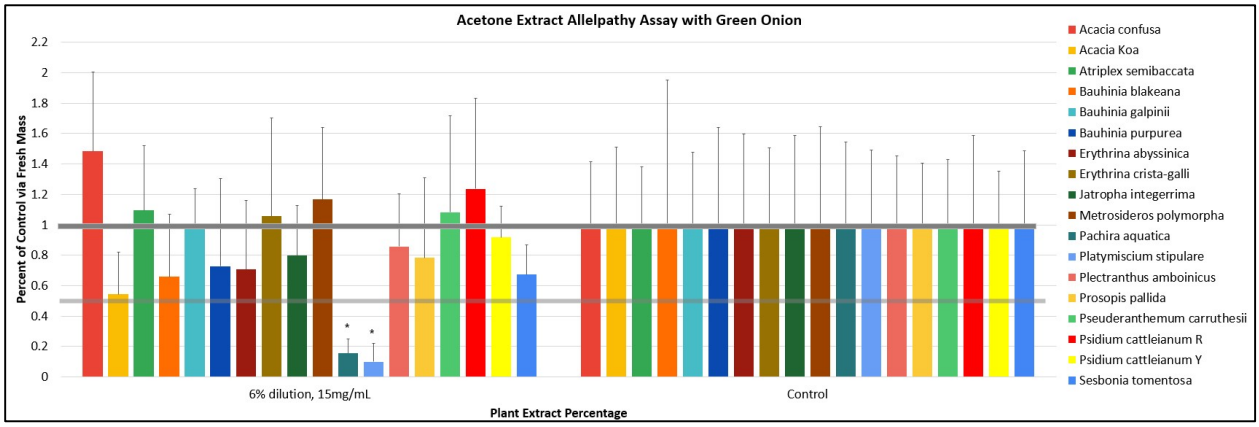


Figure 4. A comparison of the effects of various plant's macerated leaf acetone extracts at a 6% concentration (15mg/mL) on the growth of green onion, a monocot. The "*" denote a significance between the control and the experimental with $P < 0.01$ utilizing Anova: single factor. The dark horizontal line indicates 100% control growth while the smaller gray line indicates 50% control growth. N = 18

Appendix B. Plant Specific Leaf Soak Data

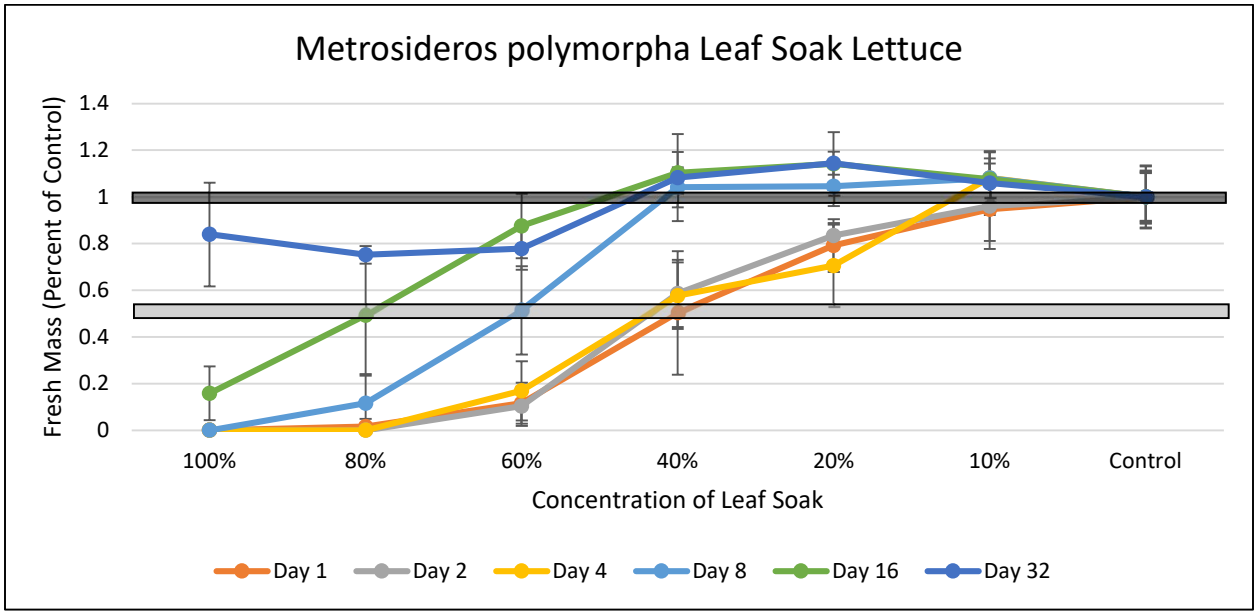


Figure 1. A time course of continuous soaking of *Metrosideros polymorpha* leaves and their effect on the growth of lettuce. The dark horizontal line indicates 100% control growth while the light grey line indicates 50% control growth. Values are fresh mass displayed as a percent of the control. Inhibition of growth was greatest in day 1, 2 and 4, with the least plant growth inhibition on day 32. N = 18

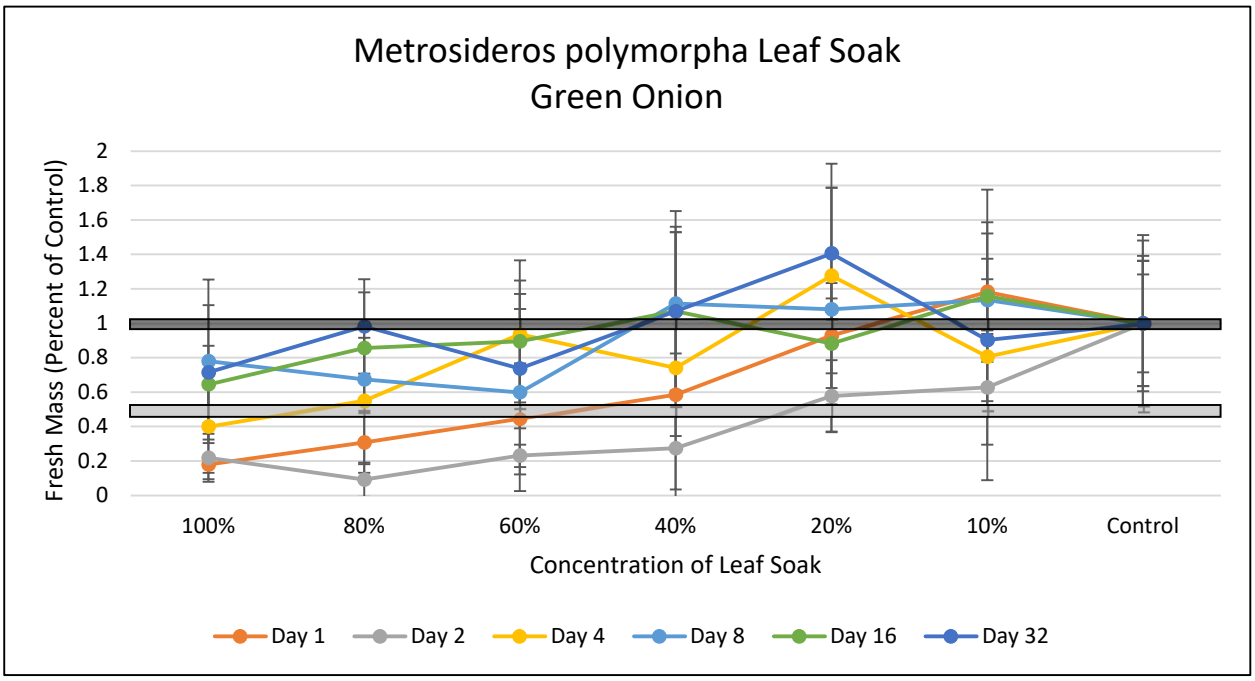


Figure 2. A time course of continuous soaking of *Metrosideros polymorpha* leaves and their effect on the growth of green onion. The dark horizontal line indicates 100% control growth while the light grey line indicates 50% control growth. Values are fresh mass displayed as a percent of the control. Inhibition of growth was greatest in day 1 and 2 and growth gradually increased as time went on. N = 18

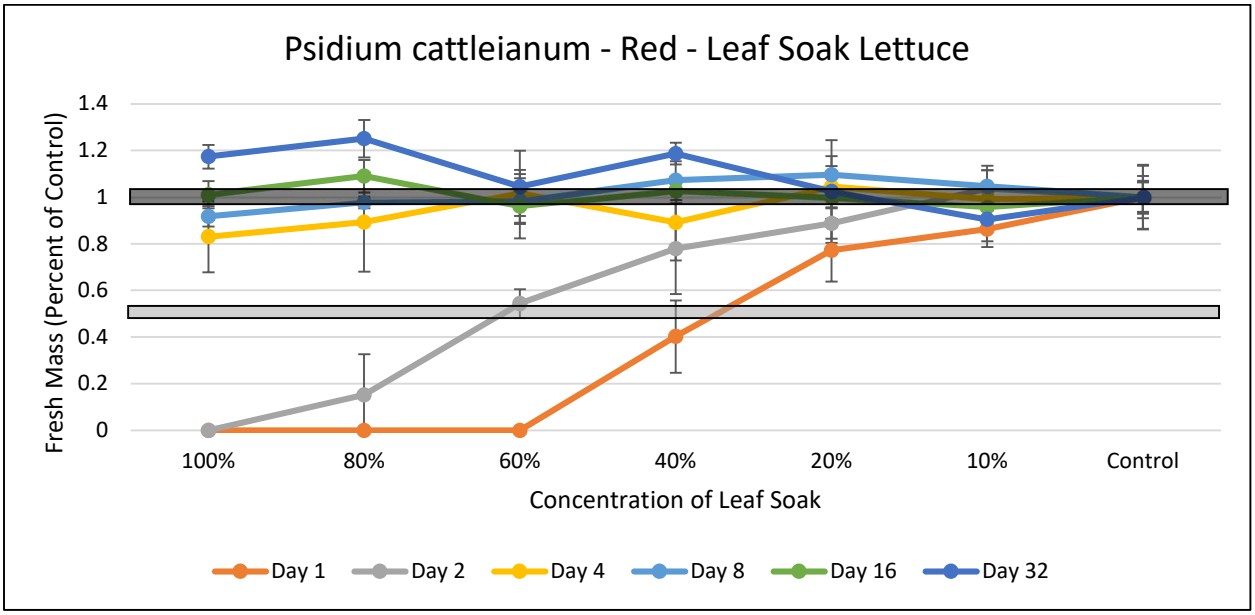


Figure 3. A time course of continuous soaking of *Psidium cattleianum* red variety leaves and their effect on the growth of lettuce. Values are fresh mass displayed as a percent of the control. The dark horizontal line indicates 100% control growth while the light grey line indicates 50% control growth. Inhibition of growth was greatest in day 1 and 2, with a large restoration of growth from day 4 onward. N = 18

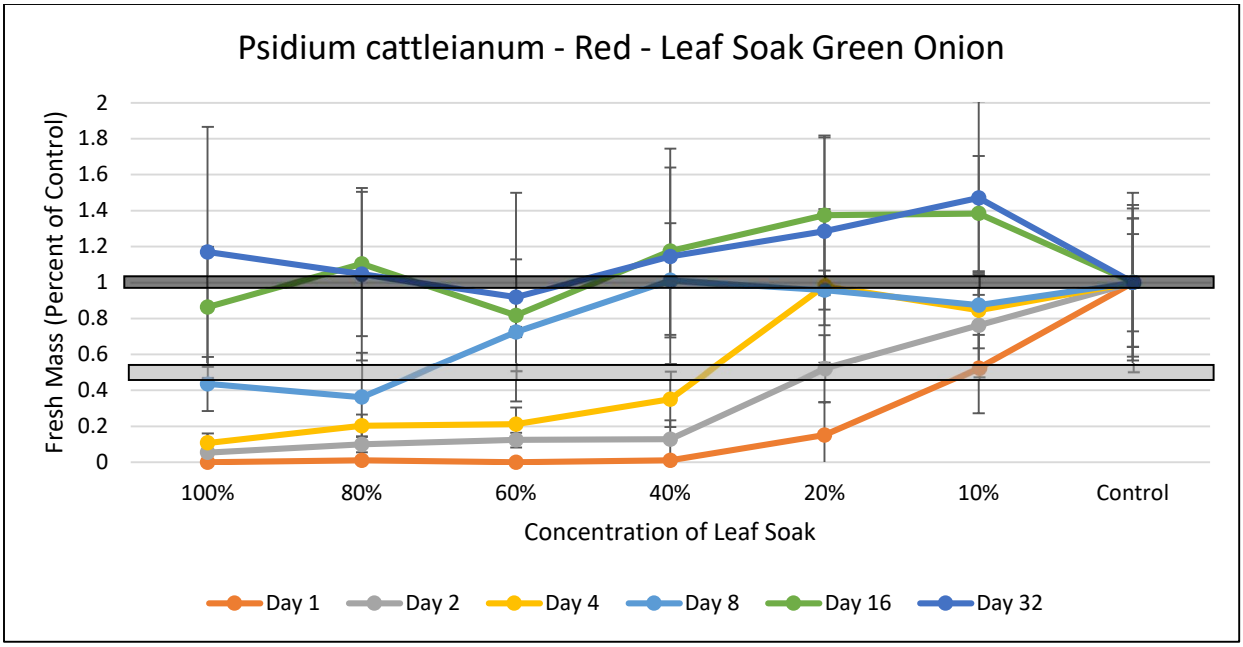


Figure 4. A time course of continuous soaking of *Psidium cattleianum* red variety leaves and their effect on the growth of green onion. Values are fresh mass displayed as a percent of the control. The dark horizontal line indicates 100% control growth while the light grey line indicates 50% control growth. Inhibition of growth was greatest in day 1, 2 and 4 with growth gradually increasing as time went on. N = 18

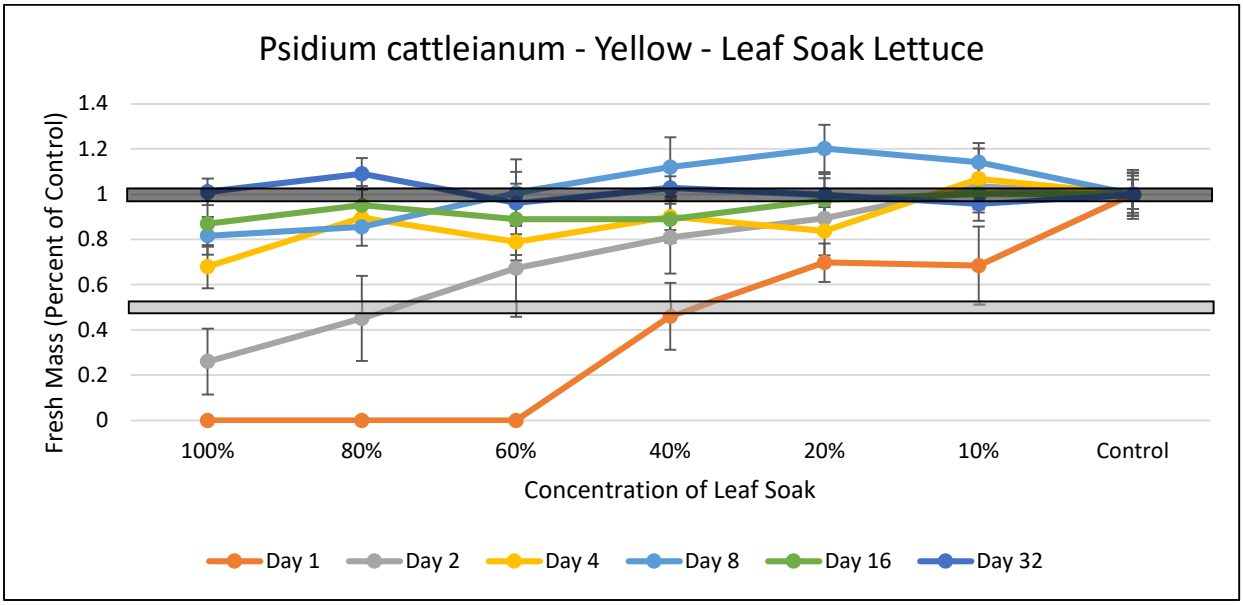


Figure 5. A time course of continuous soaking of *Psidium cattleianum* yellow variety leaves and their effect on the growth of lettuce. Values are fresh mass displayed as a percent of the control. The dark horizontal line indicates 100% control growth while the light grey line indicates 50% control growth. Inhibition of growth was greatest in day 1, with a slight restoration of growth on day 2 and a large restoration of growth from day 4 onward. N = 18

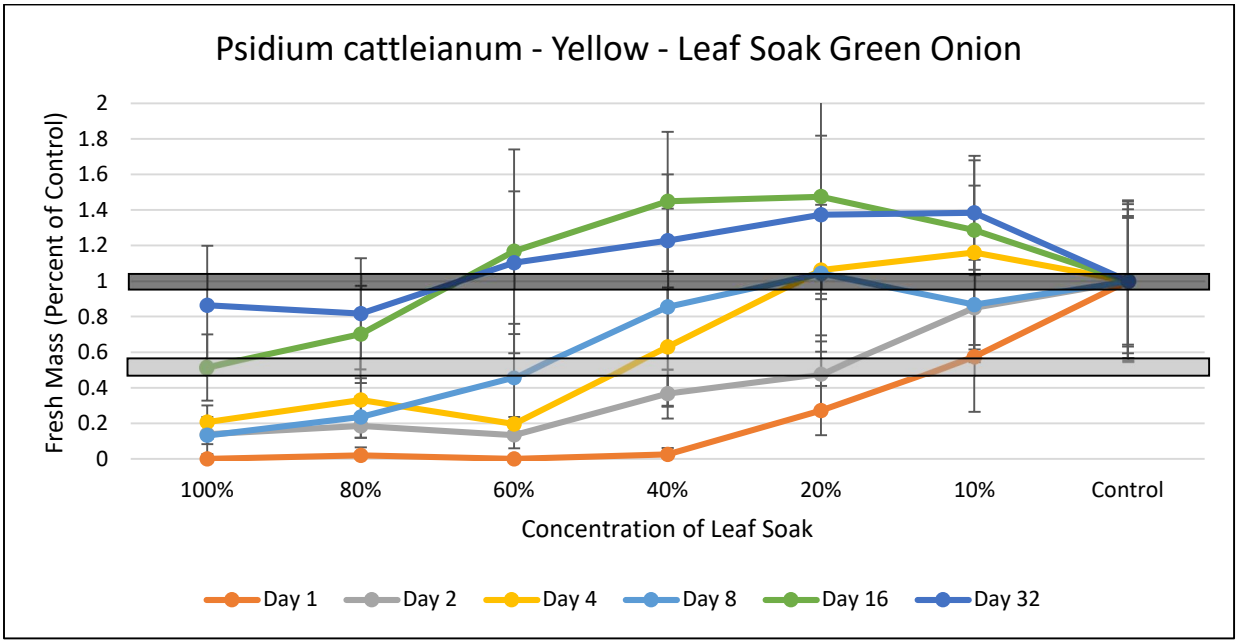


Figure 6. A time course of continuous soaking of *Psidium cattleianum* yellow variety leaves and their effect on the growth of green onion. Values are fresh mass displayed as a percent of the control. The dark horizontal line indicates 100% control growth while the light grey line indicates 50% control growth. Inhibition of growth was greatest in day 1, 2, 4 and 8, with a restoration of growth from day 16 onward. N = 18

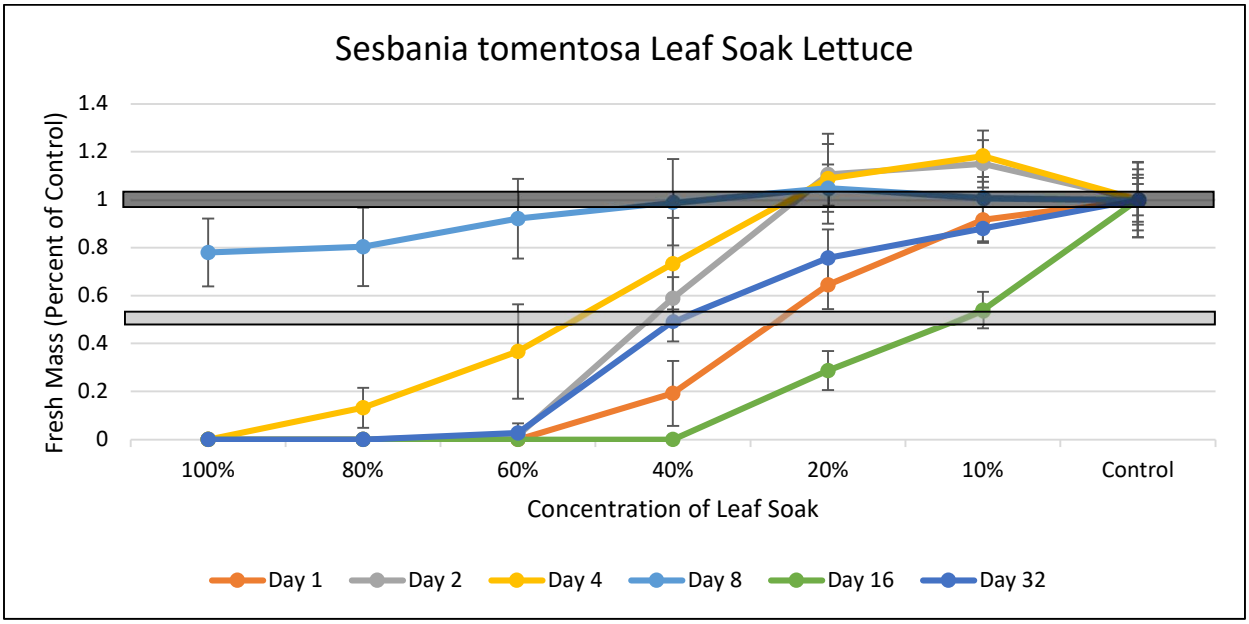


Figure 7. A time course of continuous soaking of *Sesbania tomentosa* leaves and their effect on the growth of lettuce. Values are fresh mass displayed as a percent of the control. The dark horizontal line indicates 100% control growth while the light grey line indicates 50% control growth. Inhibition of growth was greatest in day 1 and 32, with the least inhibition of growth seen on day 8. N = 18

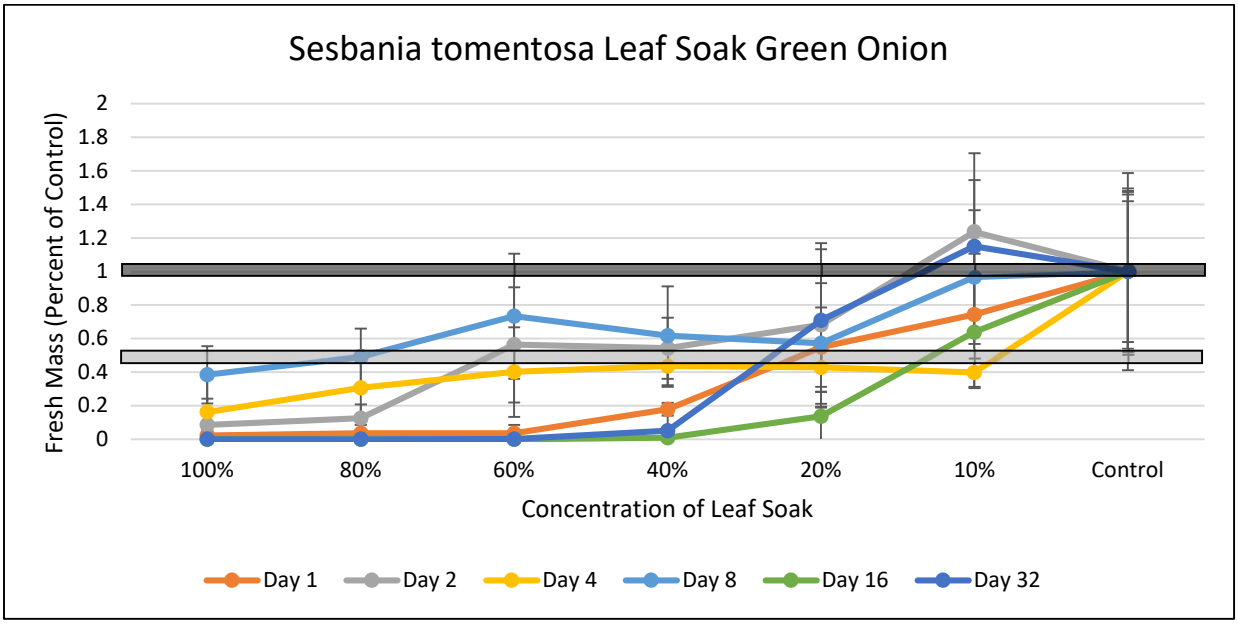


Figure 8. A time course of continuous soaking of *Sesbania tomentosa* leaves and their effect on the growth of green onion. Values are fresh mass displayed as a percent of the control. The dark horizontal line indicates 100% control growth while the light grey line indicates 50% control growth. Inhibition of growth was greatest in day 1, 2, 16 and 32, with the least inhibition of growth seen on day 8. N = 18

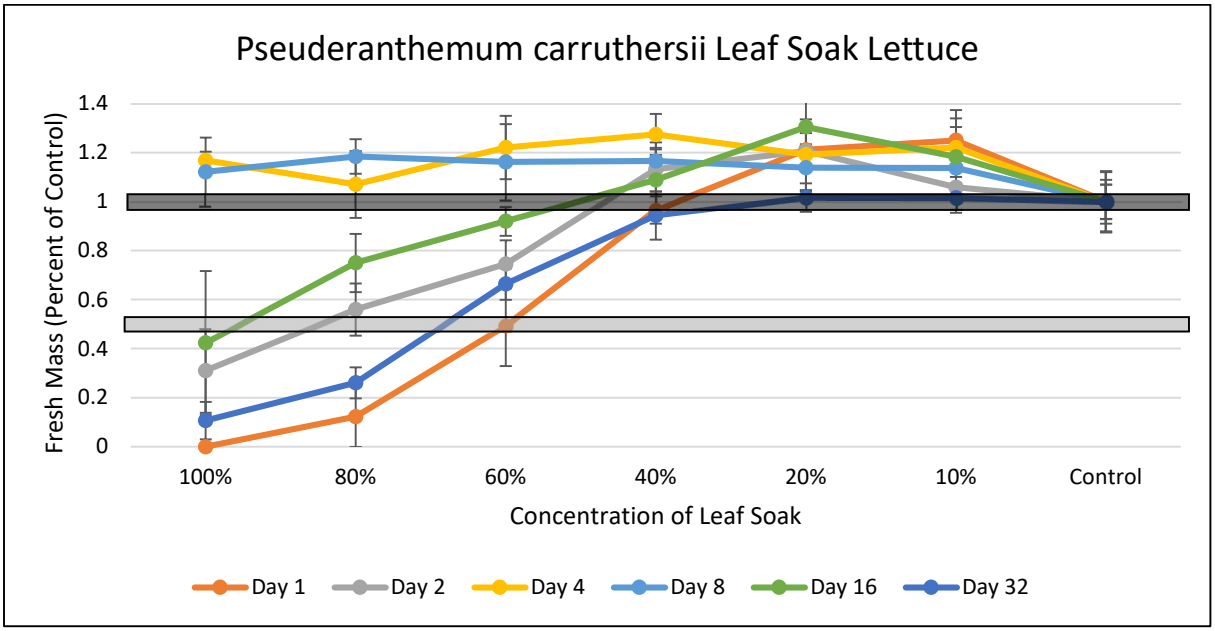


Figure 9. A time course of continuous soaking of *Pseuderanthemum carruthersii* leaves and their effect on the growth of lettuce. Values are fresh mass displayed as a percent of the control. The dark horizontal line indicates 100% control growth while the light grey line indicates 50% control growth. Inhibition of growth was greatest in day 1 and 32, with the least inhibition of growth seen on day 4 and 8. N = 18

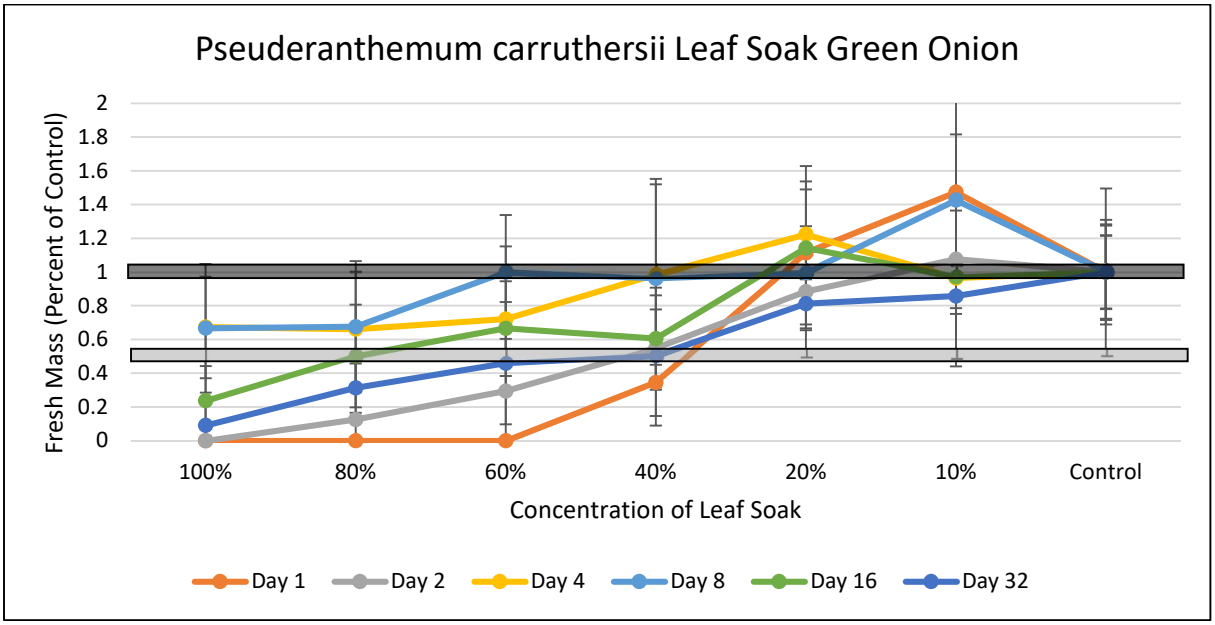


Figure 10. A time course of continuous soaking of *Pseuderanthemum carruthersii* leaves and their effect on the growth of green onion. Values are fresh mass displayed as a percent of the control. The dark horizontal line indicates 100% control growth while the light grey line indicates 50% control growth. Inhibition of growth was greatest in day 1, 2 and 32, with the least inhibition of growth seen on day 4 and 8. N = 18

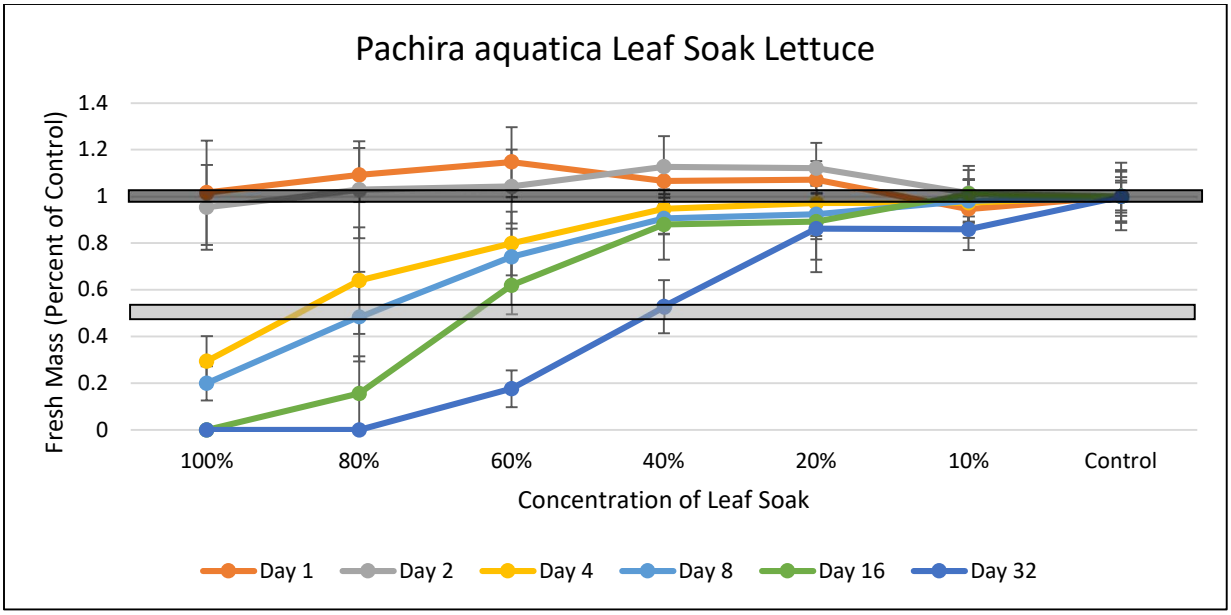


Figure 11. A time course of continuous soaking of *Pachira aquatica* leaves and their effect on the growth of lettuce. Values are fresh mass displayed as a percent of the control. The dark horizontal line indicates 100% control growth while the light grey line indicates 50% control growth. Inhibition of growth was greatest in day 16 and 32, with the least inhibition of growth seen on day 1 and 2. N = 18

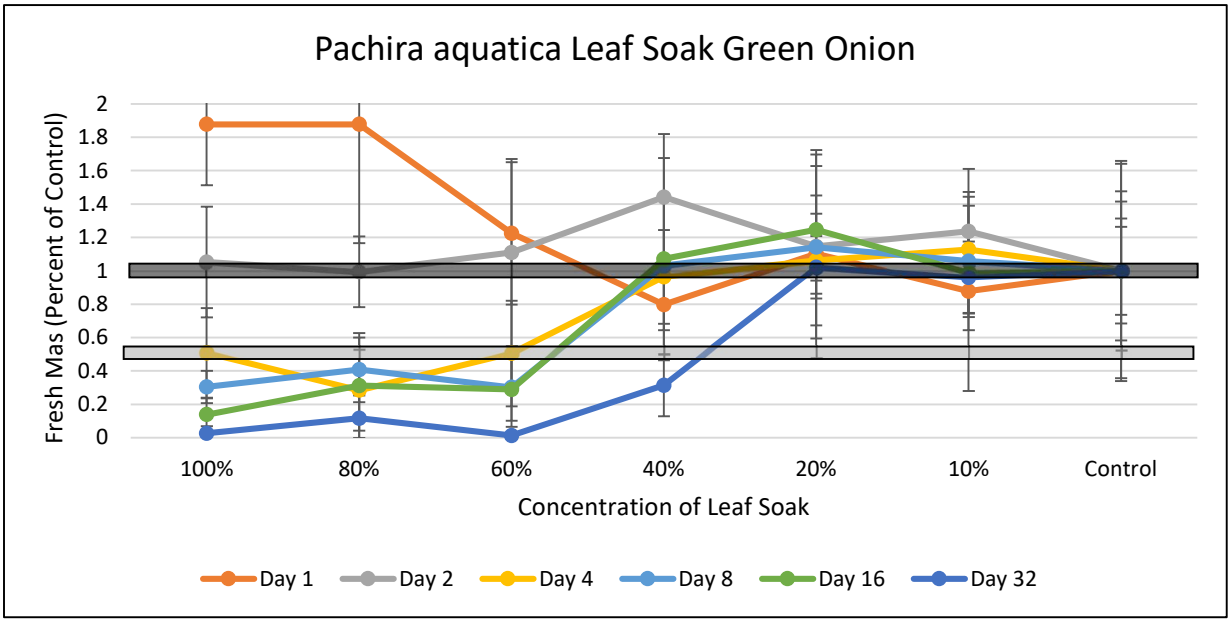


Figure 12. A time course of continuous soaking of *Pachira aquatica* leaves and their effect on the growth of green onion. Values are fresh mass displayed as a percent of the control. The dark horizontal line indicates 100% control growth while the light grey line indicates 50% control growth. Inhibition of growth was greatest in day 16 and 32, with the least inhibition of growth seen on day 1 and 2. N = 18

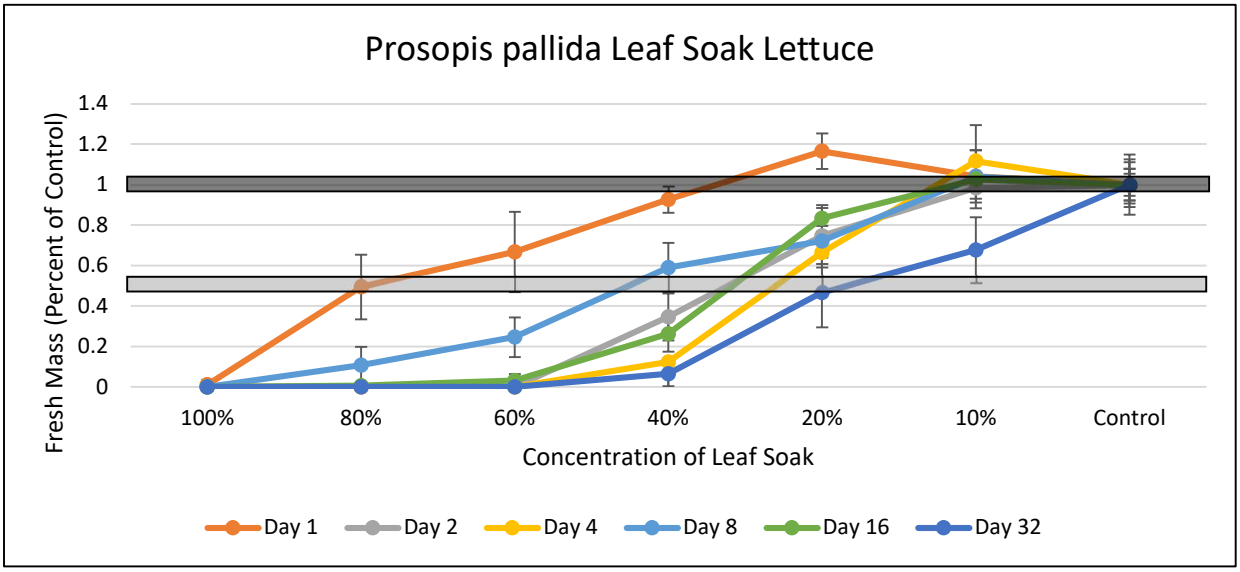


Figure 13. A time course of continuous soaking of *Prosopis pallida* leaves and their effect on the growth of lettuce. Values are fresh mass displayed as a percent of the control. The dark horizontal line indicates 100% control growth while the light grey line indicates 50% control growth. Inhibition of growth was least in day 1 and greatest in day 32, but complete inhibition of growth was found with pure 100% leaf soak solution on all days. N = 18

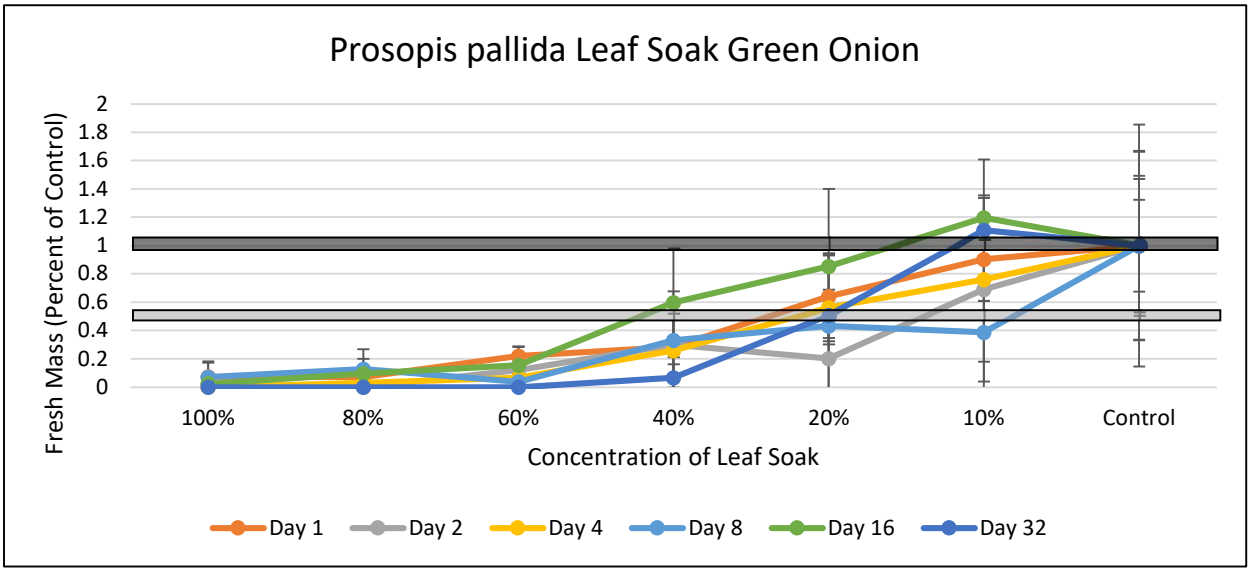


Figure 14. A time course of continuous soaking of *Prosopis pallida* leaves and their effect on the growth of green onion. Values are fresh mass displayed as a percent of the control. The dark horizontal line indicates 100% control growth while the light grey line indicates 50% control growth. Inhibition of growth was least in day 16 and greatest in day 32, but complete inhibition of growth was found with pure 100% leaf soak solution on all days. N = 18

Appendix C. pH Affect on Plant Crops.

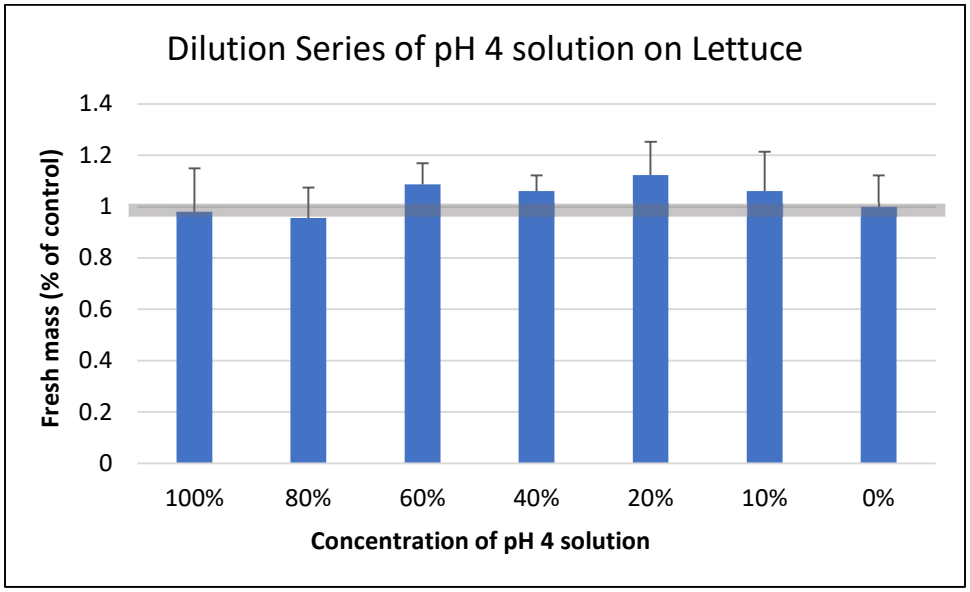


Figure 1. Dilution series of a pH4 solution (representing the most acidic plant extract) on the growth of lettuce. Even at full strength, there was no significant affect on the growth. N = 18

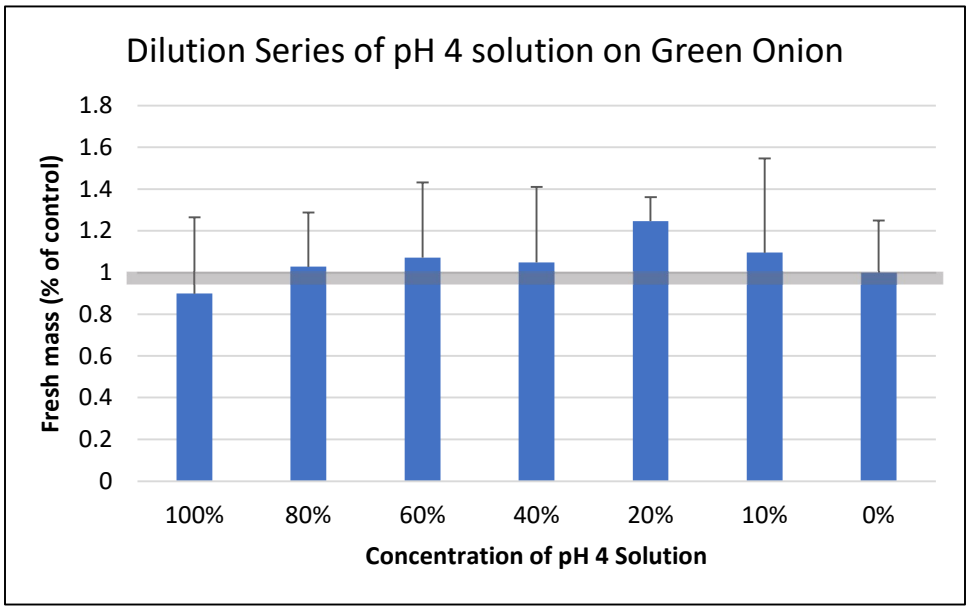


Figure 2. Dilution series of a pH4 solution (representing the most acidic plant extract) on the growth of green onion. Even at full strength, there was no significant effect on the growth. N = 18

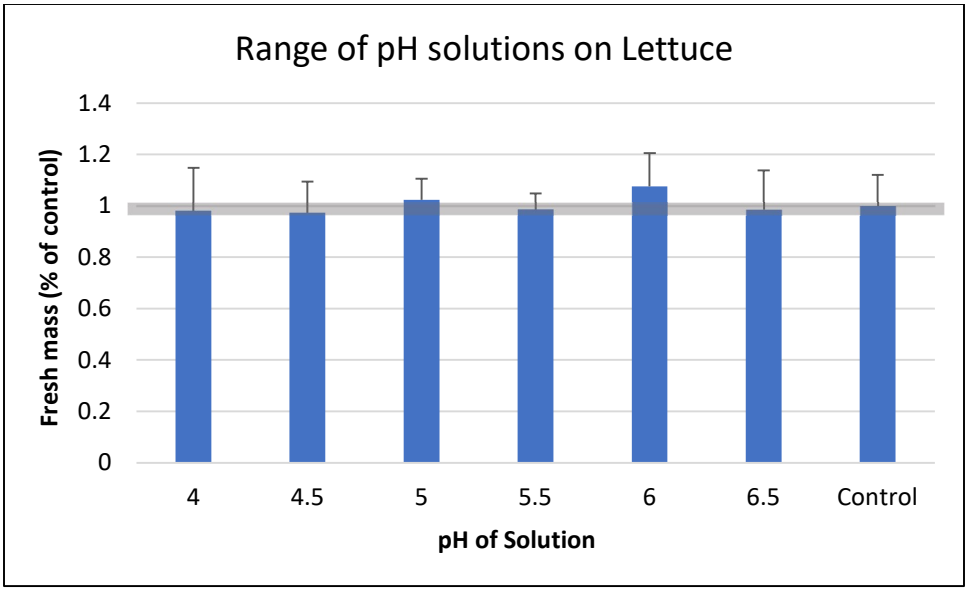


Figure 3. A range of pH solutions (representing the diversity of our plant extracts) on the growth of lettuce. There was no significant affect on the growth. N = 18

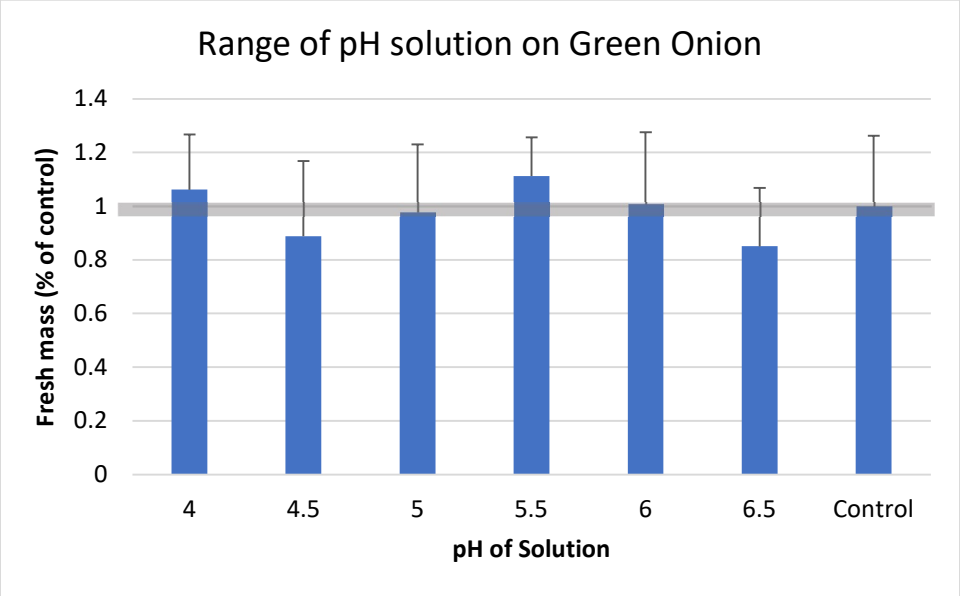


Figure 4. A range of pH solutions (representing the diversity of our plant extracts) on the growth of green onion. There was no significant affect on the growth. N = 18

Appendix D. Wa'ahila Ridge Environmental Survey

This project's very inception was due to the layman's observation that strawberry guava is reportedly allelopathic by suppression (Motooka, 2003; Wagner et al., 1999), thus allowing the investigation to begin with experiencing firsthand strawberry guava's dominance of the environment at a local park.

Wa'ahila Ridge is a State of Hawaii Park and Recreational Area located between the valleys of Manoa and Palolo. The recreational area is located at an elevation of roughly 1090ft, with both the trail and the park part of the dry forest range on the leeward side of Mount Olympus. The park is noted for its combination of Cook Pine (*Araucaria columnaris)*, Norfolk Island Pine (*Araucaria heterophylla)*, Ironwoods (*Casuarinaceae spp.)* and Strawberry Guava (*Psidium cattleianum)* of both red and yellow varieties (Hall, 2016; Nelson, 2003). Our goal was to determine what plants grow in conjunction with strawberry guava over the 4 seasons, looking specifically to see if there are any changes in undergrowth vegetation.

Two flat areas were selected, 1 with heavy strawberry guava infestation guava (mix of red and yellow variety) and 1 with no strawberry guava, to visualize the differences of biodiversity. Orientation of each square subsection of the larger area for reproducibility was determined by GPS coordinates along with path trail markers and compass directionality. Each square was 10m by 10m, broken down into a 2m by 2m grid. 5 randomly selected squares from within the 25 charted squares were chosen for each sampling date through randomly generated numbers (Random.org). Each sampling date was chosen to represent a seasonal difference (winter, summer, spring, fall) and to see changes over time, if any. Within each chosen square, plant species and number of individuals were recorded. A plant was counted if it was taller/longer than 6 inches and thus "established" (Harper, 1975).

Table 1. A display of the species found by date and location during the survey of Wa'ahila ridge.

Summer: 6/28/2019			
Undisturbed Forest:	Species Found	Strawberry Guava Infested	Species Found
3	*Araucaria columnaris* (1), *Justicia betonica* (8)	9	*Psidium cattleianum* (50)
7	*Justicia betonica* (11)	11	*Psidium cattleianum* (46)
9	*Monstera deliciosa* (1), *Justicia betonica* (8), Unknown tree (1)	18	*Psidium cattleianum* (40)
16	*Justicia betonica* (11),	19	*Psidium cattleianum* (24)
24	Unknown tree (1), *Justicia betonica* (10)	25	*Psidium cattleianum* (34)
Fall: 9/28/19			

Undisturbed Forest:	Species Found	Strawberry Guava patch:	Species Found
3	*Araucaria columnaris* (1), *Justicia betonica* (8)	1	*Psidium cattleianum* (26)
12	*Justicia betonica* (12)	2	*Psidium cattleianum* (39), *Araucaria columnaris* (1)
13	*Araucaria columnaris* (1), *Justicia betonica* (4)	4	*Psidium cattleianum* (25)
14	*Monstera deliciosa* (1), *Justicia betonica* (7),	20	*Psidium cattleianum* (49)
25	*Justicia betonica* (11)	21	*Psidium cattleianum* (32)
Winter: 01/18/2020			
Undisturbed Forest:	Species Found	Strawberry Guava patch:	Species Found
9	*Monstera deliciosa* (1), Unknown tree (1), *Justicia betonica* (8),	9	*Psidium cattleianum* (49)
13	*Araucaria columnaris* (1), *Justicia betonica* (5)	10	*Psidium cattleianum* (49)
17	*Justicia betonica* (9)	13	*Psidium cattleianum* (38)
21	*Justicia betonica* (8),	21	*Psidium cattleianum* (43), Unknown Vine (1)
25	*Justicia betonica* (10)	25	*Psidium cattleianum* (34)
Spring: 3/21/2020			
Undisturbed Forest:	Species Found	Strawberry Guava patch:	Species Found
1	*Justicia betonica* (7)	2	*Psidium cattleianum* (39)
8	*Justicia betonica* (10)	5	*Psidium cattleianum* (34)
19	*Justicia betonica* (11)	6	*Psidium cattleianum* (24)
24	Unknown tree (1), *Justicia betonica* (10),	13	*Psidium cattleianum* (38)
25	*Justicia betonica* (11)	25	*Psidium cattleianum* (34), small ironwood (1)

An enlightening count showing that, within our sample squares, even within the undisturbed forest, the ecosystem is heavily dominated in terms of plants by a singular species. We would like to note here that we do not denotate the "undisturbed forest" as being a "native

forest" because it is not. Many of the species counted are not native to the Hawaiian Islands and have been introduced over the generations. For example, *Justicia betonica* (a vine similar in growth appearance to wedelia (*Sphagneticola trilobata)*) is actually an introduced species native to Africa and is a concern for Australia where it is considered an environmental weed. In the state of Hawaii, it is considered an invasive weed, where it originated as an ornamental brought to Hawaii island in 1943. Since then, it has spread to all major islands by introduction for landscaping, and then escaping into the wild. It tolerates shade and grows dense strands wherever it becomes established, choking out other species (Bufford et al., 2016). Perhaps a problem that needs further notation at the state conservation level. Regardless, while the physical distance of the 2 areas is within 100 meters of flat land with consistent cross breeze, we see very little crossover of species. However, the white shrimp plant allowed for fellow shrubby growth while strawberry guava did not.

In terms of strawberry guava, we find a reduced level of biodiversity compared to the undisturbed forest, with no major additional trees or shrubs growing except for the "pre-existing" conifers (whose size indicated they were here much earlier than the strawberry guava invasion). However, some notable exceptions are the encroachment from the ironwoods as we can see in comparing Summer Strawberry Guava square 25 to Spring Strawberry Guava square 25. Strawberry guava can grow through even the smallest holes in the ironwood needle ground coverage, but ironwoods can also grow within loose strawberry guava patches, with more pronounced growth on the perimeter. Ironwoods are a common sight along beach communities and on hiking trails, and, while not a native species, might be considered a competitive agent against strawberry guava, able to slow down the spread of strawberry guava with its thick needle coverage. There is also the question of the vine plant growing along a strawberry guava branch. While not yet identified, this provides an interesting aspect to consider of whether parasitic or otherwise vampire plants are not affected by strawberry guava's allelopathy. pH of soil found under and around strawberry guava and regular guava groves are between 5.2-6.3, an acceptable acidic range for the Hawaiian Islands as the average soil pH is 5.4 (with rainwater being 5.6) (Adrian, 2015; Hue, 2008), thus pH is not considered to be a factor in this exhibition of plant growth inhibition. In addition, there was found to be no correlation between levels of available macrominerals (Ca, Mg, P, K, N, S and Na) in the soil under strawberry guava and regular guava groves compared to standard Hawaii soil levels despite the differences between both trees (Adrian, 2015).

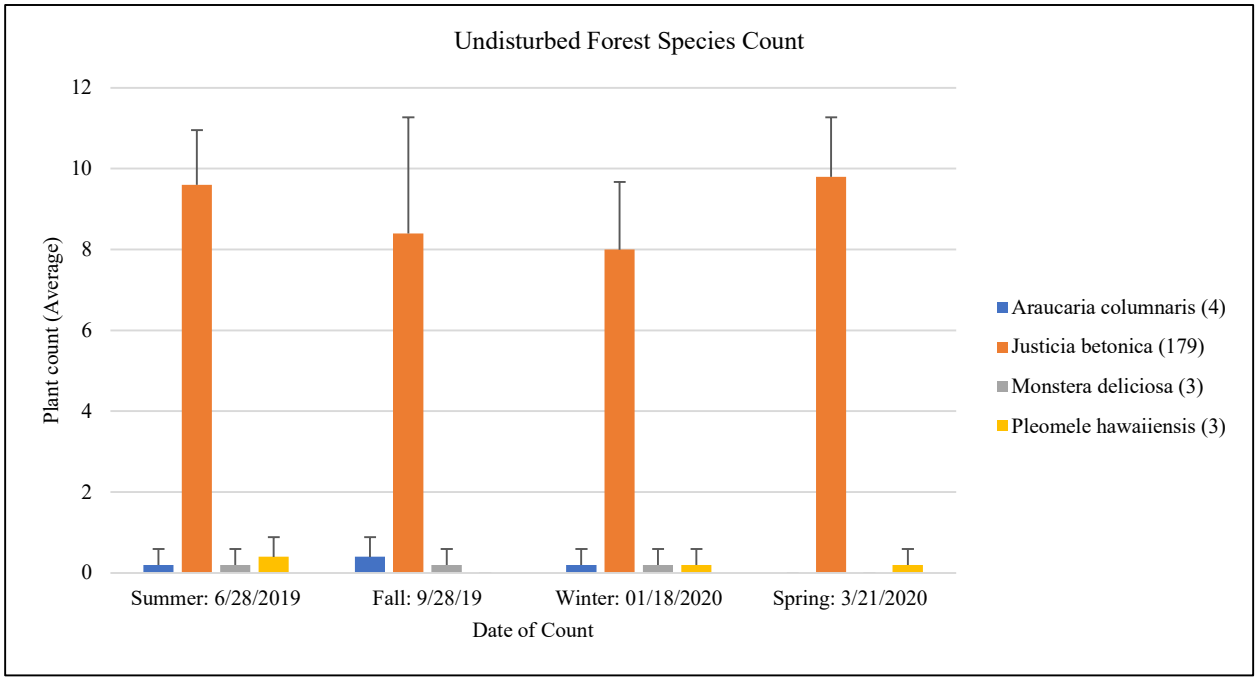


Figure 1. A summary of the Wa'ahila Ridge survey conducted over the course of a year in an non-strawberry guava infested area. The dominant species was *Justicia betonica* (white shrimp plant), with the other species present a combination of trees and shrubs. N = 5

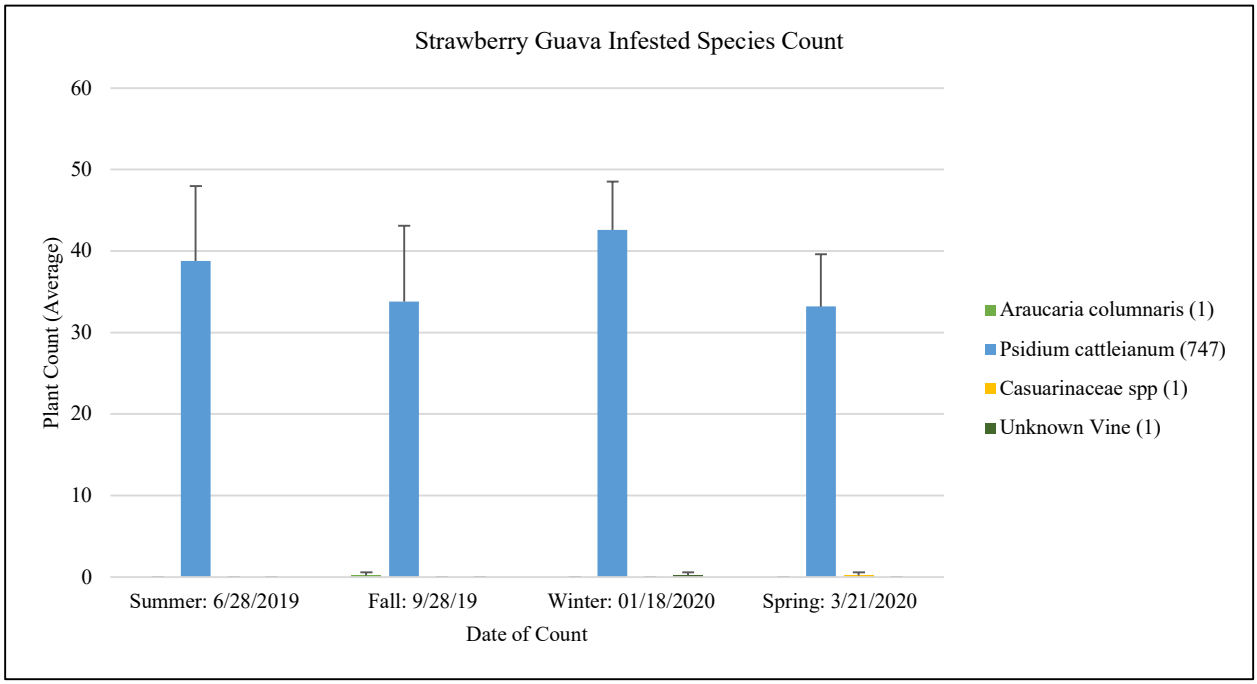


Figure 2. A summary of the Wa'ahila Ridge survey conducted over a year in an strawberry guava infested area. The dominant species was strawberry guava with the other species present only being old growth trees. Nothing was found growing under the canopy line within the center of the heavily infested area. N = 5

Appendix E. Greenhouse Assay Ratio Data

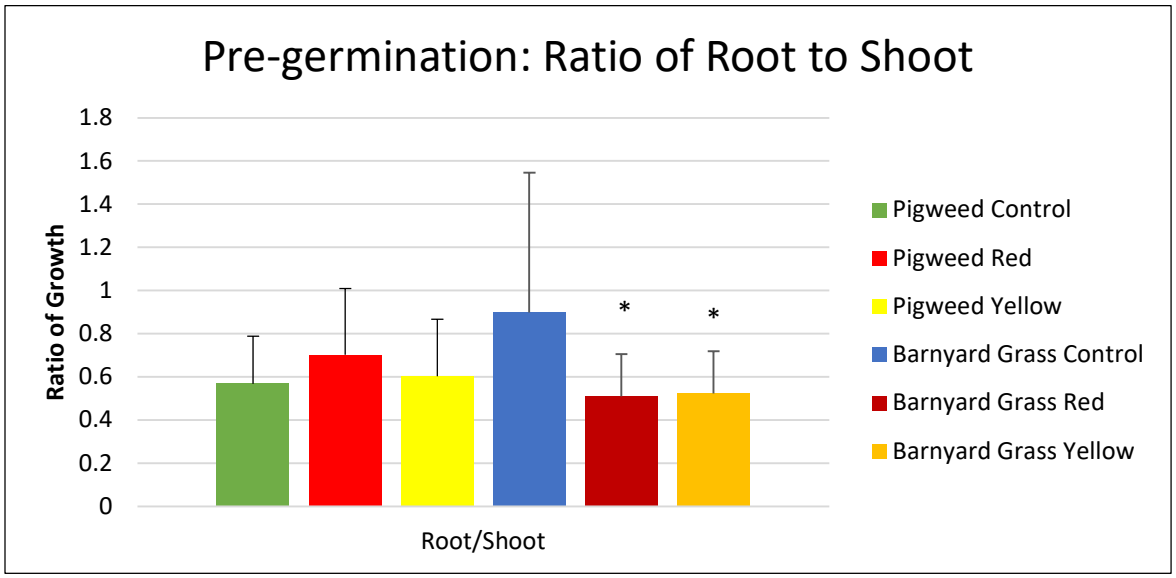


Figure 1. A comparison of the ratio of root to shoot growth with different application of strawberry guava leaf extract (red and yellow variety). A "*" denote a <0.05p value between the treatment and the control. Statistical tests were done with ANOVA: single factor. N = 18

Appendix F. Sep Pak Chromatography Data

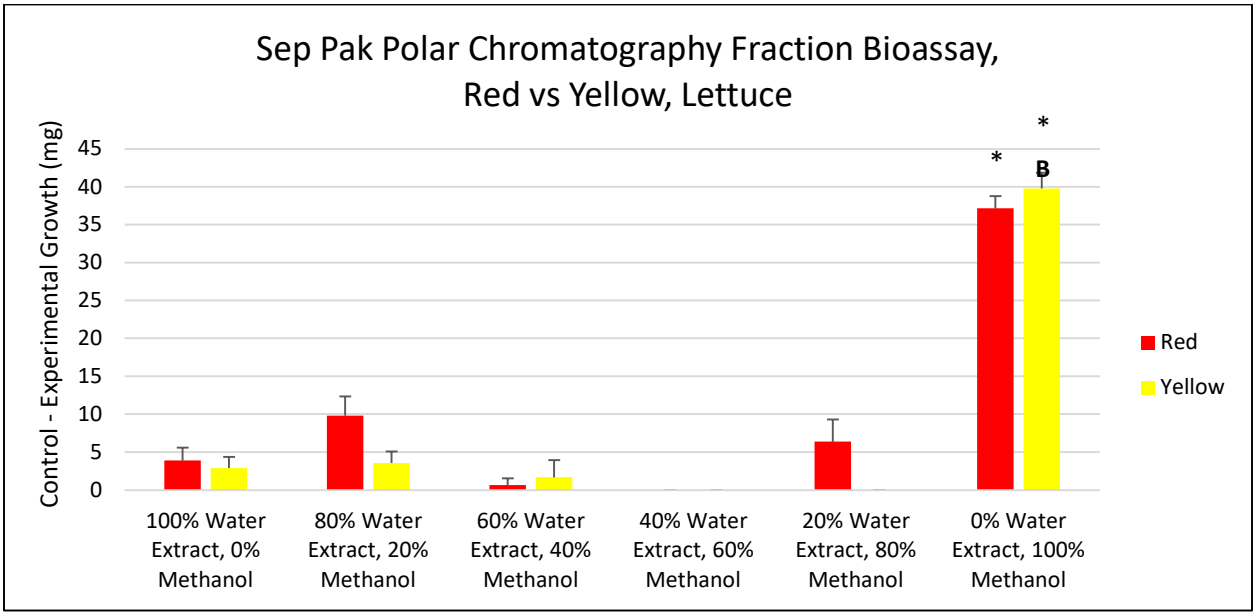


Figure 1. A comparison of the difference between the growth of lettuce in control solution or with a treatment (10% fraction solution, either red or yellow). A "*" denote a <0.05p value between the treatment and the control. Statistical tests were done with ANOVA: single factor. There was no statistical difference between red and yellow. Statistical tests were done with ANOVA: single factor. N = 18

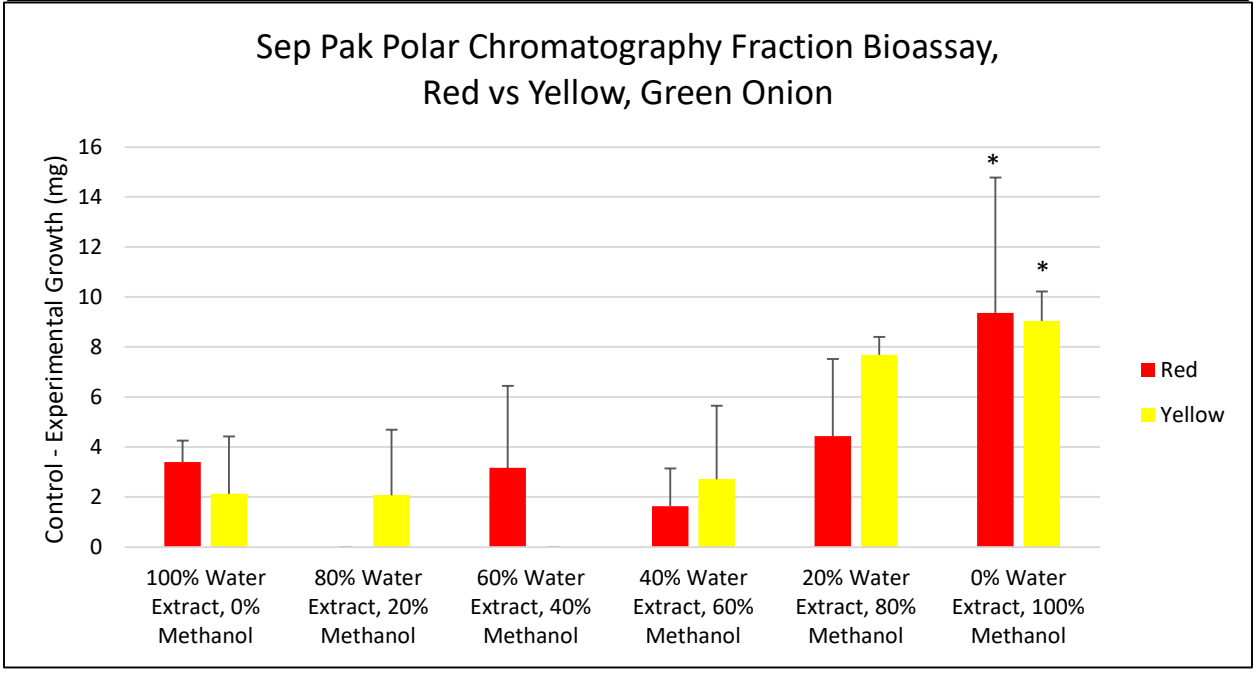


Figure 2. A comparison of the difference between the growth of green onion in control solution or with a treatment (10% fraction solution, either red or yellow). A "*" denote a <0.05p value between the treatment and the control. Statistical tests were done with ANOVA: single factor. Statistical tests were done with ANOVA: single factor. N = 18

Appendix G. HPLC Chromatography Data of Bioactive Peak

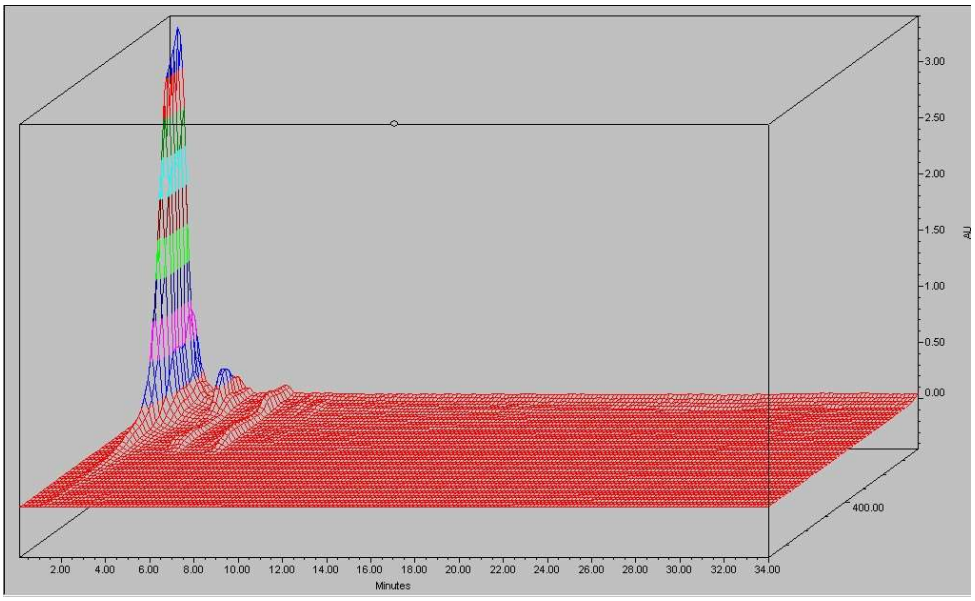


Figure 1. A 3D spectrum of a Waters Nova-Pak C18 4um 3.9mm I.D. x 150mm run with a 22 minute gradient of Acetonitrile and Water + Acetic Acid (15%) (5-65% Acetonitrile) of *Psidium cattleianum* yellow variety leaf soak. OD ranges from 200 to 800. Majority of activity is seen within the solvent front and the 2 peaks of interest.

Closer analysis of these two peaks of interest beyond the solvent front shows they have a UV maxima representative of flavonoids. Peak A, runtime 3.346 minutes, has a UV maxima at 253.6 and 364.8 wavelength while Peak B, runtime 5.347 minutes, has a UV maxima at 254.8 and 348.4 wavelength.

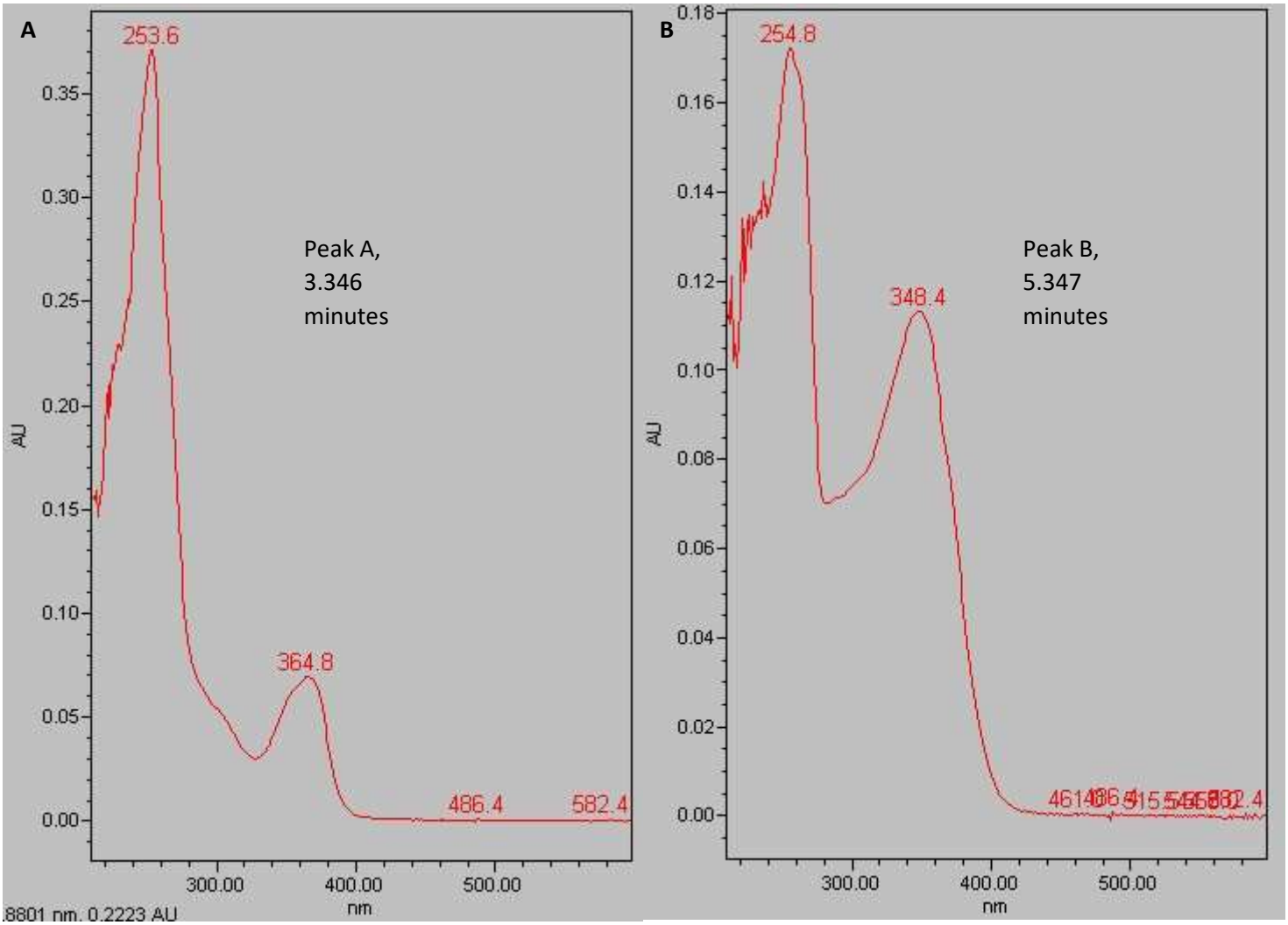
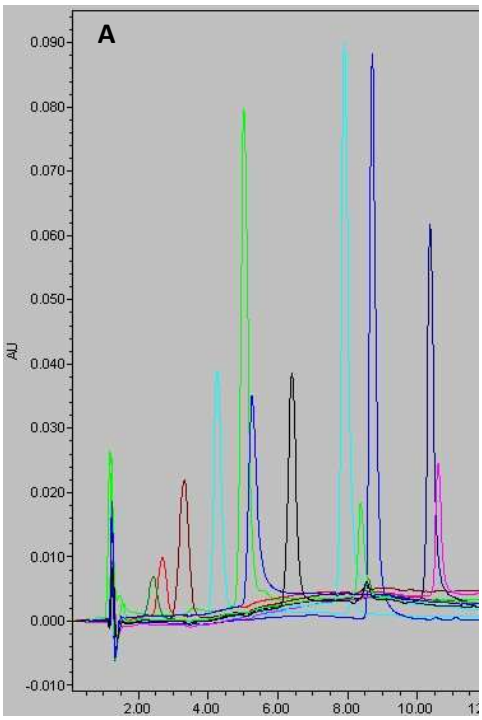


Figure 2. the UV Maxima of the two peaks found during the HPLC run as previously described of the *Psidium cattleianum* yellow variety leaf soak. Results were similar for the *Psidium cattleianum* red variety leaf soak. A – Peak A with runtime of 3.346 minutes, UV maxima at 253.6 and 364.8 wavelength. B – Peak B with runtime of 5.347 minutes, UV maxima at 254.8 and 348.4 wavelength.

Comparing the three peaks of unknown identity to our suspected allelopathic peaks from guava metabolomic studies, it was found that, while our two measurable peaks may have runtimes that are similar to constituents found in regular guava leaves, their UV maxima did not match.



B

Chemical	Runtime (min)	Peak Wavelength (nm)
Taxifolin	2.42	289
P-coumaric Acid	2.69	309
Ferulic Acid	3.316	323.4
Unknown Peak 1	3.346	253.6, 364.8
Quercetin 3-B-D Glucoside	4.257	211.3, 254.8, 354.4
Guaiaverin	5.016	254.8, 354.4
Myricetin	5.256	241.8, 373.2
Unknown Peak 2	5.347	254.8, 348.4
Liquiritigenin	6.409	240.6, 276, 311.5
Quercetin	7.905	253.6, 370.8
Luteolin	8.7	250.1, 348.1
Naringenin	8.378	289
Kaempferol	10.366	265.4, 367.2
Apigenin	10.596	266.6, 338.9

Figure 3. A: Waters Nova-Pak C18 4um 3.9mm I.D. x 150mm with a 22 minute gradient of Acetonitrile and Water + Acetic Acid (15%) (5-65% Acetonitrile) of a variety of *Psidium guajava* leaf constituents in 10 uL injects at 0.1mM concentrations. **B:** A list of potentially allelopathic *Psidium guajava* leaf constituents and their associated runtimes and UV maxima. While elution times were similar, no UV maxima data corresponded perfectly with our unknown constituents found in strawberry guava.

Appendix H. Mass Spec Additional Data

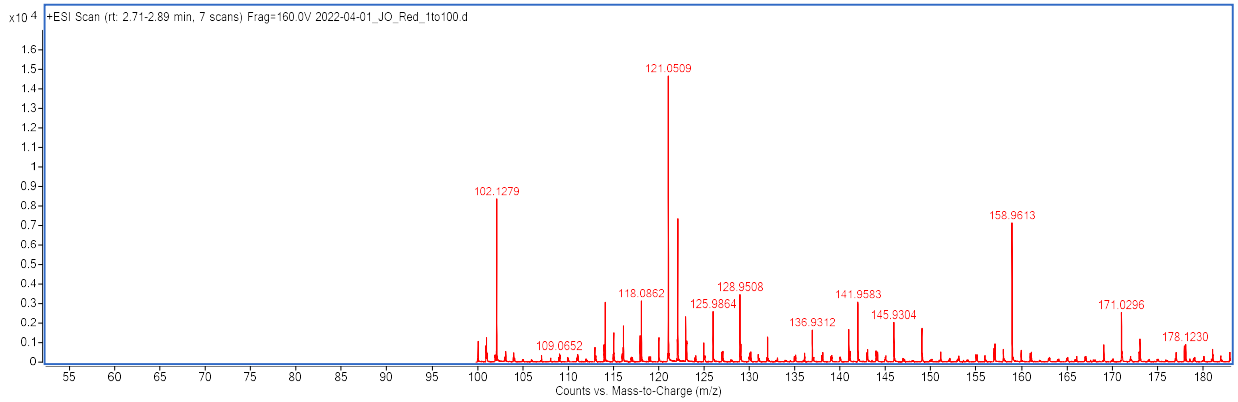


Figure 1. Potential hit of gallic acid to positive mode mass spectroscopy data when searching for H+ ion 171.0300 (m/z). Retention time of 2.63 minutes (171.0296m/z; 0.4mmu, 2.34ppm) for red strawberry guava. Mmu = milli mass error and ppm = parts per million for the molecular formula C7H6O5.

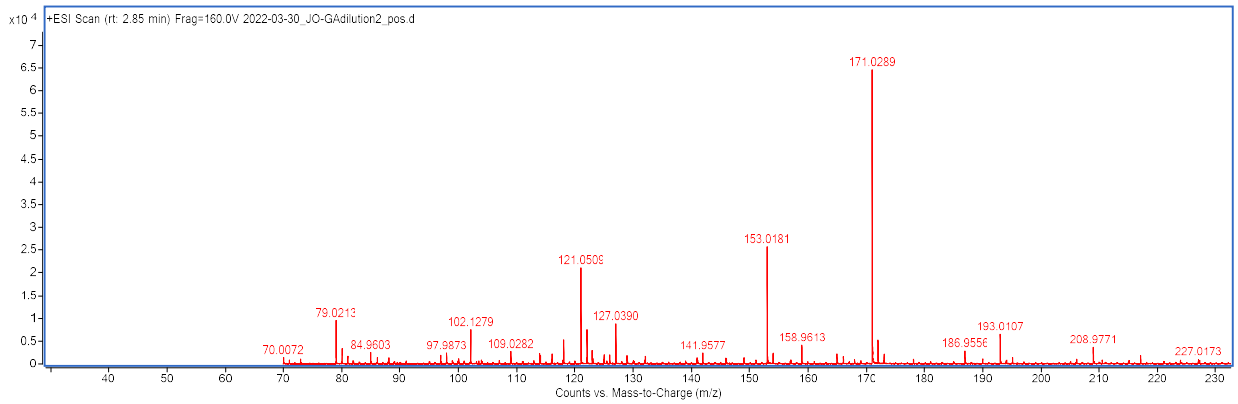


Figure 2. Mass spectrum of gallic acid standard in positive mode when searching for H+ ion 171.0300 (m/z). Retention time of 3.02 minutes (171.0289 m/z; 1.1mmu 6.43ppm) Mmu = milli mass error and ppm = parts per million for the molecular formula C7H6O5.

While the standard previously showed a retention time of 0.4 minutes later than the red and yellow samples, mixing the standard with either sample moved the retention time to match the sample retention time (2.68 minutes) without a double peak at the 0.4 minute later mark. The LCMS and MS/MS data confirmed there was no additional component added to the samples, finding only 1 peak on the LCMS chromatogram and a combination of the gallic acid fragmentation seen from the sample and the standard. The delay in the standard is hypothesized to have been caused by ions in the sample such as magnesium or calcium affecting retention time.

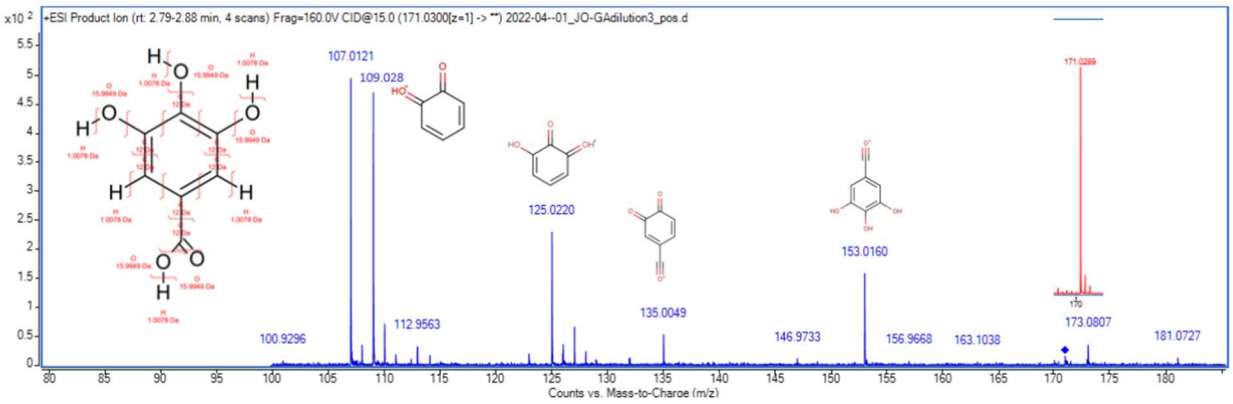


Figure 3. MS/MS analysis at 15V collision energy of suspected gallic acid peak for standard gallic acid in positive mode. Fragmentation pattern of gallic acid, predicted fragments, and close up of LCMS ionization state, are noted on the chromatogram, with special focus given to fragment 125.0220 m/z and 153.016 m/z, commonly found in both our fragment, the standard and relevant literature for gallic acid.

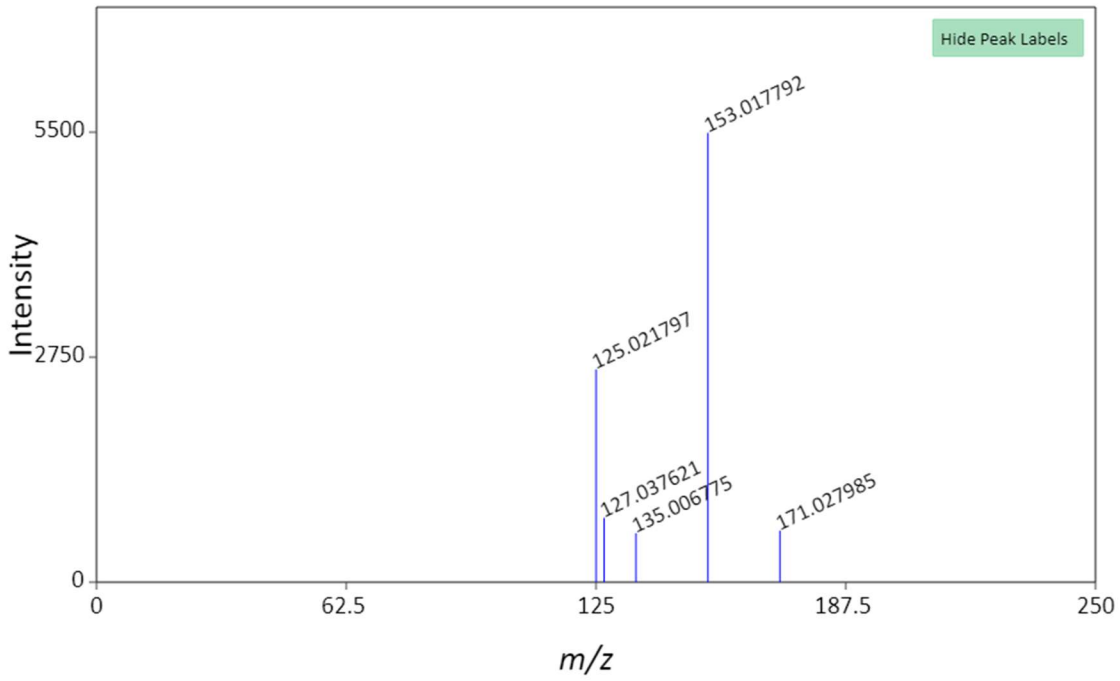


Figure 4. MS/MS analysis on a DI-ESI-qTof positive mode for the fragmentation pattern of gallic acid, as retrieved from the GNPS library spectra ID 374050. Special focus given to fragment 125.0218 m/z and 153.0178 m/z, as these are both found in both our fragment, the standard and relevant literature for gallic acid (Wishart et al 2007).

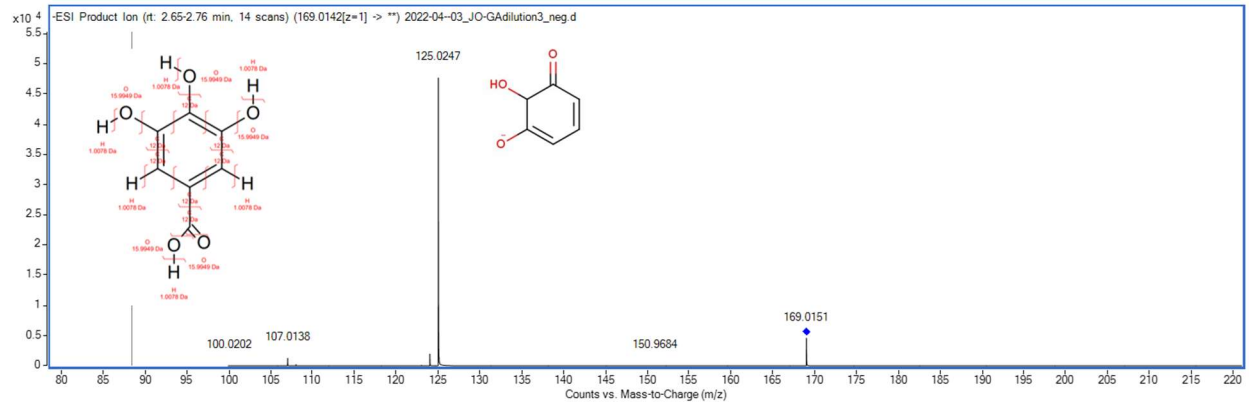


Figure 5. MS/MS analysis at 15V collision energy of suspected gallic acid peak for standard gallic acid in negative mode. Fragmentation pattern of gallic acid and predicted fragments are noted on the chromatogram, with special focus given to fragment 125.0247 m/z, commonly found in both our fragment, the standard and relevant literature for gallic acid.

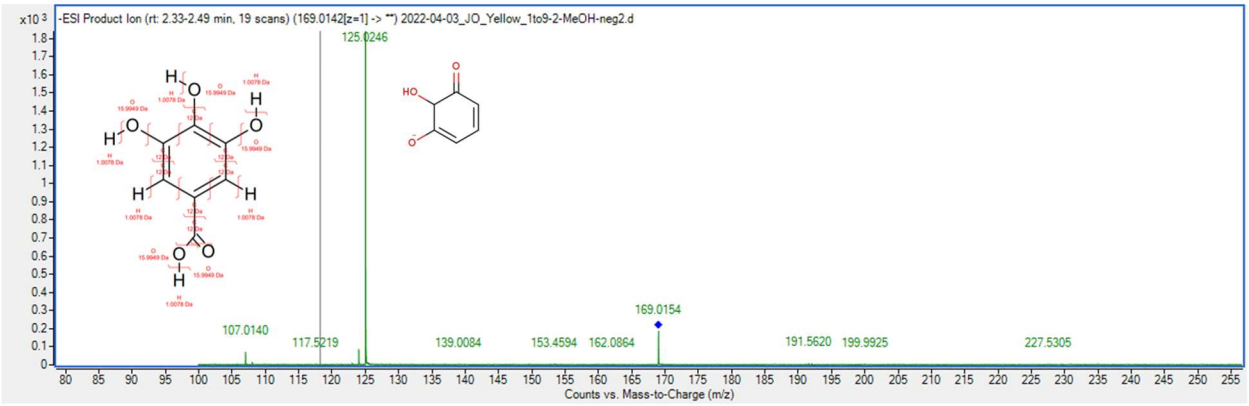


Figure 6. MS/MS analysis at 15V collision energy of suspected gallic acid peak for yello strawberry guava in negative mode. Fragmentation pattern of gallic acid and predicted fragments are noted on the chromatogram, with special focus given to fragment 125.0246m/z (0.1mmu, 0.8ppm) commonly found in both our fragment, the standard and relevant literature for gallic acid.

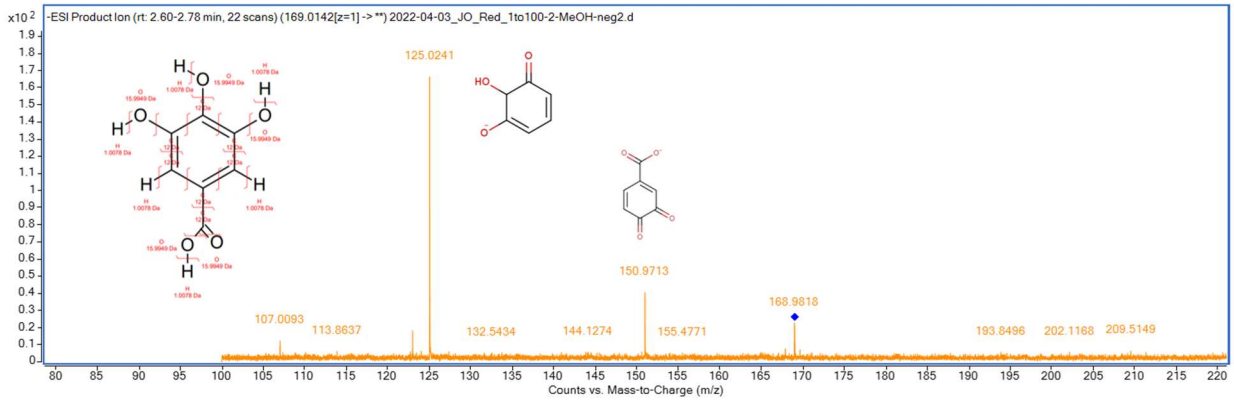
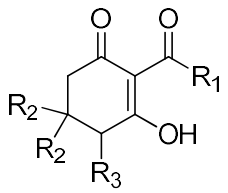


Figure 7. MS/MS analysis at 15V collision energy of suspected gallic acid peak for red strawberry guava in negative mode. Fragmentation pattern of gallic acid and predicted fragments are noted on the chromatogram, with special focus given to fragments 125.0241m/z (0.6mmu, 4.8ppm) and 150.9713m/z (2.9mmu, 19.21ppm), commonly found in both our fragment, the standard and relevant literature for gallic acid.

Appendix I. HPPD Additional Data

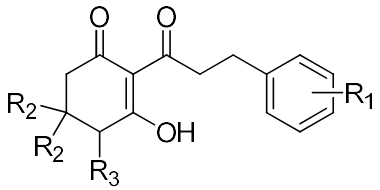
Table 1. Structures and activities of 19 2-acyl-cyclohexane-1,3-diones with simple aliphatic side chains tested in this study



Cpd #	R_1	R_2	R_3	mw	IC_{50} (μM)[ab]	Triketone Average Binding Energy (kcal/mol)[c]
1b	methyl	H	H	154	23.09±4.34	-3.85±0.0141
1f	methyl	methyl	H	182	53.12±17.09	-2.96±0.117
3b	pentyl	H	H	210	2.93±0.47	-3.97±0.1687
3e	pentyl	methyl	H	238	4.17±0.45	-3.45±0.1687
4b	nonyl	H	H	266	0.46±0.06	-4.39±0.2563
4e	nonyl	methyl	H	294	2.66±0.95	-3.44±0.3058
5b	undecyl	H	H	294	0.30±0.03	-4.55±0.3429
5f	undecyl	methyl	H	322	0.77±0.11	-3.41±0.3971
6a	hexadecyl	H	H	364	1.58±0.20	-3.8±0.4536
6b	hexadecyl	methyl	H	392	1.70±0.31	-2.83±0.4273

[a] The data represents means followed by standard error, n=3.
[b]The commercial product sulcotrione has an IC_{50}app: 0.25±0.02 μM.
[c]The data represents means followed by 1 standard deviation, n=10-25 depending on cluster population

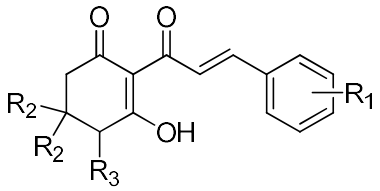
Table 2. Structures and activities of 17 cyclohexane-1,3-diones with phenyl side chains tested in this study



Cpd #	R_1	R_2	R_3	mw	IC_{50} (μM)[ab]	Triketone Average Binding Energy (kcal/mol)[c]
8b	H	H	H	244	1.27±0.09	-4.92±0.1367
8e	H	methyl	H	272	2.17±0.37	-3.84±0.215
10b	3-methoxy	H	H	274	0.31±0.04	-4.78±0.1477
10d	3-methoxy	methyl	H	302	1.50±0.17	-3.15±0.2014

[a] The data represents means followed by standard error, n=3.
[b]The commercial product sulcotrione has an IC_{50}app: 0.25±0.02 μM.
[c]The data represents means followed by 1 standard deviation, n=10-25 depending on cluster population

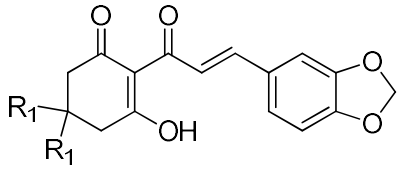
Table 3. Structures and activities of 24 cyclohexane-1,3-diones with phenylene side chains tested in this study



Cpd #	R_1	R_2	R_3	mw	IC_{50} (μM)[ab]	Triketone Average Binding Energy (kcal/mol)[c]
8a	H	H	H	242	5.43±0.58	-5.64±0.1475
8d	H	methyl	H	270	58.6±8.67	-4.11±0.0732
10a	3-methoxy	H	H	272	0.89±0.10	-4.97±0.0857
10c	3-methoxy	methyl	H	300	6.4±1.2	-3.95±0.1303

[a] The data represents means followed by standard error, n=3.
[b]The commercial product sulcotrione has an IC_{50}app: 0.25±0.02 μM.
[c]The data represents means followed by 1 standard deviation, n=10-25 depending on cluster population

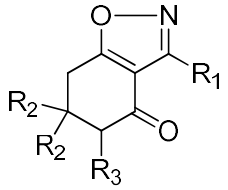
Table 4. Structures and activities of 4 cyclohexane-1,3-diones with other side chains tested in this study



Cpd #	R_1	mw	IC_{50} (μM)[ab]	Triketone Average Binding Energy (kcal/mol)[c]
13a	H	286	9.51±1.84	-5.74±0.1484
13c	methyl	314	28.7±3.72	-4.76±0.1929

[a] The data represents means followed by standard error, n=3.
[b]The commercial product sulcotrione has an IC_{50}app: 0.25±0.02 μM.
[c]The data represents means followed by 1 standard deviation, n=10-25 depending on cluster population

Table 5. Structures and activities of non-diones with simple aliphatic side chains tested in this study



Cpd #	R_1	R_2	R_3	mw	IC_{50} (μM)[ab]	Triketone Average Binding Energy (kcal/mol)[c]
1a	methyl	H	H	151	>100	-3.48
3a	pentyl	H	H	207	>100	-4.25
4a	nonyl	H	H	263	>100	-4.71
5a	undecyl	H	H	291	>100	-4.65

[a] The data represents means, n=3.
[b]The commercial product sulcotrione has an IC_{50}app: 0.25±0.02 μM
[c]The data represents means followed by 1 standard deviation, n=10-25 depending on cluster population

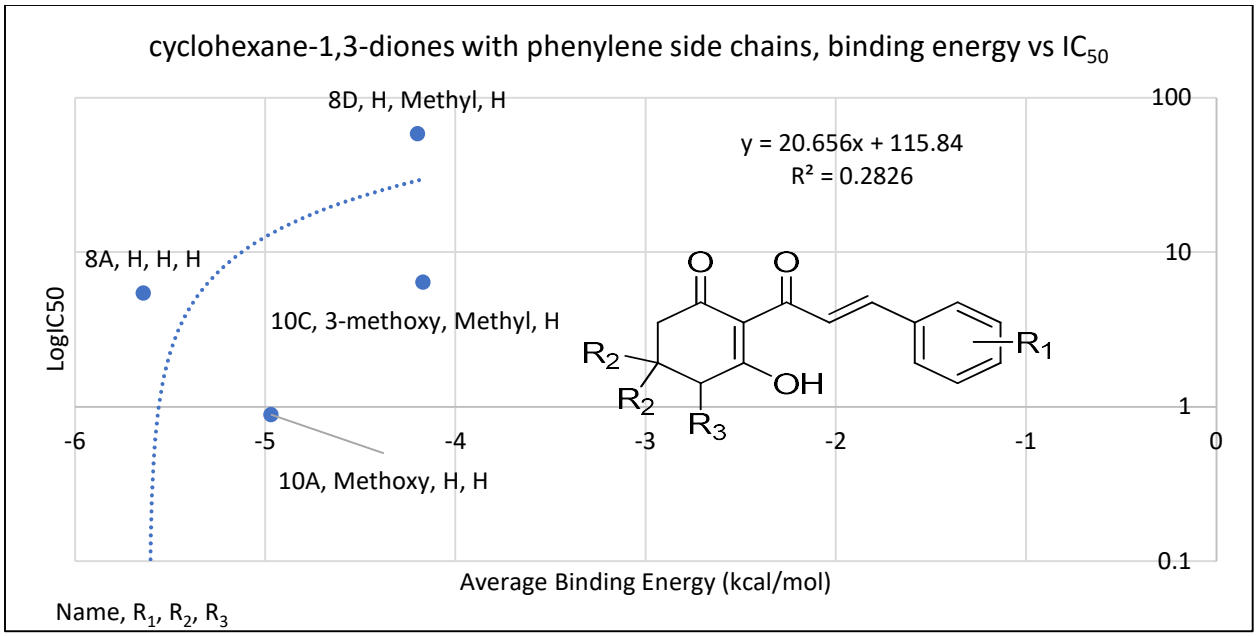


Figure 1. The relationship of binding energy and the $\log IC_{50}$ of a selection of cyclohexane-1,3-diones with phenylene side chains. Molecules are organized according to class of base structure (Grouping), seen on the bottom right. Each data label follows the pattern of "Name, Grouping, R_1, R_2, R_3", where "R_1, R_2, and R_3" refer to the functional group. Addition of a head group to R_2 reduced binding energy and was found with a less effective IC_{50}. R_1 tail addition of 3-methoxy lowered IC50 compared to predicted with binding energy.

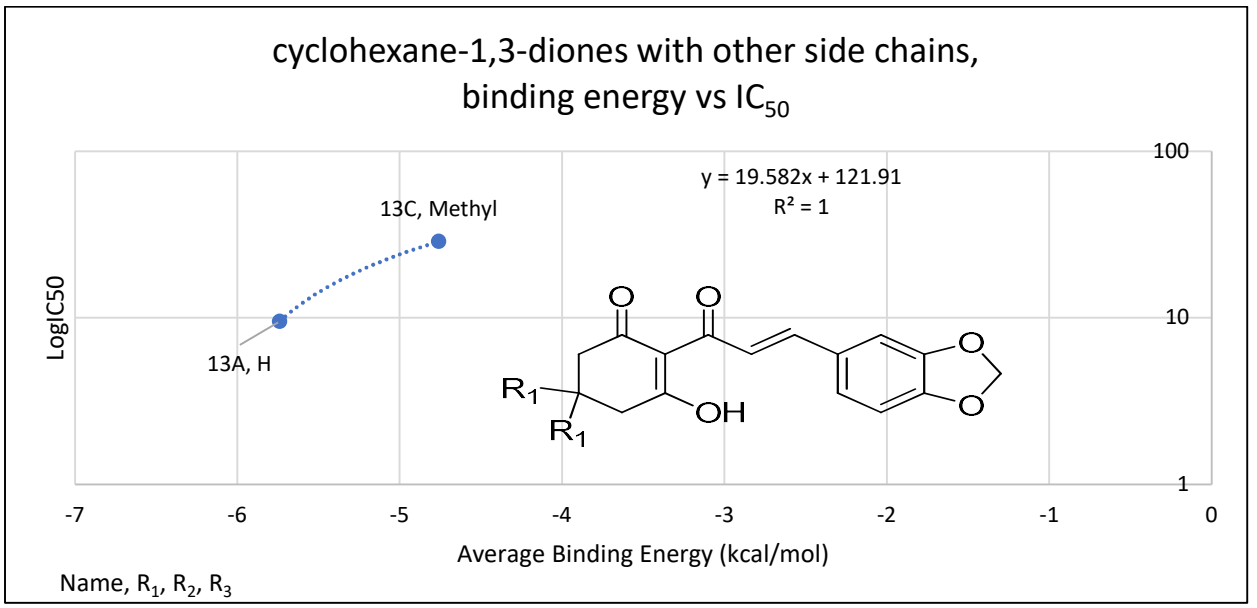


Figure 2. The relationship of binding energy and the $\log IC_{50}$ of a selection of cyclohexane-1,3-diones with other side chains. Molecules are organized according to class of base structure (Grouping), seen on the bottom right. Each data label follows the pattern of "Name, Grouping, R_1, R_2, R_3", where "R_1, R_2, and R_3" refer to the functional group. Addition of a head group to R_2 reduced binding energy and was found with a less effective IC_{50}.

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