**Testing Natural Products for Toxicity against Agricultural Pests and Pathogens**

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## Abstract

In a constant race to overcome pesticide resistance by pests and pathogens, this research aimed to discover bioactive inhibitory compounds from natural products. I tested extracts from 10 different fungal isolates that were previously shown to have antifungal activity, and 5 commercially available natural products. Assays were developed to test natural products for antibiotic activities against model pathogens and pests including fungi, insects, and molluscs. The objectives of this research were to develop and determine the effectiveness of bioassays, and to identify potentially interesting natural products. Of the 10 fungal isolates tested, reconstituted broths of *Penicillium virgatum, Ramularia vizellae,* and *Trichoderma* sp. showed pronounced anti-fungal activity. Further, *P. virgatum* and *Trichoderma* sp. shows anti-insect activity while broths of *Ramularia vizellae* and *Trichoderma* sp. exhibited anti-mollusc activity. A preliminary metabolomic study identified potentially interesting metabolites that requires further investigation to determine the chemical structure(s), mode of action, and other attributes.

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# 1. Introduction

## 1.1 Secondary Metabolites

Ecosystems encompass complex, diverse, and multispecies communities of micro- and macro-organisms. To co-exist and survive in such communities, organisms need to establish communication and interaction networks. Organisms modulate these networks, at least in part, via secondary metabolites, also known as extrolites or natural products (Netzker *et al.,* 2015; Katz & Baltz, 2016). Unlike primary metabolites, secondary metabolites are not directly involved in, or essential to, growth, development, or reproduction of the organism. Secondary metabolites represent chemically diverse metabolites that can have a wide range of biological activities. Further, secondary metabolites may improve the organism's fitness and increase its competitive strength by modulating interactions with surrounding organisms (Aly, Debbab, & Proksch, 2011; Keller, N.P., 2018).

In particular, sessile organisms synthesize or sequester diverse secondary metabolites for the purpose of communication and chemical defence. Often, these secondary metabolites are produced during stationary phase or under duress due to environmental pressure(s) (Calvo *et al.,* 2002; Keller, N.P., 2018). For example, chemical defenses are deployed as a response to the presence of predators, competitors, and/or pathogens. Secondary metabolites are produced abundantly by microorganisms in crowded environments and vary in composition and complexity depending on the circumstances and community composition (Cavalier-Smith, T., 1992). Additionally, at low concentrations, secondary metabolites can serve as a means of chemical communication between symbiotic organisms (Singh, Son, & Lee, 2016). They can also be involved in processes such as metal sequestration and transport, and in cues to initiate sexual differentiation or other life history stages (Demain & Fang, 2000; Macheleidt *et al.,* 2016).

Because of these biological activities, secondary metabolites have been employed extensively in the pharmaceutical industry. Medical applications of secondary metabolites range from fighting microbial infections and cancers to prevention of immune rejection of organ transplants. More broadly, secondary metabolites are used as pesticides to prevent crop loss or to reduce materials spoilage. Furthermore, the chemical structure of secondary metabolites serves as a source of inspiration for chemists to synthesize synthetic or semi-synthetic derivatives to be

used by the pharmaceutical or agricultural industry. Because of these applications there is a constant drive to discover novel secondary metabolites that have agricultural or medicinal applications.

Fungi, in particular, produce a variety of interesting secondary metabolites. As integral members of their ecosystems, fungi are known as decomposers, plant mutualists, or pathogens that frequently compete with other microbes, insects, and animals. Thus, to thrive in their environment fungi have evolved secondary metabolites of enormous chemical diversity with broad bioactive activities (Holighaus & Rohlfs, 2016; Aly, Debbab, & Proksch, 2011). Fungal secondary metabolites may be classified into four main groups – polyketides, terpenoids, shikimic acid derivatives, and non-ribosomal peptides. Further, hybrid secondary metabolites synthesized from moieties from different classes are commonly found such as the meroterpenoids, which are produced from the fusion of terpenes. Among the fungi, ascomycetes appear to possess more genes encoding for secondary metabolites than basidiomycetes, archeo-ascomycetes, and chytridomycetes. Furthermore, genomic analysis of hemi-ascomycetes and zygomycetes revealed a paucity of genes encoding for secondary metabolites (Pusztahelyi, Holb, & Pocsi, 2015).

Interference competition, also called antagonism, is a likely factor in shaping an ecosystem's composition. Fungal inter- and intra-species antagonism has been shown to play a role in diseases in plants and insects, animal poisoning or intoxication, biocontrol of other fungi, and resistance to fungivory and infections/infestations by other microbes (Gloer, 1995). Interspecies antagonism leads to antifungal effects whereas plant pathogenic fungi are known to cause phytotoxic effects in host plants or cause an increase in resistance against other pathogens. Furthermore, evidence of anti-insect effects is seen in insects that share a food source with fungi or prey on fungi as a food source. Similarly, soil-inhabiting fungi are dietary sources for arthropods and nematodes therefore fungi produce toxins capable of deterring fungivory (Gloer, 1995). In addition to using toxins as a direct chemical defence tactic, fungi are capable of a more indirect, subtle form of chemical defence by producing secondary metabolites which interfere with bacterial and animal communication. For example, the coprophilous ink cap mushroom, *Coprinus cinerea,* produces lactonases that interfere with quorum sensing signals used by gram-negative bacteria. Another example is from the mold *Aspergillus nidulans*, which is known to

produce secondary metabolites mimicking insect juvenile hormones that can interfere with insect development (Künzler, M., 2018).

Secondary metabolites are derived from central metabolic pathways using primary metabolites precursors. Genomic mining studies revealed genes involved in secondary metabolite synthesis are often arranged in a contiguous manner known as the biosynthetic gene cluster (BGC). Whereas genes involved in primary metabolic synthesis are usually spread throughout the fungal genome (Keller, 2018; Walsh and Fischbach, 2010). The biosynthetic gene clusters can range from a group of 2 genes such as the synthesis of valactamide to over 20 genes that are required for aflatoxin synthesis. The small clusters tend to contain genes encoding one dedicated enzyme with a few tailoring enzymes, whereas the larger clusters have several dedicated and tailoring enzymes. Dedicated enzymes include polyketide synthases, non-ribosomal protein synthases, tryptophan synthetases, and dimethylallyl tryptophan synthetases while the tailoring enzymes are responsible for a vast spectrum of bioactivity observed (Keller, 2018; Pusztahelyi, Holb, & Pocsi, 2015). The expression of such clusters is tightly regulated and only expressed when triggered by either external biotic or abiotic stimuli. Thus, most biosynthetic gene clusters remain 'cryptic' until induced by some environmental cue (Keller, 2018). Once activated, these clusters can produce numerous structurally diverse chemicals (Walsh and Fischbach, 2010).

This research project is based on identifying secondary metabolites that show activity against agriculture pests and pathogens such as fungi, insects, and molluscs. Fungi were selected as the source of secondary metabolites since their ecological niche results in diverse heterotrophic interactions among microbes and higher organisms such as insects and molluscs. Furthermore, based on genomic mining, there are still numerous undiscovered, cryptic secondary metabolite gene clusters that have yet to be utilized. Further, commercially available products were tested including Tea Tree Oil (Stockton Inc.,), Acadian (Stella Maris), Venerate (Maronne Bio Inc.), STK-53 (Stockton Group), and Grandevo (Marrone Bio Inc.). Tea Tree Oil is a plant-based product derived from leaves of *Melalueca alternifolia*, while Acadian is comprised of secondary metabolites from several different classes of chemicals derived from marine plants. Grandevo and Venerate are comprised of secondary metabolites from several different chemical classes derived from microbes. Lastly, STK-53 is considered a botanical pesticide.

## 1.2 Agricultural Pests and Pathogens

A major factor that threatens food security is product damage inflicted by pests and pathogens. Integrated Pest/Pathogen Management (IPM) refers to the use of a multi-pronged approach that focuses on supporting growth of healthy crops and natural pest control mechanisms in addition to having the least disruptive effect on agro-ecosystems (Barzman *et al.,* 2015). Synthetic pesticides have routinely been used as part of the integrated pest/pathogen protocol to prevent loss of yield however their use is under scrutiny due to the damage inflicted on both the biotic and abiotic environment (Masi *et al.,* 2018). Furthermore, there is a constant race between the development of inhibitors of pests/pathogens and the development of strains that are resistance to these inhibitors. Although alternative biocontrol methods exist such as introducing a predatory, competitive, or pathogenic species, they are not entirely effective measure and can cause additional shifts in the ecosystem. To mitigate the negative effects associated with use of pesticides, the agriculture industry is moving towards the use of biopesticides derived from natural products. Secondary metabolites tend to have a shorter half-life than synthetic chemicals, thus preventing their accumulation in the environment. Diverse secondary metabolites can have novel modes-of-action and this can reduce problems associated with the development of resistant strains. As a result, secondary metabolites are thought to be more efficient, eco-friendly, cost-effective, and pose a low risk to human health (Masi *et al.,* 2018; Hubbard *et al.,* 2014).

Although there have been steady increases in the use of biopesticides, the rate of commercial success is less than 10% due to biological, environmental, technological, and commercial challenges in the R&D sector (Hubbard *et al.,* 2014). A crucial element of commercial success of a product lies in deciphering the mode of action. The mode of action influences the efficiency of secondary metabolites as pest control agents, ease of use, regulatory approval processes, consistency of response, and susceptibility of the host target(s) and non-targets (Masi *et al.,* 2018). Further, the mode of action has an impact on the manufacturing method, the final cost of the product, and can inform predictions on whether resistance will develop in the target organism(s) (Hubbard *et al.,* 2014).

### 1.2.1 Fungal Pathogens

Fungal pathogens influence agriculture yield by causing diseases in animals and plants which in turn lowers the nutritional value hence affecting the quality of products. Additionally, phytopathogens can produce toxins within their living hosts causing adverse or lethal effects on human and animal health upon consumption (Dangl & Jones, 2001). A surge in fungal pathogens has been observed both in severity and rapid global spread, causing approximately 15% in crop production losses (McDonald & Stukenbrock, 2016). This can be attributed to the agricultural practices adopted globally leading to the creation of agro-ecosystems. These ecosystems have developed along with agriculture for the past 12,000 years to generate a constant and reliable food supply. Agricultural ecosystems enabled the rise of civilizations and now take up more than 40% (~540 million ha) of the land surface across the globe. By comparison, tropical rainforests emerged over millions of years and take up approximately 600 million ha (McDonald & Stukenbrock, 2016).

As a consequence of feeding a rapidly increasing human population, agro-ecosystems shifted from growing locally adapted, genetically diverse crops to growing monocultures of high-yielding varieties that are easily integrated into modern production systems. Further, the reduction in both genetic and environmental heterogeneity has created a favorable environment for the emergence of new varieties of pathogens. These pathogens are host-specific, able to evolve more rapidly and are more virulent than their "wild" ancestors (McDonald & Stukenbrock, 2016). Additionally, fungal pathogens have spread rapidly across the globe due to the recent increase in agricultural trade between countries.

### 1.2.2 Insect Pests

Insect pests are also known to cause a loss in yield by causing physical damage to plants, by vectoring viral, bacterial and fungal diseases and by reducing the nutritional value of crops. Insects can also be serious pests in farm animals. For example, fly larvae can be either obligatory or facultative parasites of animals causing a myriad of diseases known as Traumatic Myiasis (Hall *et al.,* 2016). These insects can create and/or enlarge wounds of the dermal and subdermal layers in addition to body orifices and sinuses. Similar to toxin-producing plant pathogens, flies of the Muscidae family are known to attack and bite animals leading to the release of toxins which cause anxiety, itching, skin inflammation, and stress. Such symptoms are known to

decrease the productivity of livestock and the quality of products such as milk, eggs, and meat. Additionally, these flies are carriers of disease-causing pathogens which may be harmful to animals and humans (Shevchenko *et al.,* 2017). Other insect pests of livestock include the blood-sucking insects from the Muscidae family and other members of the Diptera order. These can lead to the deterioration of the animal's health and are carriers of pathogens (Shevchenko *et al.,* 2017). In terms of crops alone, there are more than 10,000 species of insects known to be pests worldwide of which only 10% are considered major pests. An annual global investment of 40 Billion USD into preventative and management strategies including chemical and biological methods, insect pests are responsible for destroying one-fifths of the global crop production annually (Dhaliwal, Jindal, & Mohindru, 2015).

### 1.2.3 Mollusc Pests

Molluscs such as slugs and snails are among the most damaging pests found in gardens, are known to cause serious damage to economically important crops and can also act as vectors of animal pathogens (Wilen & Flint, 2018; Le Gall & Tooker, 2017; McDonnell *et al.,* 2018). Many aspects of basic ecology of molluscs are poorly understood but, generally molluscs can either be terrestrial or aquatic and are known to be generalist herbivores. They play a crucial role in defining the composition of plant communities and may become invasive species leading to the displacement of native species (Le Gall & Tooker, 2017; McDonnell *et al.,* 2018). A small number of molluscs have been deliberately or accidentally spread worldwide via the horticultural and pet trades, leading to the homogenization of this malacofauna across the planet. Their broad distribution, generalist feeding strategies, and ability to estivate/hibernate during drought/cold conditions have made it difficult to prevent spread and eliminate established colonies of invasive molluscs.

Since snails and slugs generally prefer succulent foliage or flowers, they are primary pests of seedlings and herbaceous plants (Wilen & Flint, 2018). For example, among terrestrial pests, the European brown garden snail, *Cantareus aspersum*, is a known pest of citrus trees and horticultural and ornamental crops in Europe, the US, and New Zealand (Cordoba, Millar, & McDonnell, 2018; Le Gall & Tooker, 2017). Meanwhile, the gray field slug, *Deroceras reticulatum*, is a known pest of most crops in temperate and tropical areas of the world (Cordoba, Millar, & McDonnell, 2018). One of the most destructive aquatic snails is the golden apple snail,

*Pomacea canaliculata*, which is on the top 100 list of invasive species responsible for causing devastations in rice and taro fields in addition to other aquatic habitats.

The golden apple snails affect the agriculture industry by consuming significant amounts of rice and taro crops leading to economic losses including a decrease in crop yield and increase in preventative and management costs. Rice bowl countries are especially affected by this invasive species since they are the main producers of rice and rely on rice as their primary source of nourishment (Olivier *et al.,* 2016; Prabhkaran, Bhore, & Ravichandran, 2017). These countries have seen economic losses of up to $1.47 Billion USD per annum due to crop damage – not including funds directed towards preventing and eradicating the mollusc pest population (Prabhkaran, Bhore, & Ravichandran, 2017). Currently, these snails have spread as far as the US, Japan, China, Malaysia, the Philippines, and Taiwan and are predicted to spread to Australia, India, and Bangladesh in the next 5 years (Carvalho de Brito & Joshi, 2016).

### 1.3 Metabolomics & Bioassay-Guided Fractionation

The globalization of agro-ecosystems has lead to the emergence of host-specific, rapidly evolving, and highly virulent pests and pathogens that threaten our food security. To mitigate such agricultural losses, a combination of control and preventative methods are needed to support the growth of healthy crops. Furthermore, pesticides which naturally reduce pest/pathogen growth and prevent accumulation of chemicals in the environment are in demand. Secondary metabolites produced by fungi are gaining attention as a source of natural pesticides based on their broad activity spectrum, their capacity to produce chemically diverse secondary metabolites that can be degraded quickly in the environment. Here we attempt to design bioassays that are easy and cost-efficient to implement while generating data to identify secondary metabolites showing inhibitory effects against fungal, insect, and mollusc pests.

Fungal bioassays were key to this process. They were used to assess consistent activity among reconstituted broths grown at different times. A set of preliminary bioassays was performed using all 15 natural products against all 3 bioassays however the second set of bioassays relied on using the fungal bioassay as a guide. If reconstituted broths from source fungi did not exhibit antifungal activity, we did not test these extracts against our insect or mollusc models. A subset of reconstituted broths was further chemically refined using Liquid-Liquid Extraction (LLE). A portion of the refined broths were run through HPLC with the

chromatograms detected using HRMS while the remaining portion was used to conduct a final fungal bioassay to follow activity. The data generated via HPLC-HRMS was used to perform a metabolomic analysis in an attempt to identify potentially active secondary metabolite(s) while using the results from the refined fungal bioassay as a guide for the metabolomic analysis.

### 1.4. Research Objectives

The over- and mis-use of chemical pesticides against fungal pathogens and pests such as insects and molluscs have rendered them ineffective due to the formation of resistant strains. Additionally, the use of such chemicals has caused adverse effects in humans, non-target organisms, and an accumulation of toxins in the environment. Thus, a constant search for eco-friendly and cost-efficient pesticides with novel mode-of-actions is essential to limit the damage inflicted upon economically important crops. The seemingly unlimited source of diverse chemicals structures and range of biological activities of secondary metabolites serve as a rich source for natural product-derived pesticides.

Our research sought to test 10 fungal isolates and 5 commercially available natural products against fungal pathogens and insect and mollusc pests. We created or modified bioassays representing all three types of organisms and hypothesized that the fungal bioassay would be able to predict inhibitory activity in either the insect bioassay and/or mollusc bioassay. The 10 fungal isolates chosen were previously described as producing anti-fungal metabolites at 13 °C (Micalizzi *et al.,* 2017), thus we further hypothesized the use of all three bioassays would give insights on the presence of target-specific or general toxins.

Although any number of stressors can be used, we used a sub-optimal temperature and a long incubation period to trigger the production of a novel metabolite profile in each isolate. In addition to the cold, a long incubation period would capture metabolites produced during stationary or senescence stage and thus may potentially be defensive compounds. To test for antifungal activity, *Saccharomyces cerevisiae* was used to represent fungal pathogens whereas *drosophila melanogaster* was used to represent insect pests to test for anti-insect activity. Lastly, *P. maculata and P. canaliculata* were used to represent molluscs to test for anti-mollusc activity.

## 2. Materials and Methods

### 2.1 Fungal Strains used as Secondary Metabolite Producers

Table 1 summarizes the 10 isolates used in this study along with describing the type of isolate, site of collection, and isolate ID. These fungal isolates were obtained in a previous study in a screen for microbes that inhibit *Pseudogymnoascus destructans*, a fungal pathogen of bats (Micalizzi *et al.,* 2017). These isolates were stored on agar slants of Potato Dextrose Agar (PDA; Difco™ Laboratories Limited) at 13°C in the dark. Cultures were transferred to fresh slants 6-9 months. Species were identified, where possible, by morphological examination and based on ITS and β-tubulin sequences (Micalizzi *et al.,* 2017). For simplicity, isolates will be referred to their numbers, thus S8I2ACS will be referred to as Isolate 1, RW2I1a as Isolate 2, etc.

Table 1. Type, source, and identification of fungal isolates

| No. | Isolate | Growth Form | Organism Name | Source | GENBANK No. |
|---|---|---|---|---|---|
| **1** | S8I2ACS | Filamentous | *Penicillium virgatum* | Soil site 8 | AM901674.1 |
| **2** | RW3I1a | Yeast | *Cystofilobasidium capitatum* | Rotten wood | NR_111042.1 |
| **3** | RW6A1P | Filamentous | *Boeremia exigua* var. *exigua* | Rotten wood | GU237707.1 |
| **4** | RW3A2Pa | Filamentous | *Phacidium grevilleae* | Rotten wood | KR476718.1 |
| **5** | PCA5P | Filamentous | *Phialemonium inflatum* | Plate contaminant | KY305083.1 |
| **6** | OA1I5Mb | Filamentous | *Ramularia vizellae* | Outdoor air | KP894307.1 |
| **7** | PCA20P | Filamentous | *Oidiodendron rhodogenum* | Plate contaminant | NR_119425.1 |
| **8** | PCA22M | Filamentous | *Aspergillus caninus* | Plate contaminant | KY305081.1 |
| **9** | S3A2ACS | Filamentous | *Trichoderma* sp. | Soil site 3 | HM000037.1 |
| **10** | S9A1R | Filamentous | *Penicillium herquei* | Soil site 9 | NR_103659.1 |

### 2.2 Growth Conditions

Inoculum from each isolate was transferred from agar slant to 250 ml conical flasks containing 50 ml Potato Dextrose Broth. Inoculated flasks were incubated at 13 °C in the dark for a period of 6 weeks. This process was repeated using 4 x 50 ml culture flasks of each isolate to generate biological replicates of fermented broths.

### 2.3 Preparation of Reconstituted Broths

Mycelia of the fermented broths was removed by passing the supernatants through a cheesecloth. Broths of each isolate were pooled into a single 500 ml conical flask that was capped using double-layered aluminium foil. Pooled broths for each Isolate 1, 3, and 5-10 were vacuum filtered through P5 filter paper (Fisher Scientific, Pittsburg PA 15275). Isolates 2 and 4 were first vacuum filtered through P8 filter paper (Fisher Scientific) before vacuum filtered through P5 paper. Once filtered, the broths were divided into 30 ml aliquots and stored at -20 °C in sterile 50 ml Falcon tubes. The reconstituted broths were either used directly in the mollusc bioassay or freeze dried at -84 °C, 0.03 mbar for 2-3 days before resuspending to the desired concentration for fungal and insect bioassays (Labconco, FreeZone 2.5 L -84 °C).

### 2.4 Fungal Bioassay – Reconstituted Broths

The freeze-dried reconstituted broths were resuspended to 10× concentration using 1.25 % Tween 80 (Bio Basic; Markham, ON, Canada) dissolved in distilled water. The broths were transferred to 1.5 ml centrifuge tubes to be centrifuged at 13,200 RPM for 5 minutes (Eppendorf Centrifuge 5415 D, Germany). The supernatant was transferred to a 5 ml syringe to be filter sterilized using PES filter discs (UltiDent Scientific; 30 mm diameter; 0.22 µl; St-Laurent, Québec, Canada).

To test for anti-fungal activity, the S288C yeast strain was used to carry out a minimum inhibitory concentration (MIC) bioassay. YPD broth was used as the base medium of which 100 µl was added to each well in a microtiter plate. Next, 50 µl of each broth, including the carrier solvent control (1.25 % Tween 80 in PDB), was added to the first well of its designated row. Serial dilution was performed across 6 wells of each treatment by transferring 50 µl of mixture from the first well to the second from which 50 µl was transferred to the next well. This was

repeated until well number 6 from which 50 µl was discarded. Thus, all wells had 100 µl of mixture. Hygromycin B (BioShop® Burlington, ON, Canada; stock concentration: 50 mg/ml) inhibits yeast at 0.25 mg/ml, therefore it was used as a positive control by adding 1.25 µl of the stock solution to the first well and performing serial dilution as previously described using 1.25 µl instead of 50 µl. The number of yeast cell in suspension were estimated using a hemocytometer and cell density was adjusted to 1,500 cells/mL before 150 µl was added to each well (i.e. ~150 - 200 CFU/well). The CFU calculation method is described in Appendix I. The microtitre plate was sealed with parafilm and incubated at 30 °C for 48 hours. MIC was recorded as the lowest concentration that showed complete inhibition of growth. To confirm yeast cell count, 50 µl of the diluted cell suspension was spread over a YPD-agar petri dish. The agar plate was sealed and placed in the incubator along with the microtiter plate. The MIC values can be found in Appendix II based on batch number.

To determine anti-fungal activity of the natural products, the protocol mentioned above was used. 100 µl of YPD was added to all the wells before adding 50 µl of the natural product to the first well in its designated row. 50 mg of Grandevo pellets were dissolved in 1 ml distilled water before transferring 50 µl to the first well in the Grandevo designated row. 50 µl of distilled water was added to the first well of the negative carrier control row before performing serial dilution by 33.3 % across each treatment as described above. Hygromycin B was used as the positive control and was prepared as described above. Next, 150 µl of cell suspension was added to each well (i.e. ~150 - 200 CFU/well). The microtitre plate was sealed using parafilm and incubated at 30 °C for 48 hours before results were noted.

**2.5 Insect Bioassay – Reconstituted Broths**

To test the broths for presence of anti-insect metabolites, 1.25 % Tween 80 solution was used to resuspend freeze-dried extracts to the desired concentration (1×, 2×) and then sterilized using syringe filters (30 mm, PES 0.22 µm). The bioassays were carried out in replicates of 2 or 3 depending on the initial volume of extracts. Each replicate consisted of 1 g of fruit fly food (Formula 4-24; Carolina Biological Supply Company, Burlington, North Carolina 27215) dissolved in 5 ml of 1× or 2× broth. Granules of Bakers yeast (Fleischmann's Traditional Active Dry Yeast) were sprinkled onto the surface of the fly food before adding a 1:1 sex ratio of flies with a total number of either 8 or 10. This was carried out by using $CO_2$ to knock out the flies

and sex them under a microscope. Each group created was transferred to a vial containing 5 ml of cool tap water, 1 g of fly food, and a pinch of yeast. The vials were then placed in an incubator at 24 ± 1°C for a 24-hour period to condition the flies and eliminate false results generated by $CO_2$ poisoning. After the 24-hour period, the flies were transferred to their designated experimental vials by tapping each vial containing flies on a hard surface which disorients the flies. The vial was quickly inverted and placed on top of the experimental vial. The experimental vial was tapped on a hard surface gently until all flies were transferred. A stopper was quickly placed to seal the vial and prevent flies from escaping. The vials used for conditioning were sealed and kept under ambient light and temperature as a means to propagate the colony. Tea Tree Oil was used as the positive control by adding 200 µl to 5 ml of cool tap water before mixing in with 1 g of feed. 1× and 2× PDB with 1.25% Tween were used as negative controls. The vials were placed in an incubator at 24 ± 1°C for 2 weeks on a 12-hour dark/light cycle. The number of adult flies and pupae were noted every 24 hours starting from the time when the flies were transferred into the experimental vials, for a 2-week period. Time taken for the original adults to perish would indicate acute toxicity while the number of pupae successfully undergoing transformation would indicate signs of developmental toxicity. To note the number of pupae, the shell remnants of the pupae attached to the wall of the vials were counted. For details regarding the fly colony, refer to Appendix I. For details regarding the reconstituted broths concentrations, refer to Appendix II. For a detailed description of the insect bioassay protocol, please refer to Appendix III.

Anti-insect activity of the natural products was determined using the protocol described above however the bioassay was carried out under ambient laboratory light and room temperature. Additionally, the 24-hour conditioning and recovery period was not included in this bioassay. 100 µl of Tea Tree Oil, Acadian, Venerate and STK-53 were mixed in 4.9 ml cool tap water before adding 1 g of dry fly food. 10 mg of Grandevo pellets were dissolved in 5 ml of cool tap water before mixing with 1 g of dry fly food. Duplicates of each treatment were used. The bioassay was carried out in ambient light and room temperature.

The data collected was tabulated in Microsoft Excel spreadsheets where the average, standard deviation, and standard error was calculated before creating line graphs showing differences between treatment and controls. The standard deviation and error were calculated for

each day thus *n* reflected the number of replicates. To determine statistical significance, a two-tail t-test was performed with 0.05 as the cut-off. The 'Data Analysis' tab was selected under the 'Data' tab present in the tool bar. From the list of Analysis Tools, 't-Test: Two Sample Assuming Equal Variances' was selected. The last 4 values of the carrier control were selected as the first variable range while the last 4 values of the experimental treatment were selected as the second variable range. An area next to the graph was selected as the output range before selecting 'OK'. Contradicting p-values were indicated.

**2.6 Snail Bioassay – Reconstituted Broths**

To test the broths for anti-mollusc activity, snails of 1 ± 01cm in length were selected and placed on a grid sheet with labels designating each broth. Pictures of the snails were taken before exposure to broths and at the end of the bioassay using a smartphone placed at a set height and angle with respect to the snails. Snail assays were carried out in 2.1 L tanks with 2 snails per tank (Kis Omni Clear Containers, Italy). The tanks had 12 holes drilled into the lids to allow for airflow and prevent snails from escaping. The snails were transferred to their "experimental" tank containing 500 ml de-chlorinated water and allowed to acclimatize for 24 hrs without food. Once the picture was taken, 15, 30, 60, or 90 ml of broths were added to the experimental tanks and the snails were exposed for 6 hours before being transferred to their "observation" tanks. These tanks contained 1 L of fresh dechlorinated water along with a baby spinach leaf. Behavioural and other responses were noted during exposure to the reconstituted broths and within the first hours of transfer. Behavioral changes included staying in shell with no movement, staying in shell with tentacle twitching, coming out of shell with no movement, and coming out of shell with movement. Whereas responses noted included formation of mucus layer over shell and body and body falling out of shell after death. Appetite was monitored over a 2-week period by taking pictures of spinach leaves before and after 24-hour intervals starting immediately after transfer into observation tank. The pictures were taken using a smartphone placed at a set height and angle with respect to the leaves which were placed on a grid sheet with labels indicated the broths used. The leaves were pressed flat using a glass panel. On day 15, pictures of snails were taken to capture the final size of the snails. To take images of the snails, they were placed with their operculum closed and facing down. GIMP, a free photoshop software package, was used to calculate percentage of leaves eaten and percentage growth by snails.

Based on the aerial view of the images, a grid was imposed on top of the image using the centimeter ruler as a guide to standardize the grid. The surface area of snail shell and leaves was determined by counting the grid squares. Once the amount eaten was calculated for each day, a frame-shift average of 4 days was calculated along with the standard deviation and standard error. To determine statistical significance between the experimental amount eaten and carrier control, a two-tail t-test was carried out in Microsoft Excel similar to the analysis performed on insect bioassay data. For details regarding the formulae used to determine average growth and amount eaten, refer to Appendix I. For details regarding the concentration of each extract, refer to Appendix II. For a detailed description of the snail bioassay protocol, please refer to Appendix III.

Anti-mollusc activity of the natural products was tested using the protocol described above. 1 ml of the liquid products was added to their designated “experimental” tanks containing 1 L of fresh de-chlorinated water while 1 g of Grandevo pellets were added. The negative control used for the natural products was 500 ml of de-chlorinated water.

**2.7 Correlation Graphs**

To observe a correlation between the fungal and insect bioassay, the MIC values were plotted against the standardized values representing the F1 generation in the treatments. These values were calculated by determining the difference between the number of F1 adults on day 14 and the adults exposed to the treatment on day 14. Once the difference was calculated, the average and standard deviation was calculated in Excel to standardize the values. Each graph represented the MIC and F1 values belonging to the same batch of broth of each Isolate. To observe a correlation between the fungal and mollusc bioassay, a similar approach was used where the MIC values of the same batch of broth were plotted against the difference in size between the carrier and treatment molluscs. This difference in size was standardized similar to the F1 generation by using the “average”, “stdev”, and “standardize” functions in Excel. A correlation graph between insect and mollusc bioassay was constructed using change in size and F1 generation adults exposed to reconstituted broths of Isolate 9 from different batches.

## 2.8 Liquid-Liquid Extraction (LLE)

To perform liquid-liquid extraction, an aliquot of each reconstituted broth from each batch was freeze-dried before resuspending in distilled water to 10× concentration. Glass vials capable of containing 9 ml volume were used for the extraction process. Each broth would be split into 4 fractions thus 4 glass vials were designated to each extract. The glass vials were weighed and labeled with the batch number followed by the isolate number and solvent type. The broth was transferred into the $H_2O$ labelled glass vials and placed in the fume hood to perform the extraction using 3 solvents with increasing polarity. All extractions were performed by mixing the less polar solvent with the water fraction before transferring the solvent layer on top into its designated glass vial and repeating twice more. First, hexane (ACS SPECTRO grade Caledon Laboratory Chemicals; Georgetown, ON, Canada) was used as the least polar solvent. Next, ethyl acetate (SPECTRO grade; Caledon Laboratory Chemicals Georgetown, ON, Canada) was used followed by 1-butanol (Certified A.C.S, class 1C 192, Fisher Scientific Company; New Jersey, U.S.A). A 1:1 volume ratio of broth and solvent was mixed by inverting the glass vials. To eliminate emulsions the mixture was spun using a benchtop centrifuge (Eppendorf Centrifuge 5804R, Germany) at 4 - 6 °C and 400RPM for 5 minutes. Once the extractions were complete, the solvents were dried using a Nitrogen Blower (Reacti-Vap III No. 18785; Reacti-Therm III Heathing Module) set at 37 °C. The dried solvents, or fractions, were stored at -20 °C until ready for resuspension to a concentration of 0.5 mg/ml in 2 ml Amber Vials (Virtuoso SureStop, ThermoScience, Pittsburg PA 15275). The water fractions were resuspended in distilled water whereas butanol and ethyl acetate fractions were resuspended in 50 % methanol. For the LC-MS analysis an injection volume of 5 µl of each sample was run through. The hexane fractions were not included in this comparison since they require GC-MS and glass tips to resuspend using hexane. Thus, the fractions remain in storage until the equipment becomes available. Once LC-MS samples were taken, the fractions were dried under vacuum and stored at -20 °C. The data files generated were labelled in a similar manner with the first number identifying the batch number followed by the isolate number and solvent type separated by underscores. For details regarding the protocol used to chemically clean the glass vials and the method used to label the fractions, please refer to Appendix I. For details regarding the dried mass of each fraction please refer to Appendix IV.

### 2.9 Fungal Bioassay – Refined Broths

The refined broths, or fractions, were resuspended in 1.25 % Tween 80 to 2× their MIC values as calculated in the fungal bioassay of the reconstituted broths. The fractions that didn't have enough dried material to be resuspended to a 2× concentration, were resuspended to a 1× concentration. To perform the fungal bioassay, each well was labelled according to the fraction being tested. 100 µl of YPD media was added to each well followed by 50 µl of the fraction and 150 µl of cell suspension, identical to the protocol used when performing the bioassay on reconstituted broths without using serial dilution. Fractions of PDB were used as the negative control while Hygromycin B was used as the positive control. The microtitre plate was sealed using parafilm and placed in the incubator at 30 °C for 48 hours before determining presence of growth. For details regarding the calculations used to determine the volume needed to resuspend to the MIC, please refer to Appendix I.

### 2.10 Metabolomic Analysis

#### 2.10.1 Data Pre-Processing – Xcalibur and MZmine 2.4

To determine noise threshold, the Qual Browser in Xcalibur 2.2 was used. The NL (noise level) value of the spectrum view was observed for each sample that showed anti-fungal activity in the pure fraction MIC bioassay. Xcalibur was also used to convert the .raw files to .cdf using the file converter option available in the Tools drop-down menu. The .cdf files of samples from 6-week incubation period along with the methanol blanks and reserpine were uploaded to MZmine 2.4. Once imported the files underwent mass detection followed by chromatogram building, chromatogram deconvolution, isotopic peak grouper, alignment, gap-filling, and normalization. Lastly, the peak-height values of aligned and gap-filled files were exported while the peak-area values of the normalized file were exported. For details regarding the parameters used for each module, refer to the metadata present in Appendix V.

#### 2.10.2 Microsoft Excel – Data Manipulation

The aligned, gap-filled, and normalized dataset underwent data manipulation using Microsoft excel. The matrix was highlighted and transposed to a new sheet thus sample names were in column 1 while m/z and RT values were in rows 1 and 2, respectively. The RT values were rounded off to 2 decimal places (row 4) while m/z values were rounded off to 5 decimal

places (row 2). Rounded values of RT were in row 5 while those of m/z were in row 1. An extra row was inserted above row 1 to combine both rounded values using the formula available in Appendix I. Once the RT and m/z values were combined, the gap-filled and normalized data matrices were moved below the aligned data matrix. Conditional formatting was used to highlight values above noise threshold. Variables with peaks in blanks, controls, and reserpine were removed from the analysis along with variables lacking peaks in the experimental samples. The normalized data matrix was transferred into a new excel sheet and saved as both an .xlsx and .csv format for statistical analysis in RStudio.

### 2.10.3 RStudio – Multivariate Analysis using *muma*

A multivariate analysis was carried out based on the results generated from the MIC of the refined broths. To identify significant RT and m/z variables, the active fractions of a single isolate were compared to their inactive counterparts of the same isolate and fraction from different broths. Groups were created based on isolate and type of fraction before performing multivariate analysis in addition to further dividing each group into 2 classes. For example, active ethyl acetate fractions of isolate 9 from batch numbers 11, 12, & 13 were classified in class 1 while the inactive ethyl acetate fractions of isolate 9 from batch numbers 10 and 14 were classified in class 2. Multiple principle component analyses (PCA) were performed on for each isolate's active and inactive fractions to identify principle components which likely separate samples based on activity. To further enhance the difference between active and inactive samples, an OPLS-DA analysis was carried out. The pareto scaling was used for both PCA and OPLSDA analyses. The script used to perform a multivariate analysis using the *muma* package can be found in Appendix VI.

### 2.10.4 Significant Variables

To assess and identify significant variables, the loading matrix in the OPLS-DA folder was sorted from lowest RT_m/z value to highest. The data matrix of the normalized samples' peak area that were exported from MZmine was sorted in the same manner. The normalized peak area matrix was edited to remove samples not included in the analysis before pasting the matrix next to the loading matrix. As a result, the RT_m/z column was adjacent to the PC1 column, PC2 column, and the columns containing peak area of each sample in the group in question. An outline was created around active samples to distinguish from inactive samples in addition to

highlighting active samples in bold font. The score plot was assessed before sorting the new matrix according to the principle component responsible for separating samples based on activity. The top most variables were considered significant and were confirmed by assessing the peaks from the .raw data files in xcalibur. Further, the ion chromatographs of each peak were extracted to confirm whether the peaks were from the same molecule. A new data matrix was created containing information on labelled peaks including the RT_m/z value and the peak area of each sample at that RT_m/z point. Variables from the same peak were grouped while the samples showing inhibitory activity were highlighted in bold.

To construct a heatmap showing differences in metabolite abundance, the peak area values were standardized in a similar manner as the F1 adults and difference is size values calculated for the correlation graphs. Once standardized, the maximum and minimum value was selected to create an abundance range that could be used for all heatmaps. The average value of the maximum and minimum value was calculated before dividing by 6 and the resulting value was used to construct each range by adding to the minimum value. This was repeated until the maximum value was reached. Once the abundance range was created, conditional formatting was used to create the heatmaps.

## 3. Results

### 3.1 Reconstituted Broth Consistency and Texture

Inoculation of Isolate 1 lead to the formation of a single mycelial mass covering the surface of a turbid broth with a yellow color. The reconstituted broth appeared slightly frothy – easily formed bubbles when shaken and during vacuum filtration but passed through filtration steps with relative ease. Isolate 2 did not form any solid mycelial bodies, the reconstituted broth became a cloudy suspension which was difficult to vacuum filter. Thus, it was first vacuum filtered using P8 filter paper and then through P5 filter paper. Fermentation of Isolate 3 resulted in the formation of a single, jet-black mycelial body covering the surface of a dark-yellow colored broth which passed with ease through P5 during vacuum filtration. Both Isolates 2 and 3 appeared frothy during the harvest process, however only Isolate 2 appeared to have a viscous consistency. Isolate 4 appeared to have a slimy, viscous textured broth with a yellow tint and a single mycelial mass. Similar to Isolate 2, isolate 4 was difficult to filter and thus P8 was used before P5 for vacuum filtration. Fermentation of Isolate 5 resulted in formation of very small mycelial colonies which appeared to be floating throughout the yellow colored broth which easily passed through P5. Isolate 5 did not have a frothy consistency. Isolate 6 formed numerous, large, semi-circular mycelial bodies at the bottom of the flasks under a dark yellow colored extract. Isolate 6 extract was easy to filter sterilize using P5 filter paper and did not appear frothy. Isolate 7 formed a burgundy colored mycelial mass floating above a similarly colored broth which easily passed through P5 filter paper and did not appear to have a frothy consistency. Fermentation of Isolate 8 resulted in a single mycelial mass floating above a yellow colored broth which was easily filter sterilized using P5 filter paper. Isolate 9 formed a single light-yellow mycelial mass covering the surface of yellow medium. The texture of the mycelial mass was different from the smooth, rubbery texture of the rest of the isolates since it was stiffer and brittle. The broth appeared frothier than other broths but passed through P5 filter paper with relative ease. Lastly, Isolate 10 also had a single mycelial mass spanning the surface of the yellow colored broth. The broth was frothy during filter sterilization but was not viscous and therefore passed through P5 filter paper easily.

### 3.2 Fungal Bioassays

To determine anti-fungal activity in biological replicates of the reconstituted broths, the MIC protocol was performed with each set of replicated designated by batch numbers. An aliquot of each extract was freeze-dried and resuspended to a 10× concentration using 1.25 % Tween. Thus, the carrier control (PDB) was the Potato Dextrose Broth resuspended in the same manner in 1.25 % Tween. The positive control used was Hygromycin B (MIC: 0.25 mg/ml). The MIC fungal bioassay was also performed to determine presence of anti-fungal activity in commercially available natural products.

The MIC fungal bioassay was repeated using refined broths of the biological replicates to determine which fraction the active metabolite(s) appears in. The carrier controls used were purified fractions of Potato Dextrose Broth. The purified fractions were dried under vacuum and resuspended in 1.25 % Tween to twice the MIC values calculated from the reconstituted broth results. Hygromycin B was used as the positive control at a concentration of 0.025 mg/ml.

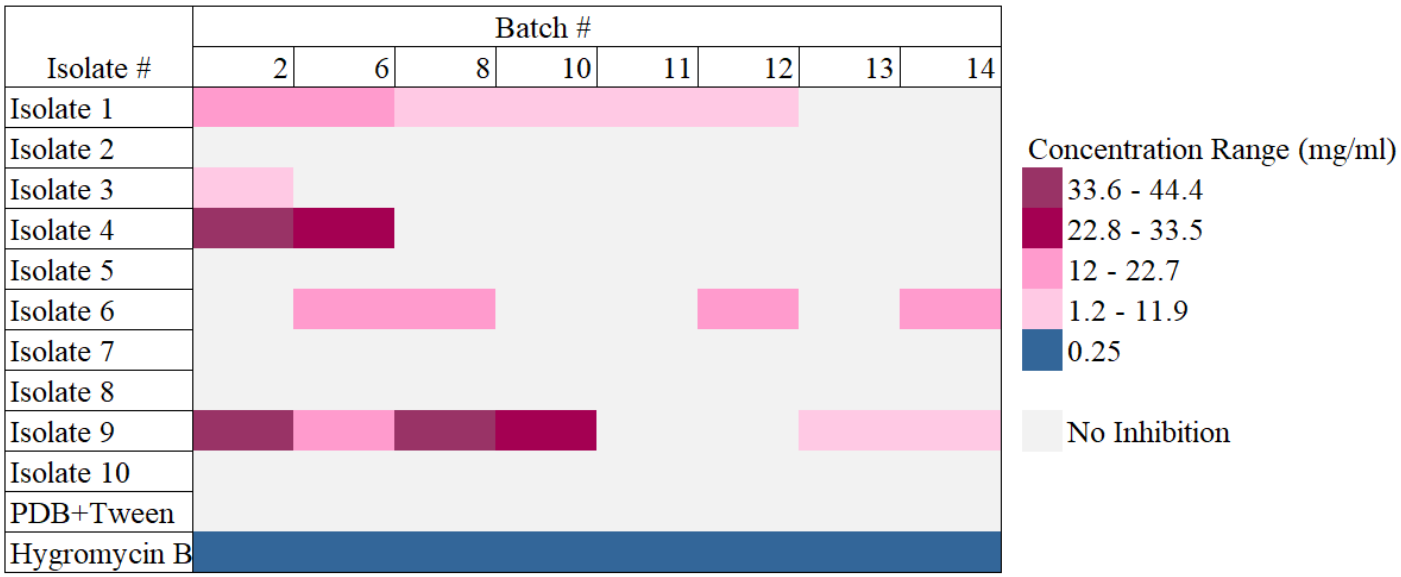


Figure 1. Heatmap of inhibitory activity of different batches of reconstituted broths, hygromycin B (positive control) and PDB+Tween (carrier control) against *S. cerevisiae* S288C. Treatment types (i.e. isolate number and controls) are present on the left while batch numbers are across the top. The legend placed on the right indicates the concentration range of reconstituted broths. Isolates which did not have inhibitory activity are indicated in a light grey color while activity of Hygromycin B at 0.25 mg/ml is highlighted in blue.

Figure 1 shows the heatmap created by the MIC values of the reconstituted broths from isolates 1-10, PDB+Tween (carrier control) and Hygromycin B (positive control). The inhibition of yeast S288C varied depending on isolate and, more interestingly, on the batch. The negative carrier control (PDB with 1.25 % Tween) and broths of Isolates 2, 5, 7, 8, and 10 consistently showed no inhibition of yeast growth, whereas the hygromycin positive control consistently inhibited yeast growth, as expected. Depending on the batch, broths from five Isolates, 1, 3, 4, 6, and 9, were completely inhibitory or did not inhibit yeast growth at the indicated concentration ranges (Figure 1). Differences in inhibitory activity between batches may be due to slight changes in the fermentation process. Regardless, broths of isolates 1, 6, and 9 were overall most inhibitory towards yeast and were thus selected for further investigations. For MIC values please refer to Appendix IV.

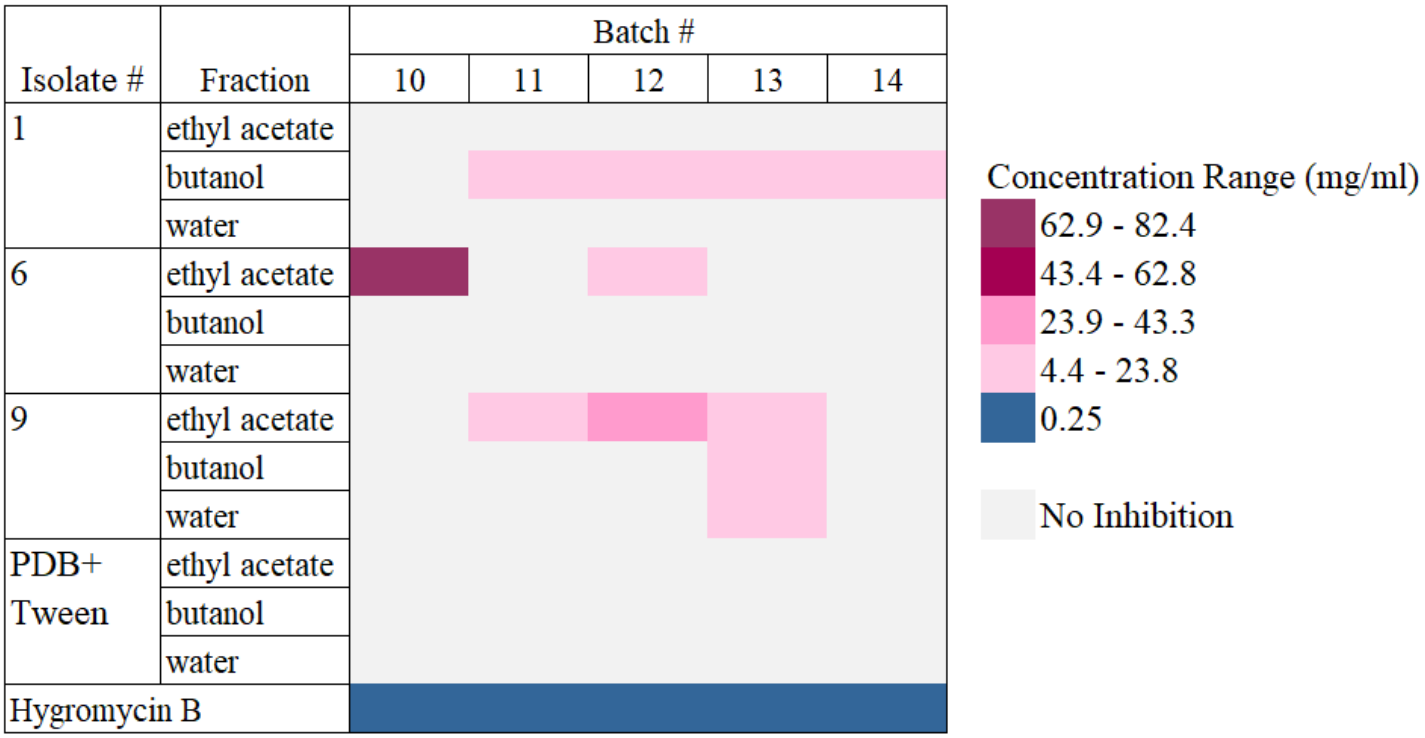


Figure 2. Heatmap of inhibitory activity of refined fractions of fungal Isolate 1, 6 and 9 from different batches, hygromycin B (positive control) and PDB fractions+Tween (carrier control) against *S. cerevisiae* S288C. Isolate number, fraction (EA = ethyl acetate, Bu = butanol, and H2O = water), and control designations are given on the left while batch numbers are across the top. The concentration legend is place on the right. Isolate fractions not exerting any inhibitory effects were indicated in light grey while hygromycin is highlighted in blue.

Figure 2 shows that inhibition of yeast S288C was carried forward from the reconstituted broths to specific refined fractions in some broths but not others. For example, batch 10 of Isolate 1 showed activity with the reconstituted broth but not with water, ethyl acetate or butanol extract. This loss of activity during fractionation could be associated with separation of synergistic inhibitors, degradation of inhibitors during chemical extraction, or the presence of inhibitors in the hexane fraction. Contrarily, reconstituted broths from batches 13 and 14 of Isolate 1 did not show anti-fungal activity but butanol extract of these same fractions did inhibit yeast growth. The presence of activity in refined fractions but lack of activity in their broth counterpart may be a results of masking effects present in reconstituted broths. In any case, fraction specificity is evident for Isolates 1 and 6, which exhibited activity in the butanol and ethyl acetate fractions only, respectively. This may suggest the presence of a single active metabolite showing toxicity towards fungi, or the presence of multiple active metabolites with similar polarity. Antifungal activity from Isolate 9 was consistently detected in batches 11-13 although batch 13 shows inhibitory activity of yeast S288C in all three fractions (butanol, ethyl acetate, and water) suggesting the presence of multiple active metabolites with varying polarity or incomplete separation during fractionation of batch 13. The MIC values are present in Appendix IV.

The result from the anti-fungal assay performed to assess inhibitory activity of commercially available natural products are presented below. Each treatment was diluted by 33.3 % across their designated row before adding ~150-200 yeast S288C cells in each well. Hygromycin B (0.25 mg/ml) was used as the positive control while distilled water was used as the carrier control.

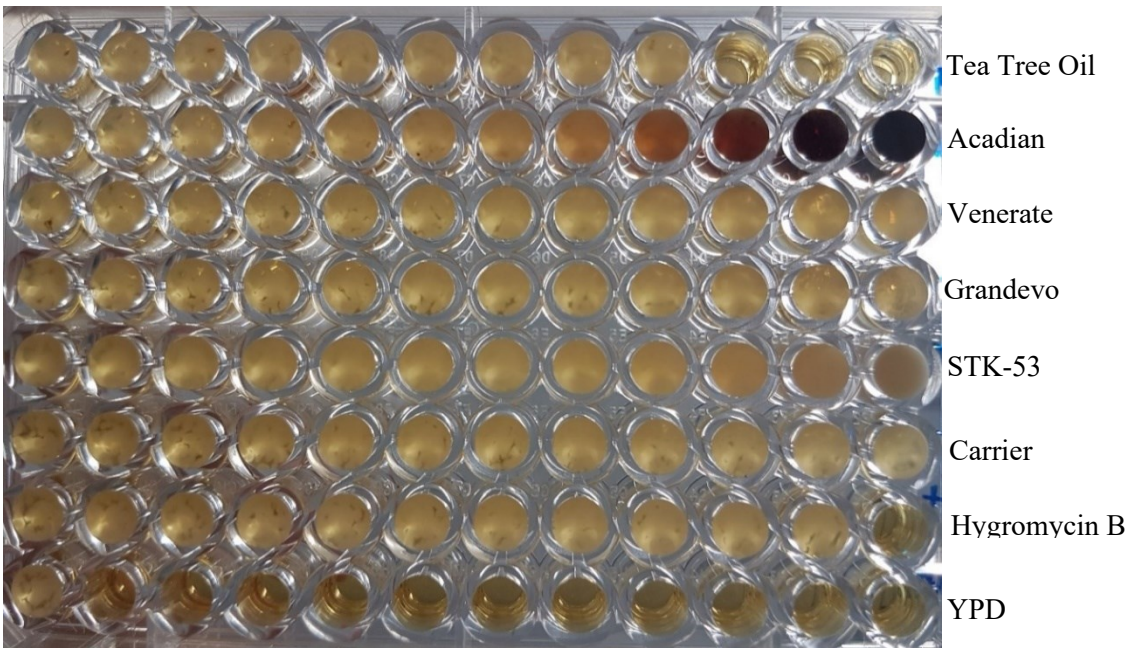


Figure 3. Fungal bioassay testing natural products, carrier control (distilled water) and hygromycin B (positive control) against *S. cerevisiae* S288C. The concentration of each treatment decreases by 33.3 % in each well from the right to left as indicated by the blue concentration gradient above the image.

Figure 3 shows the anti-fungal bioassay to test commercially available natural products, hygromycin B (positive control), and distilled water (carrier control) against *S. cerevisiae*. Tea Tree Oil exhibited inhibitory effect in the first 3 wells at 3.7 % concentration. Wells treated with Grandevo appeared to have slightly less growth than the carrier control suggesting that it may have anti-fungal properties at higher concentrations. Acadian may also have potential inhibitory properties at 33.3 % concentration, however the heavy pigmentation prevented accurate assessment of growth. Venerate, STK-53, and the carrier control did not show any inhibitory effects at 33.3 % concentration or less.

### 3.3 Insect Bioassay

Reconstituted broths of Isolates 1-10 from batches 2 and 6 were tested for anti-insect activity. These preliminary tests were used to identify isolates that showed both anti-fungal and anti-insect activity. Thus, Isolates 1 and 9 were selected for further investigation. The bioassay was performed using twice the concentration of Isolates 1 and 9 from batches 8, 12, 13, and 14. While the protocol underwent several key, fine-tuning steps through batches 6-8, the results presented below are from the final protocol used to test reconstituted broths of Isolates 1 and 9 from batch 12 – 14. These results serve as examples of the full set of results present in Appendix VII. PDB+Tween was used as the carrier control while Tea Tree Oil was used as the positive control. A two-tail t-test was used to determine the difference in F1 population and number of pupae between experimental treatment and carrier control from days 11-14 with 0.05 as the cut-off value.

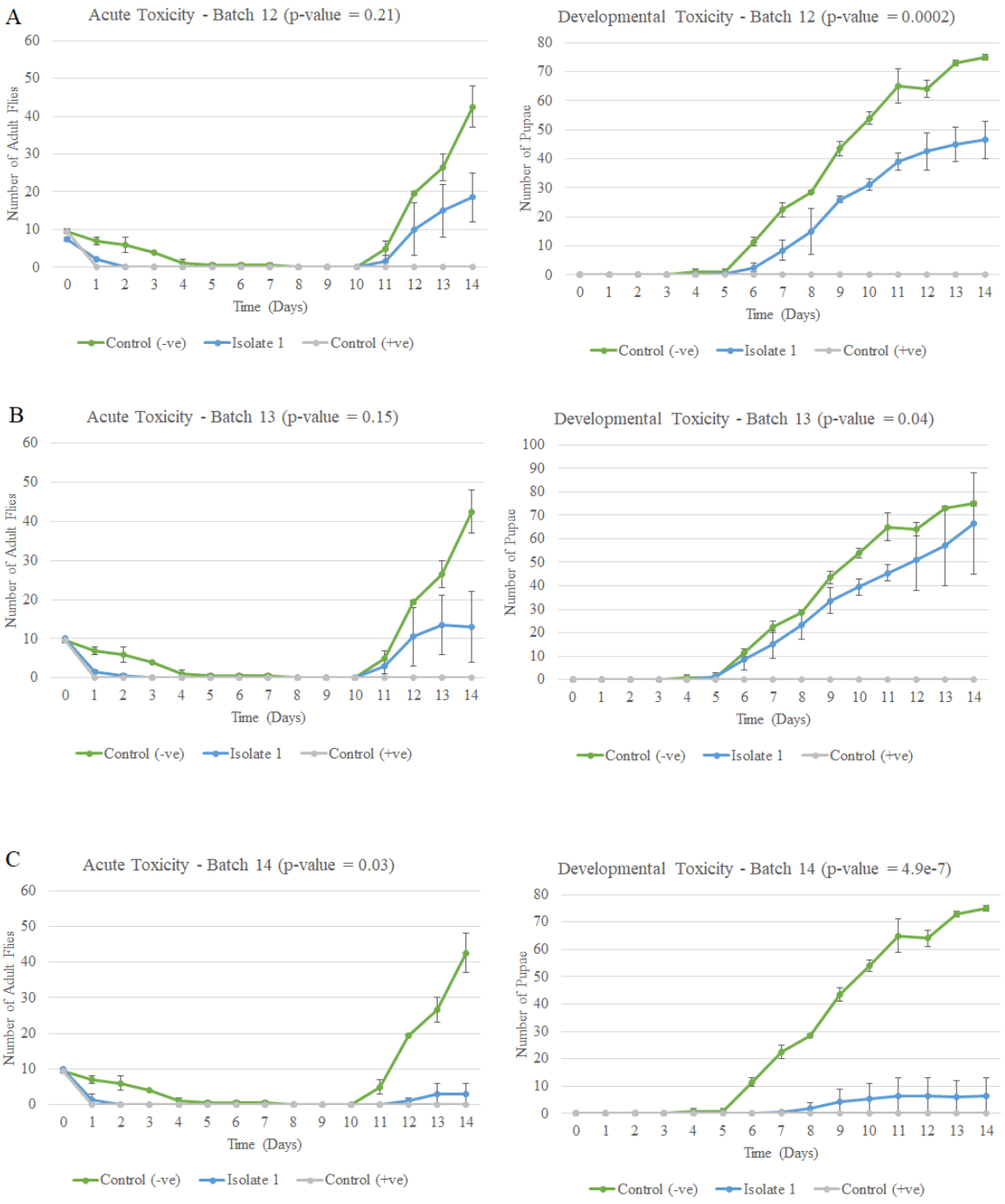


Figure 4. Acute and developmental toxicity in fruit flies observed daily when exposed to reconstituted broths of Isolate 1 from (A) batch 12, (B) batch 13, and (C) batch 14 (blue line). Positive control used was Tea Tree Oil (grey line) while PDB+Tween served as the negative carrier control (green line). T-test performed on data from days 11-14; (A) p-values = 0.21 and 0.0002, (B) 0.15 and 0.04, and (C) 0.03 and $4.9e^{-7}$.

Figure 4 illustrates the insect inhibitory effects when exposed to 2× concentration of reconstituted broth of Isolate 1 from batch 12, 13, and 14. The acute toxicity graphs present in Figure 4A, B, and C indicate a shorter life-span of parental adults in experimental treatments as compared to the carrier control, similar to when 200 µl of TTO is used. Further, the F1 generation of flies exposed to carrier control increased rapidly while the F1 generation of TTO was absent and a small number of adults were observed in the experimental treatments. A shorter life-span may suggest that Isolate 1 exhibits acute toxicity in the parental generation before they have a chance to reproduce. This may explain the low numbers of F1 generation when Tea Tree Oil and Isolate 1 extract are used. The developmental toxicity graphs in Figure 4A, B, and C show the presence of Isolate 1 extract results in fewer F1 generation pupae and perhaps in the case of Figure 4A and 4C, a slight (1-2 day) delay in the formation of pupae in comparison to the negative (carrier) control treatment.

The differences in the number of F1 generation and pupae between the three batches is also observed to determine if the pupae are able to successfully develop into adults. Further, a variation in the intensity of the toxic effect is also observed between different batches. This may be due to a similar phenomenon observed in the fungal bioassay. The concentration of metabolites between the three batches may differ thus resulting in the variation. In this case, batch 14 appears to have a higher concentration of the inhibitory metabolite(s) than batch 12 and 13. This may also give insights on the level of potency of the metabolite(s) present in Isolate 1.

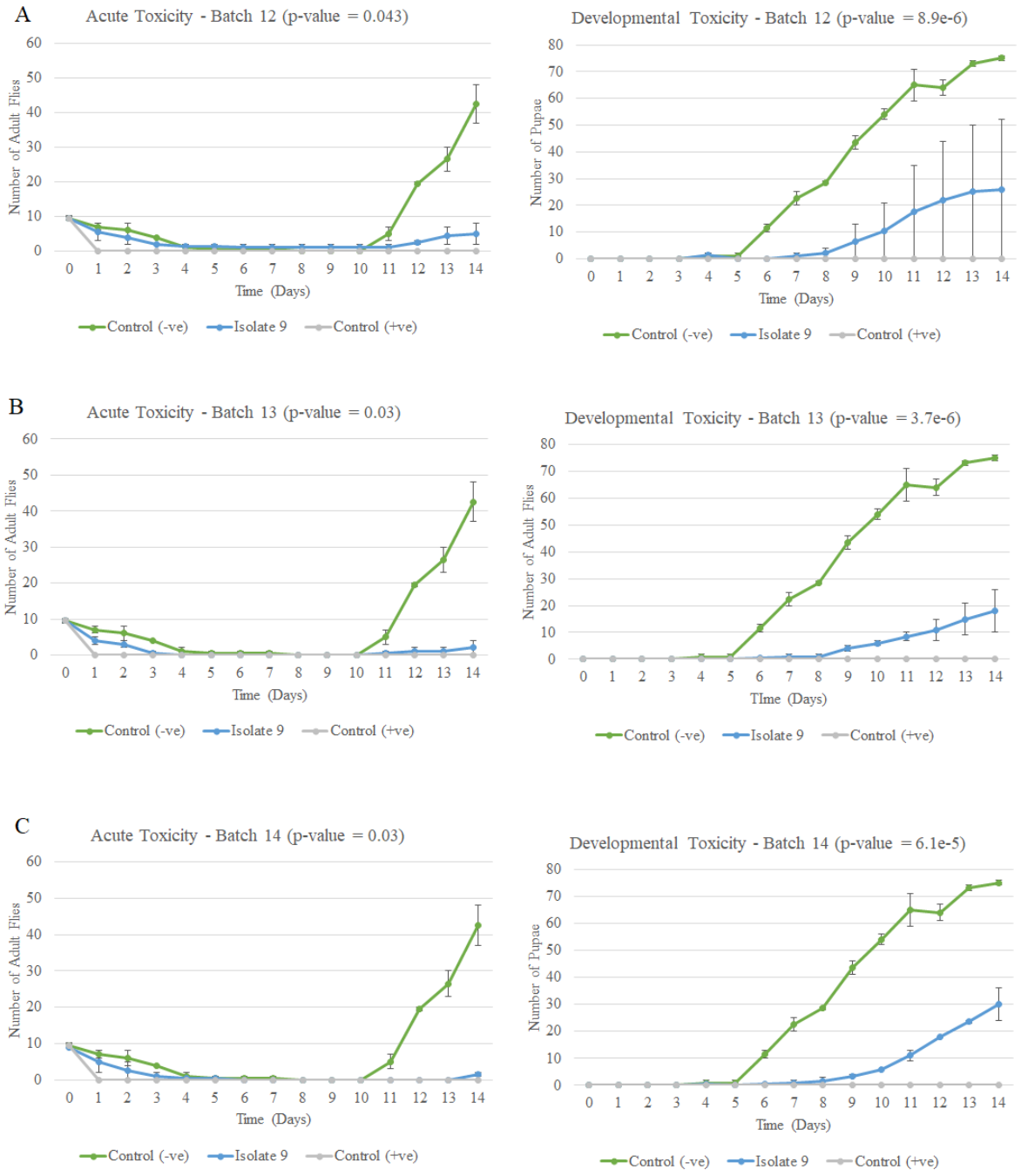


Figure 5. Acute and developmental toxicity in fruit flies observed daily when exposed to reconstituted broths of Isolate 9 from (A) batch 12, (B) batch 13, and batch 14 (blue line). Tea Tree Oil was used as the positive control (grey line) while PDB+Tween served as the carrier control (green line). ANOVA test performed on data from days 11-14; (A) p-values = 0.043 and $8.9e^{-6}$, (B) 0.03 and $3.7e^{-6}$, and (C) 0.03 and $6.1e^{-5}$.

Figure 5 shows insect inhibitory effects when exposed to 2× concentration of reconstituted broths of Isolate 9 from batch 12, 13, and 14. The acute toxicity graphs present in Figure 4A, B, and C appear to show a shorter life-span of parental adults exposed to Isolate 9 similar to when Tea Tree Oil is used. Further, while the F1 generation of flies exposed to carrier control increased rapidly, the F1 generation exposed to TTO was absent and a small number of adults were observed in the experimental treatments. The shorter life-span may suggest acute toxicity affect the parental generation before they have had a chance to reproduce thus leading to a smaller F1 generation. The developmental toxicity graphs present in Figure 4A, B, and C suggests a possible delay in the formation of pupae by 2-4 days in comparison to the negative (carrier) control. Further, based on the number of pupae and F1 generation, a pathway(s) responsible for causing the development of pupae to adult may be affected.

The subsequent results assess the anti-insect inhibitory effects of the commercially available natural products with tap water as the carrier control. This assay did not include a 24-hr recovery period and was carried out in ambient light and room temperature.

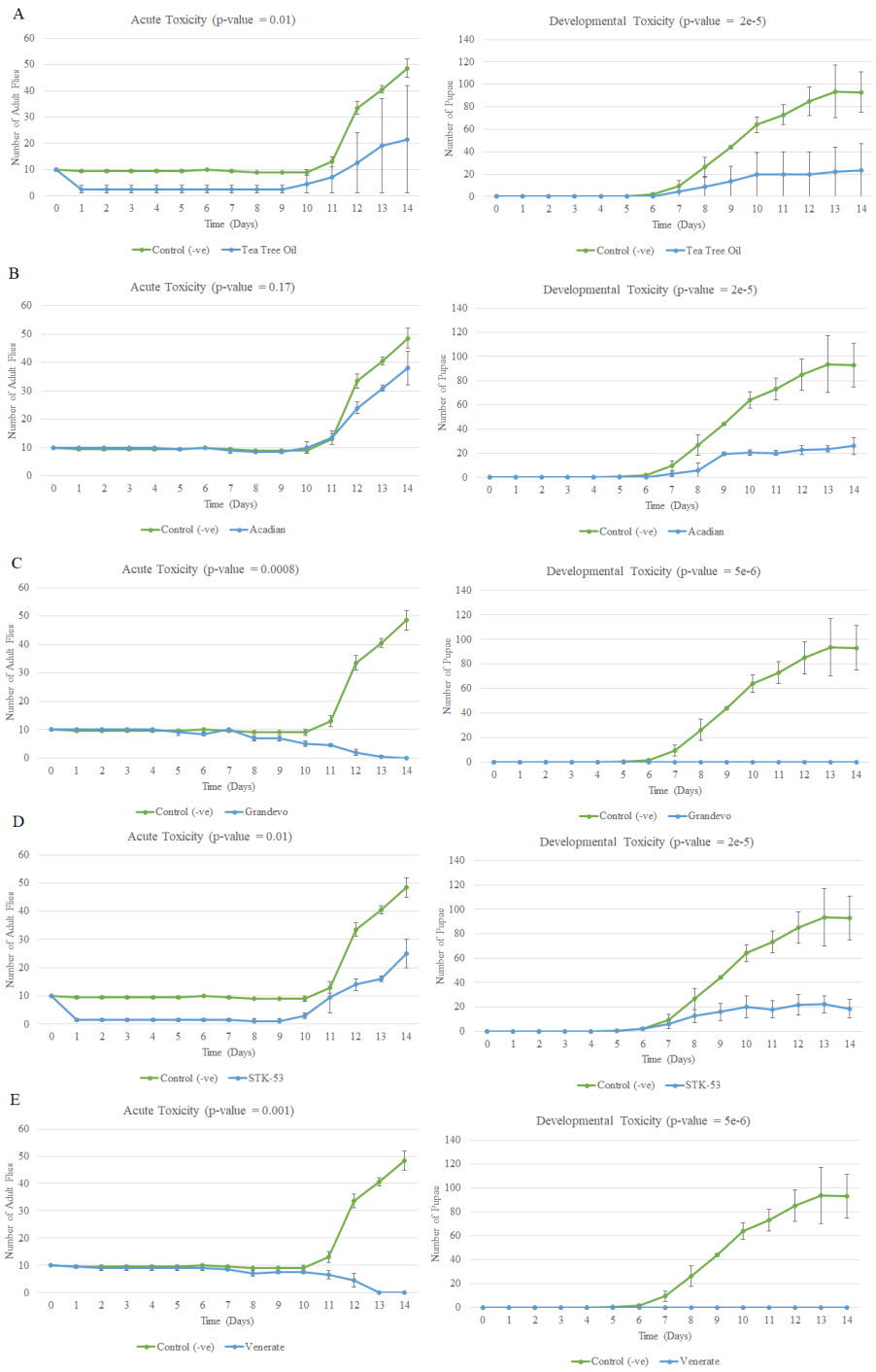


Figure 6. Acute and developmental toxicity in fruit flies observed daily when exposed to (A) Tea Tree Oil, (B) Acadian, (C) Grandevo, (D) STK-53 and (E) Venerate (blue line). Tap water was used as the carrier control (green line). ANOVA test was performed on data from days 11-14; p-values (A) 0.01 and $2e^{-5}$, (B) 0.17 and $5e^{-6}$, (C) 0.0008 and $5e^{-6}$, (D) 0.01 and $2e^{-5}$, and (E) 0.001 and $5e^{-6}$.

Figure 6 shows a short life-span of parental adults exposed to TTO and STK-53 as compared to the carrier control. This may suggest acute toxicity is caused by these two natural products causing death of the parental generation before they have had a chance to reproduce. The F1 population of these two products shows a slow increase however it is significantly less than the steep increase in F1 generation exposed to the carrier control. Figure B shows a peculiar phenomenon where the number of adults increases similar to the increase observed in the carrier control however pupae were not observed throughout the bioassay duration. Since the protocol required counting the shell remnants of instars to indirectly observe the number of pupae, the pupae were not observed since they were deposited on top of the food instead of on the side of the vial walls. Thus, while Acadian does not exhibit any toxic effects on insects it may be affecting pupae behaviour resulting in slight changes during development. Figures 6C and E show a similar pattern of a slow decline of parental generation and a lack of pupae and thus F1 generation. This may indicate an inhibitory effect that prevents reproduction in the parental generation.

### 3.4 Mollusc Bioassay

Reconstituted broths of Isolates 1-10 from batches 3 and 6 were tested for anti-mollusc activity. Batch 3 test was performed by exposing the snails to 30% mass of dried reconstituted broths while the subsequent bioassays were performed by exposing a select volume of reconstituted broths. These preliminary tests were used to identify isolates that showed both anti-fungal and anti-mollusc activity. Thus, Isolates 6 and 9 were selected for further investigation. Increasing volumes of reconstituted broths were used to gain insights on the potency of each Isolate. Therefore, bioassay for batch 6 used 15 ml of reconstituted broths, while batch 8, 12, and 14 used 30, 60, and 90 ml, respectively. Since saponins have an inhibitory effect on molluscs, Tween was not mixed with the reconstituted broths, thus the carrier control was simple PDB while 6 ml of TTO was used as the negative control. The results presented below are from the final protocol used and therefore a sample of the full dataset that is present in Appendix VIII.

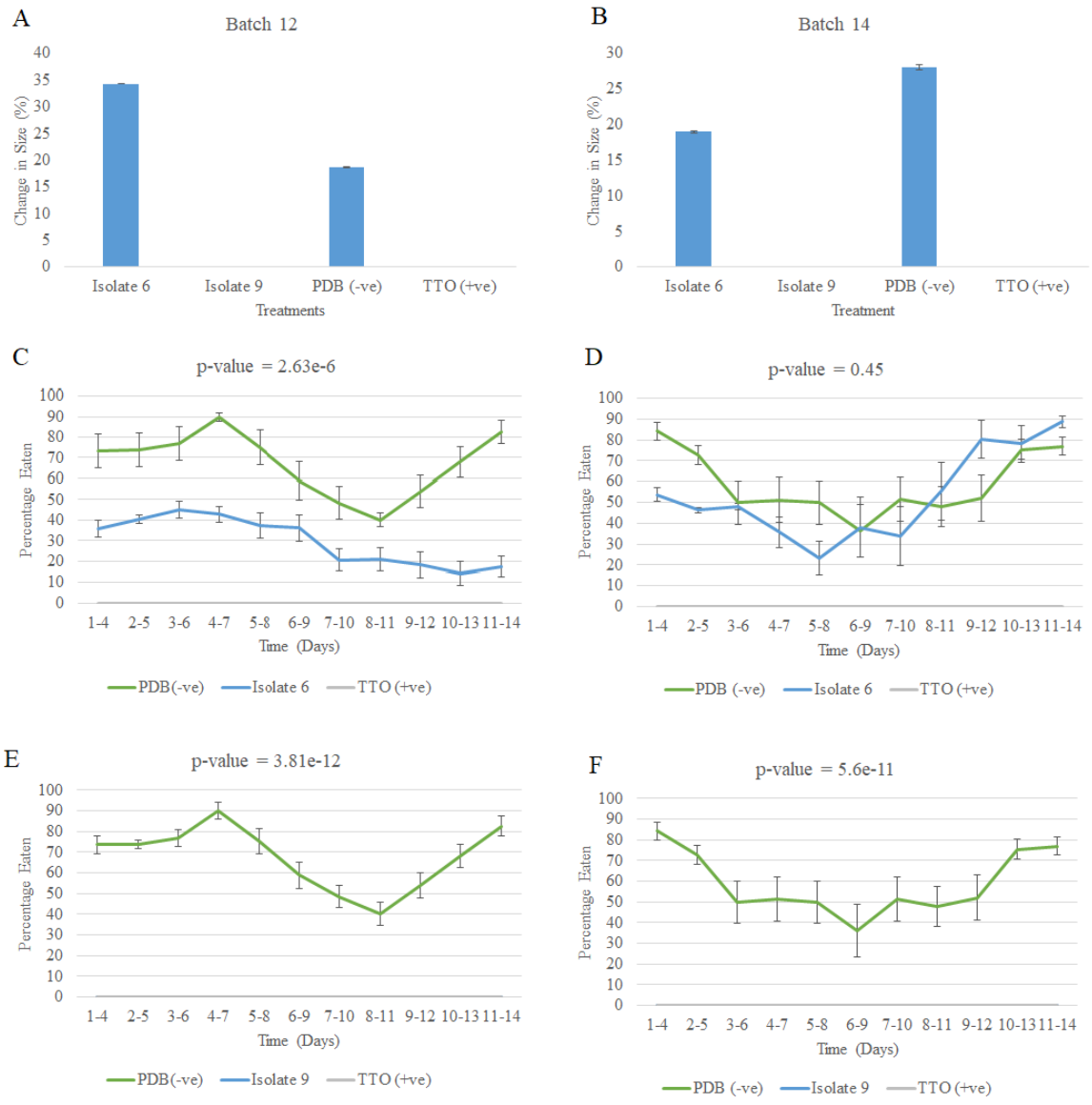


Figure 7. Average growth rate and feeding behaviour of snails exposed to 60- and 90 ml of reconstituted broths from Isolates 6 and 9. Growth rate was expressed as a percentage change in size after 14 days by snails exposed to (A) 60 ml and (B) 90 ml of Isolate 6 and 9 broths along with carrier control (PDB only) and 6 ml of TTO. The feeding pattern was expressed as a percentage amount eaten of a 4-day frameshift average. Percentage eaten by snails exposed to (C) 60 ml reconstituted broth of Isolate 6 ($p = 2.63e^{-6}$) , (D) 90 ml reconstituted broth of Isolate 6 ($p = 0.45$), (E) 60 ml reconstituted broth of Isolate 9 ($p = 3.81e^{-12}$ , and (F) 90 ml reconstituted broth of Isolate 9 ($p = 5.6e^{-11}$) . The p-values calculated using a t-test of the entire data matrix.

Figure 7 illustrates the average growth rates of 2 snails for each treatment with reconstituted broths of Isolate 6 and Isolate 9 in comparison to the negative (carrier) control and Tea Tree Oil as a positive control. Figure 7A shows a greater change in size of snails exposed to Isolate 6 than the carrier control while Figure 7B shows a greater change in size of snails in the carrier control. This may be caused by the batch-effect we have been observing throughout the bioassays since a few of the preliminary bioassays indicated a suppression of growth and/or appetite. The growth rate of snails exposed to reconstituted broth of Isolate 9 was more similar to that of Tea Tree Oil than the carrier control in both Figure 7A and B. Snails exposed to Isolate 9 remained inside their shells during the exposure time and a mucus layer formed around the shells a few hours after transferring them to the 'observation' tank. The snails exposed to TTO behaved in a similar manner however the mucus layers formed a 2-3 days after transfer into the 'observation' tank.

The feeding behaviour observed in Figure 7C and D of snails exposed to broths of Isolate 6 suggest some suppression in of appetite in the Figure 7C possibly due to the presence of metabolite(s) that slightly reduce the appetite of snails but do not cause any short-term detrimental effects. Further, due to the snails highly selective appetites, this phenomenon may still be explained by batch variation coupled with the snails' refusal to eat the food provided. Figure 7D shows an insignificant difference between the feeding behaviour of experimental treatment and carrier control but a smaller increase in growth suggesting the presence of inhibitory metabolite(s). Figures 7E and F give insights to the high level of potency of the metabolite(s) present in Isolate 9.

The following results assess potential anti-mollusc effects of commercially available natural products on snails following the same protocol used to assess the natural products previously discussed. The bioassay was carried out in ambient light and room temperature.

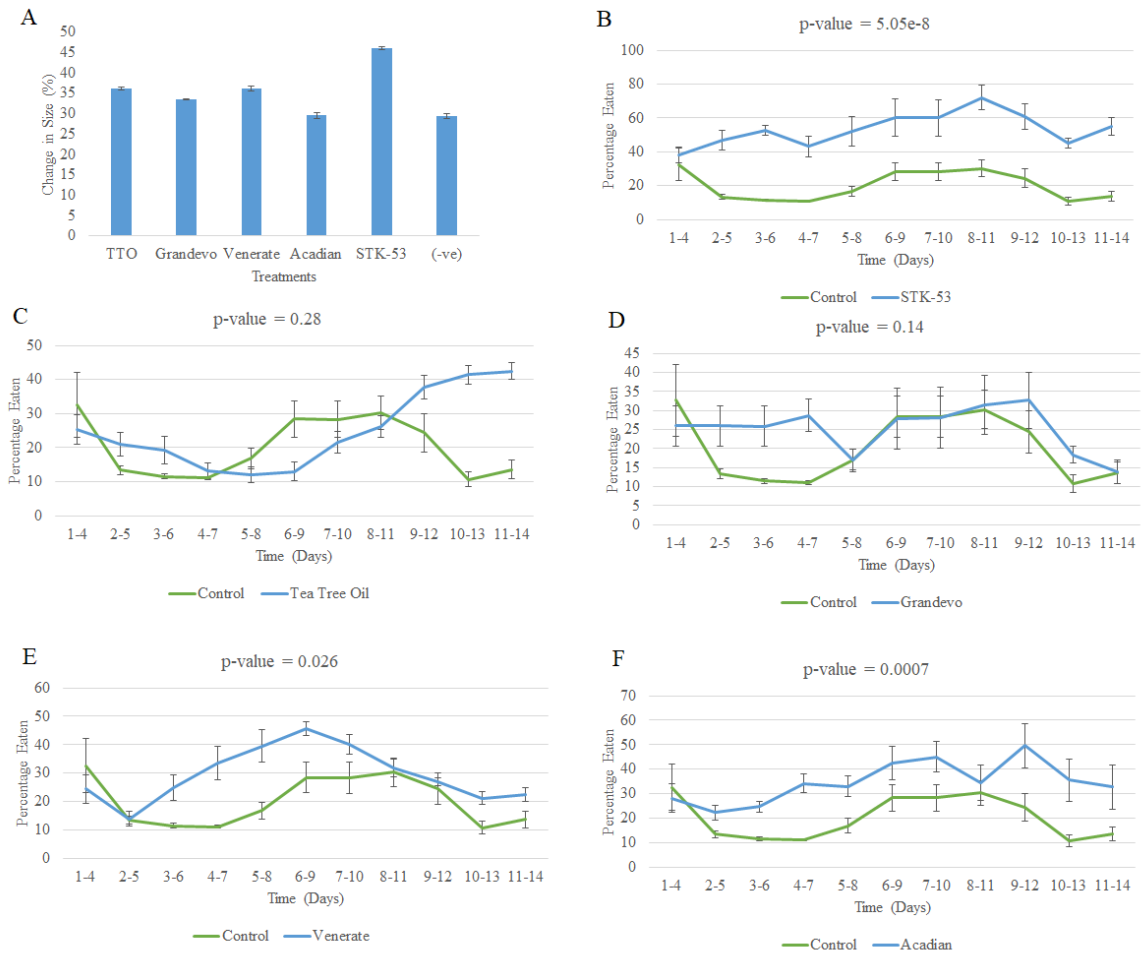


Figure 8. Average growth rate and feeding behaviour of snails exposed to commercially available natural products. (A) Growth rate was expressed as a percentage change in size after 14 days by snails exposed to 1 ml of TTO, Venerate, Acadian, and STK-53 and 1 g of Grandevo. The feeding behaviour was expressed as a percentage amount eaten by snails exposed to (B) STK-53 ($p = 5.05e^{-8}$), (C) Tea Tree Oil ($p = 0.28$), (D) Grandevo ($p = 0.14$), (E) Venerate ($p = 0.026$), and (F) Acadian ($p = 0.0007$). The p-values calculated using t-test confirmation of entire data matrix.

Figure 8 illustrates a greater change in size of snails exposed to experimental treatments compared to the carrier control. Further, the feeding behaviours of Figure 8B, E, and F suggest an increase in food consumption by snails exposed to the experimental treatment as compared to the carrier control while Figures 8C and D suggest no significant difference between the two comparisons. Based on observations during exposure and transfer, snails exposed to TTO

retrieved inside their shells 5-10 minutes after TTO was added to the 'experimental' tank while snails exposed to STK-53, Acadian, Grandevo, and Venerate retrieved inside their shells within the first hour of exposure. Once transferred, snails exposed to Acadian, Grandevo, and Venerate came out of their shells within the first 5-10 minutes while snails exposed to STK-53 and TTO remained inside for 1-2 hours. This information coupled with the use of TTO as a positive control for the bioassays involving fungal isolates may give insights on the level of sensitivity of the bioassay.

### 3.5 Bioassay Correlations

To determine whether fungal bioassays can be used to predict anti-insect and anti-mollusc activity, graphs were constructed to observe the correlation between the MIC values, number of F1 adults in insect bioassays, and change in snail size in snail bioassays. Further, the correlation graph between insect and snail bioassay was also constructed to observe any associations between the two bioassays.

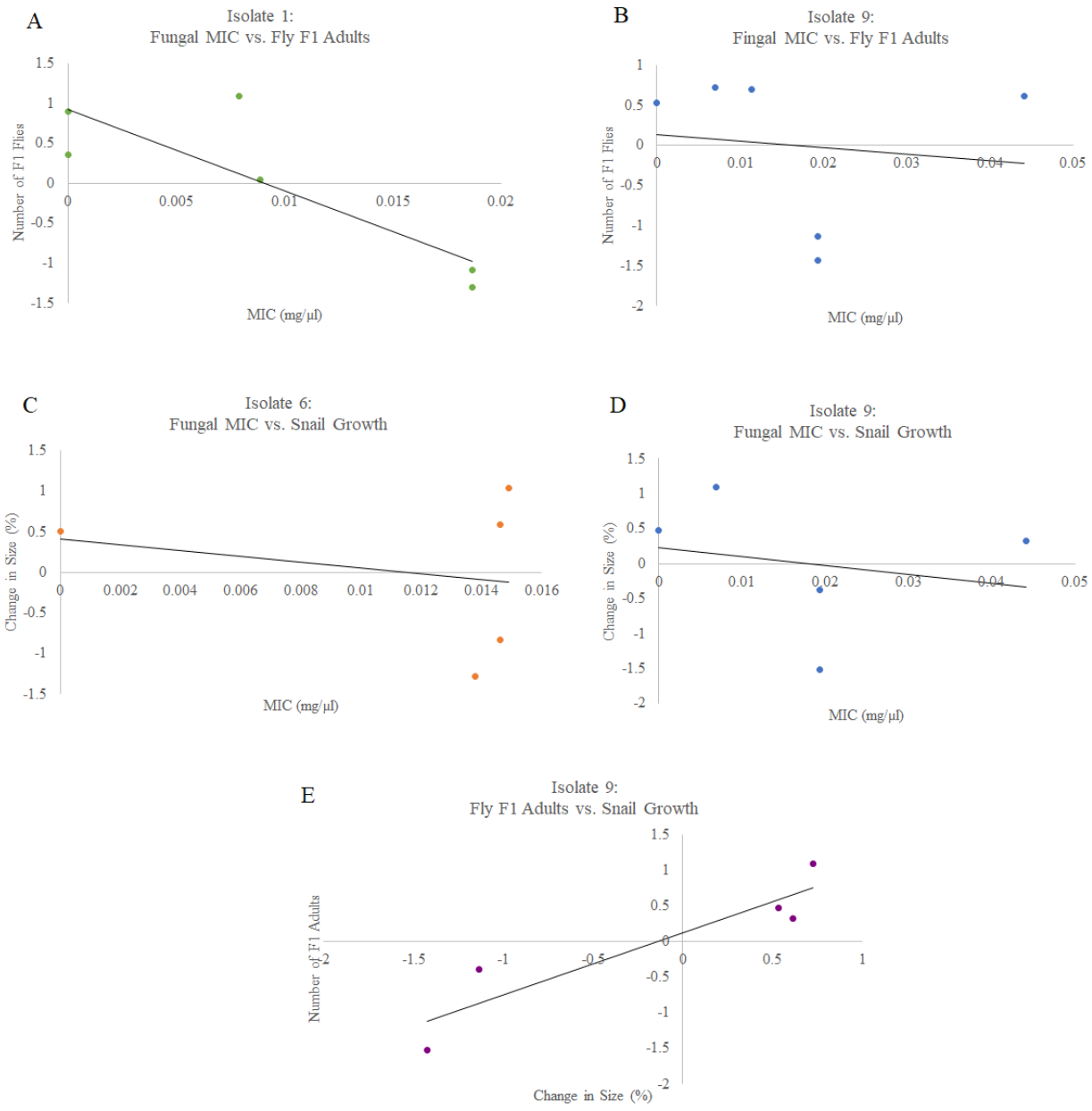


Figure 9. Correlation between (A) fungal MIC and insect F1 generation of Isolate 1, (B) fungal MIC and insect F1 generation of Isolate 9, (C) fungal MIC and snail growth rate of Isolate 6, (D) fungal MIC and snail growth rate of Isolate 9, and ( E) insect F1 generation and snail growth rate of Isolate 9.

Figure 9A and 9B show a negative correlation between fungal and insect bioassays thus suggesting the fungal bioassay cannot be used to predict insect inhibitory effects when testing reconstituted broths of Isolates 1 and 9. The difference in intensity of both negative correlations

may suggest the predictability is dependent on the presence of target-specific or general toxins. Figure 9C and 9D also show a negative correlation between fungal and mollusc bioassay when testing reconstituted broths of Isolates 6 and 9. Further, Figure 9E shows a strong positive correlation between the insect and mollusc bioassay suggesting either bioassay can be used to predict inhibitory activity in the other. Although a positive correlation is observed, it may not be indicative of any relations between the two bioassays and simple the presence of a general toxin.

### 3.6 Metabolomic Analysis

Based on the MIC of refined fractions, the fractions showing activity were compared to their inactive counterparts using multivariate analyses. The active vs. inactive groups created for such an analysis are shown in table 2 where the first number refers to the batch number while the second refers to the isolate number followed by the type of solvent used to create the fraction. Fractions exhibiting inhibitory activity were compared to inactive fractions from the same isolate and fraction but different batches.

PCA and OPLS-DA were performed on these groups to determine the metabolites that are responsible for the differences between the two groups. PC1 and PC2 value were used to highlight potentially significant variable that represented the same peak. The abundance of metabolite at these specific points were determined by extracting peak area data from the normalized data matrix generated via MZmine. A heatmap was created using peak area to determine the abundance of the metabolites in active and inactive samples.

Table 2 shows the groups created based on the results from the MIC bioassay performed using the refined broths. These groups were created based on the activity variations between different batches in an attempt to cause sample separation based on inhibitory activity or the lack of, in the PCA and OPLS-DA analyses.

Table 2. Active vs. inactive fractions used in a comparative metabolomic analysis of batches fermented for a 6-week period.

| **Active Fractions** | **Inactive Fractions** |
|---|---|
| 11.9.EA<br>12.9.EA<br>13.9.EA | 14.9.EA |
| 11.1.Bu<br>12.1.Bu<br>13.1.Bu<br>14.1.Bu | 10.1.Bu |
| 10.6.EA<br>12.6.EA | 11.6.EA<br>14.6.EA |
| 13.9.Bu | 11.9.Bu<br>12.9.Bu<br>14.9.Bu |
| 13.9.$H_2O$ | 11.9.$H_2O$<br>12.9.$H_2O$<br>14.9.$H_2O$ |

### 3.6.1 Isolate 9: Ethyl Acetate

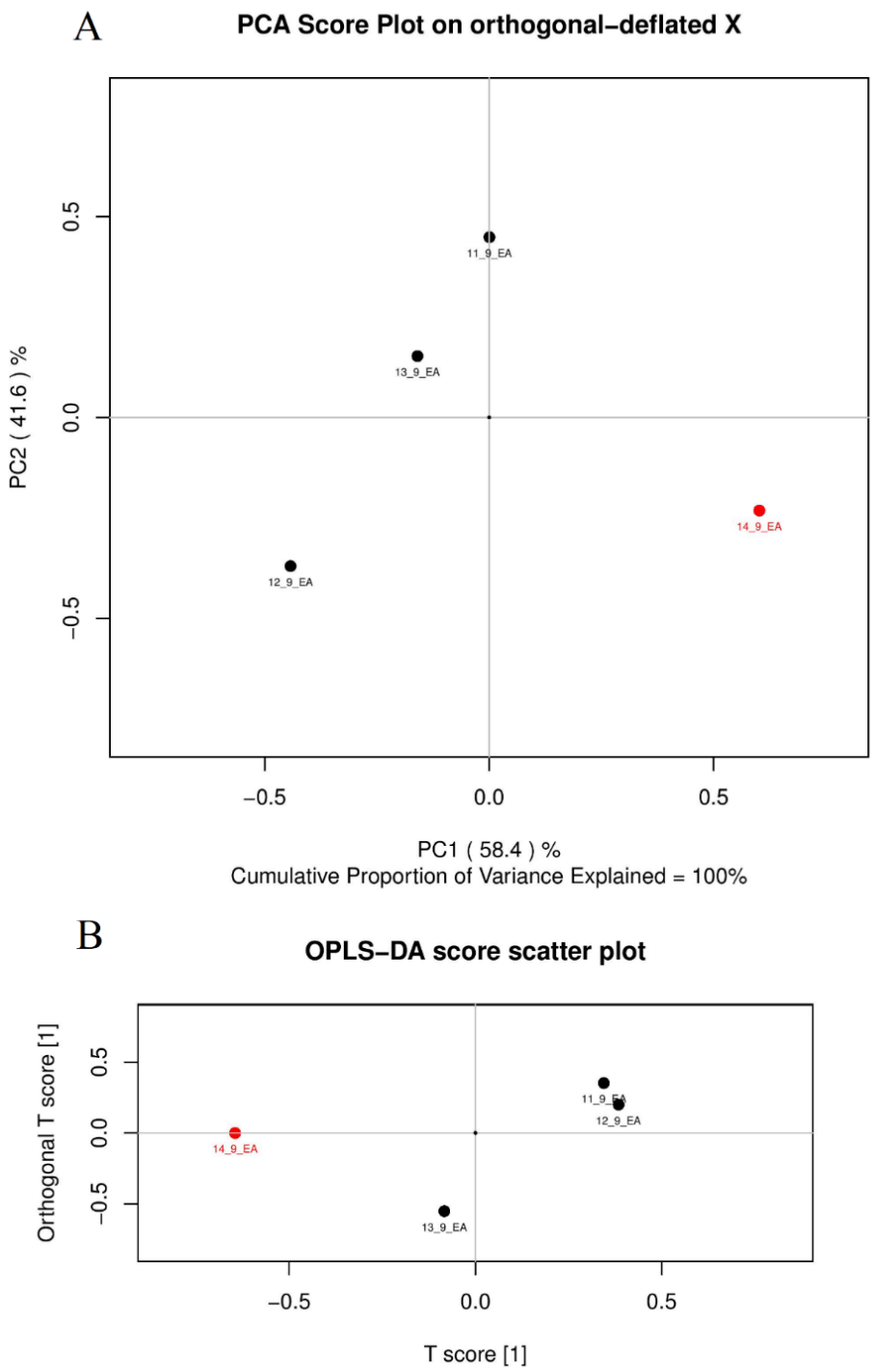


Figure 10. (A) PCA analysis of isolate 9 active ethyl acetate fractions (black) vs. inactive fraction (red). PC1 explains 58.4% variance while PC2 explains 41.6% with a cumulative variance of 100% explained. (B) OPLS-DA analysis of active vs inactive samples.

Figure 10 image A highlights the difference between active and inactive samples based on PC1 where metabolites responsible for activity may be pulling the active samples in the negative direction on the PC1 axis. Image B highlights the presence of within group variation of active samples. This may be due to differences between individual batches.

| | RT_m/z | 14_9_EA | **11_9_EA** | **12_9_EA** | **13_9_EA** |
|---|---|---|---|---|---|
| 1 | RT4.33_596.30803 | | | | |
| 2 | RT4.33_579.28153 | | | | |
| 3 | RT4.32_601.26332 | | | | |
| 4 | RT4.33_561.27093 | | | | |
| 5 | RT3.54_299.1495 | | | | |
| 6 | RT3.54_281.13915 | | | | |
| 7 | RT3.54_619.27438 | | | | |
| 8 | RT3.56_321.13226 | | | | |
| 9 | RT3.57_221.08146 | | | | |
| 10 | RT4.87_597.24828 | | | | |

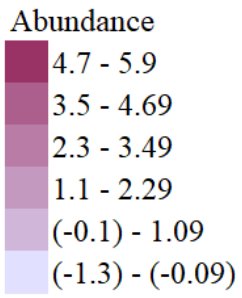


Figure 11. Heatmap of potential significant variables based on negative PC1 values.

Figure 11 illustrates a heatmap generated using significant RT_m/z with negative PC1 values. Active samples 12_9_EA and 13_9_EA (ethyl acetate fractions of Isolate 9 from batch 12 and 13, respectively) appear to have metabolites at relatively higher abundance than sample 11_9_EA and 14_9_EA, the latter being the only inactive sample in this group. However, RT4.32_601.26332 appears to represent a metabolite which occurs in higher abundance in the active samples than the inactive sample.

### 3.6.2 Isolate 9: Butanol

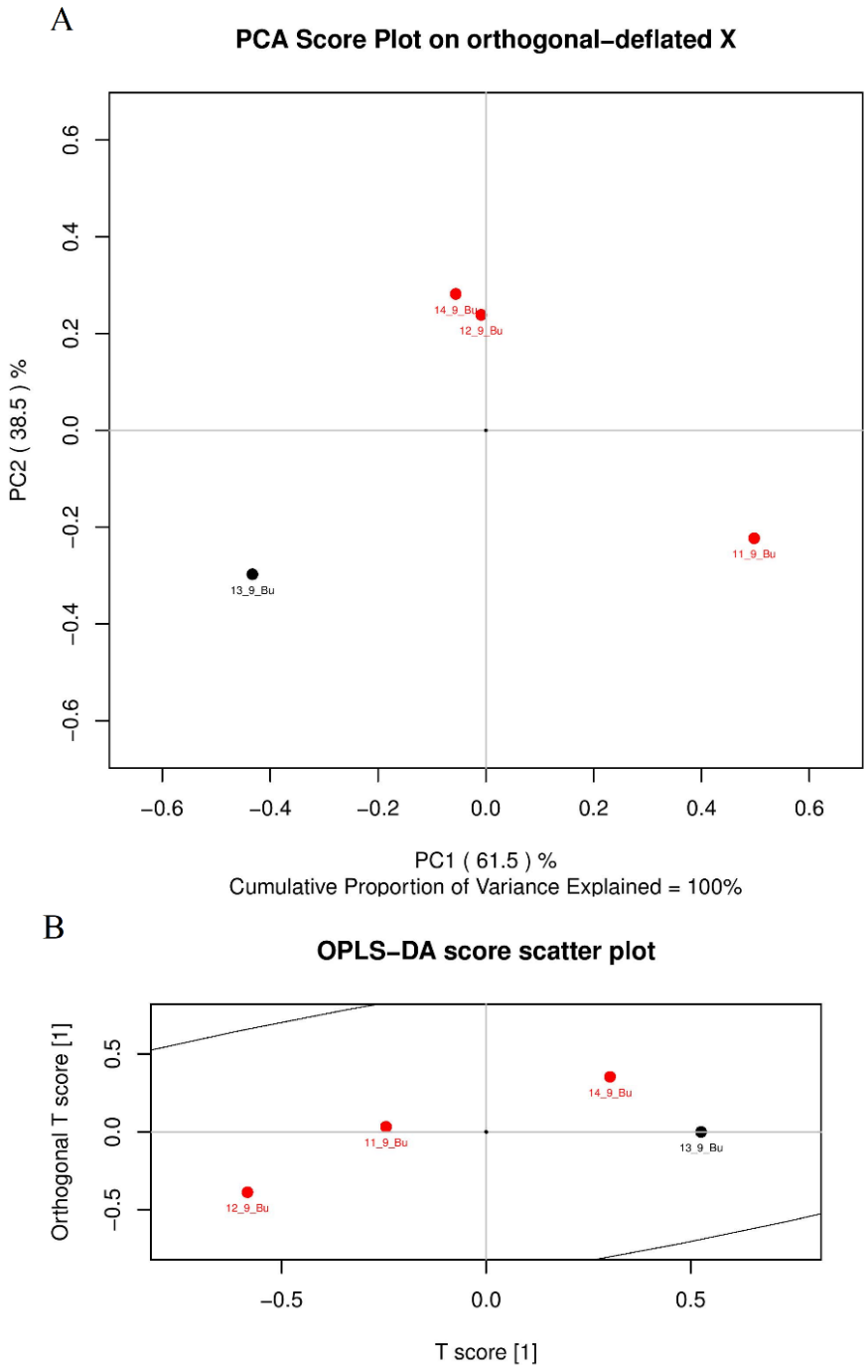


Figure 12. (A) PCA score plot (orthogonal-deflated X) of isolate 9 active butanol fraction (black) vs. inactive fractions (red). PC1 explains 61.5% variance while PC2 explains 38.5% with a cumulative variance of 100% explained. (B) An OPLS-DA score scatter plot of active vs inactive samples.

Figure 12 doesn't clearly show the separation of samples based on activity however the presence of the active metabolite in the negative quadrant of both PC1 and PC2 may suggest the significant metabolites in the active sample are pulling the sample towards the bottom left side. Thus, variable with negative PC1 and PC2 values were extracted from the loading matrix. Further, the OPLS-DS in image B highlights the within group variation present among the inactive samples. As previously mention, this may be due to a batch effect.

| | PC1 - RT_m/z | 11_9_Bu | 12_9_Bu | 14_9_Bu | **13_9_Bu** |
|---|---|---|---|---|---|
| 1 | RT4.01_263.12839 | | | | |
| 2 | RT4.03_281.13911 | | | | |
| 3 | RT4.7_543.25992 | | | | |
| 4 | RT4.03_245.11786 | | | | |
| 5 | RT4.01_303.12147 | | | | |
| 6 | RT4.03_203.07078 | | | | |
| 7 | RT4.33_561.27093 | | | | |
| 8 | RT4.23_255.15984 | | | | |
| 9 | RT4.74_876.44053 | | | | |
| 10 | RT4.87_597.24828 | | | | |
| 11 | RT4.7_561.2703 | | | | |
| 12 | RT4.74_263.12852 | | | | |

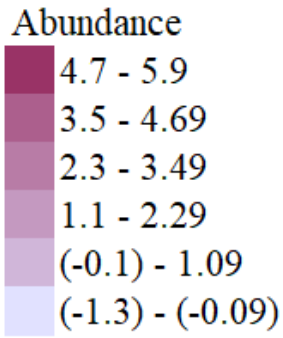


Figure 13. Heatmap of potential significant variables based on negative PC1 values.

Figure 13 shows the abundance of each potentially significant metabolite with respect to their samples. With sample 13_9_Bu being the only active sample, metabolites at most RT_m/z values appear to be in higher abundance than its inactive counterparts. Exceptions to this rule are RT4.7_561.2703 and RT4.74_263.12852.

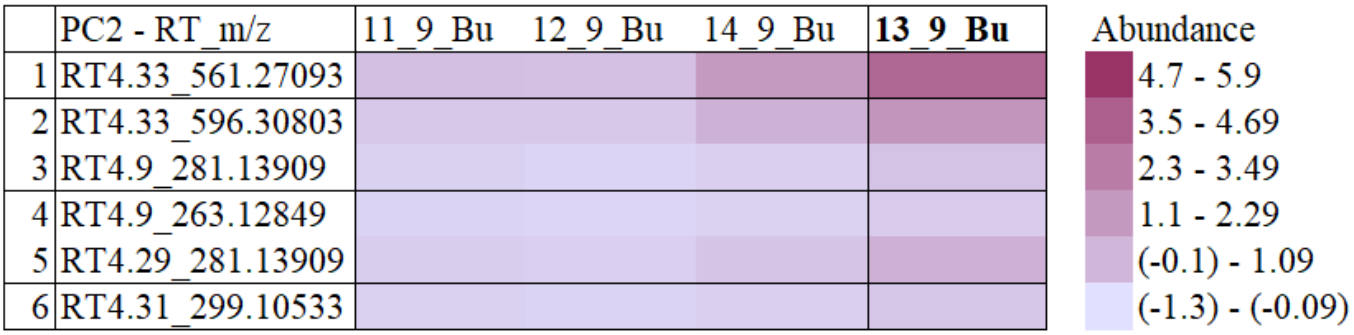


Figure 14. Heatmap of potential significant variables based on negative PC2 values.

Figure 14 shows a similar abundance pattern where metabolites are in high abundance in the active sample comparatively to the inactive samples. The inactive sample, 14_9_Bu appears to have metabolites at higher abundance than the other two inactive samples which is indicative of the large within group variation observed in PCA and OPLS-DA score plots in Figure 12.

### 3.6.3 Isolate 9: Water

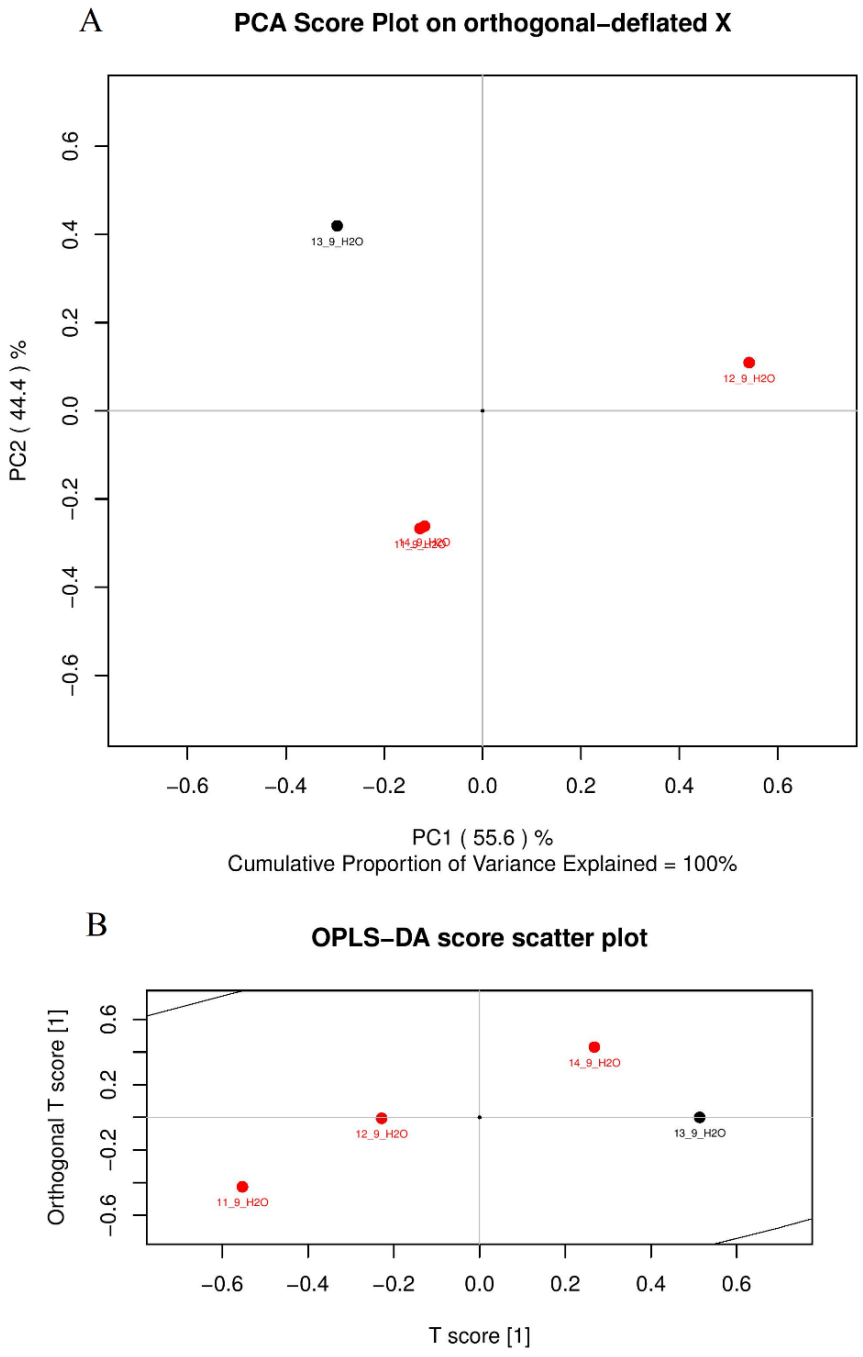


Figure 15. (A) PCA score plot (orthogonal-deflated X) of isolate 9 active water fraction (black) vs. inactive fractions (red). PC1 explains 55.6% variance while PC2 explains 44.4% with a cumulative variance of 100% explained. (B) An OPLS-DA score scatter plot of active vs inactive samples.

Figure 15 doesn't clearly show the separation of samples based on activity however the active sample position on the graph can be indicative of which variables to highlight in the loading data matrix. Significant variables can be found based on negative PC1 values and positive PC2 values. Similar to previous OPLS-DA, there is high within group variance among the inactive samples.

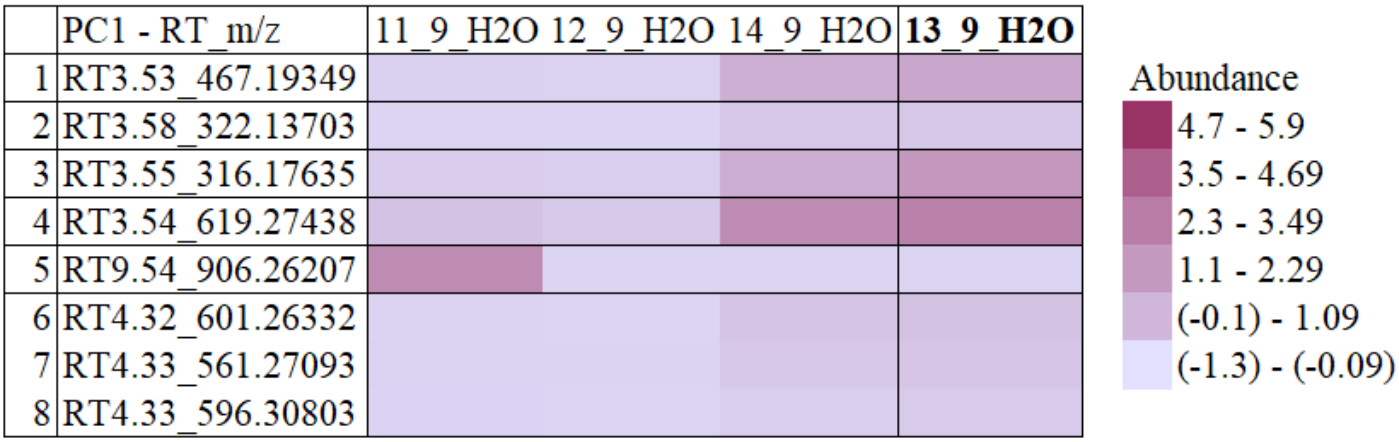


Figure 16. Heatmap of potential significant variables based on negative PC1 values.

Figure 16 shows the heatmap used to observe the abundance of potentially significant metabolites in active and inactive samples. The active sample from batch 13 shows the highest abundance in all variables except RT9.54_906.26207. The relative abundance of each metabolite in samples 14_9_H2O is similar to the active sample than its inactive counterpart thus suggesting the reason for the large within group variance observed in the score plots present in Figure 15.

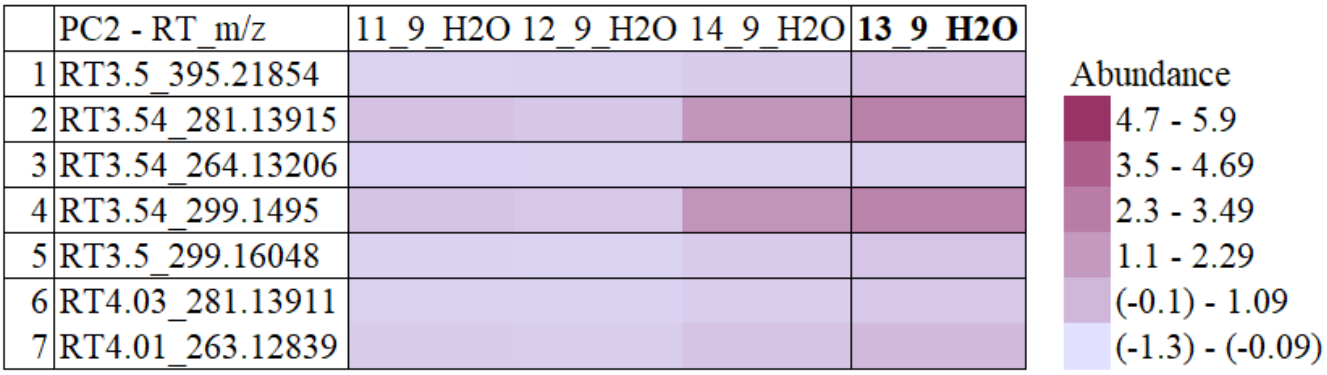


Figure 17. Heatmap of potential significant variables based on positive PC2 values.

Figure 17. shows the heatmap based on positive PC2 values which also indicates the presence of metabolites in high abundance in the active samples compared to the moderate to low amounts present in the inactive samples. A similar trend to the one seen in Figure 16 is observed in Figure 17. Although all metabolites occur in higher abundance in the active samples as compared to the inactive samples, the inactive sample – 14_9_H2O appears to contain metabolites in similar yet lower abundance to the active sample. Both variation within the inactive group and between the active and inactive groups can be due to the batch effect or degradation of some metabolites during chemical extraction.

### 3.6.4 Isolate 1: Butanol

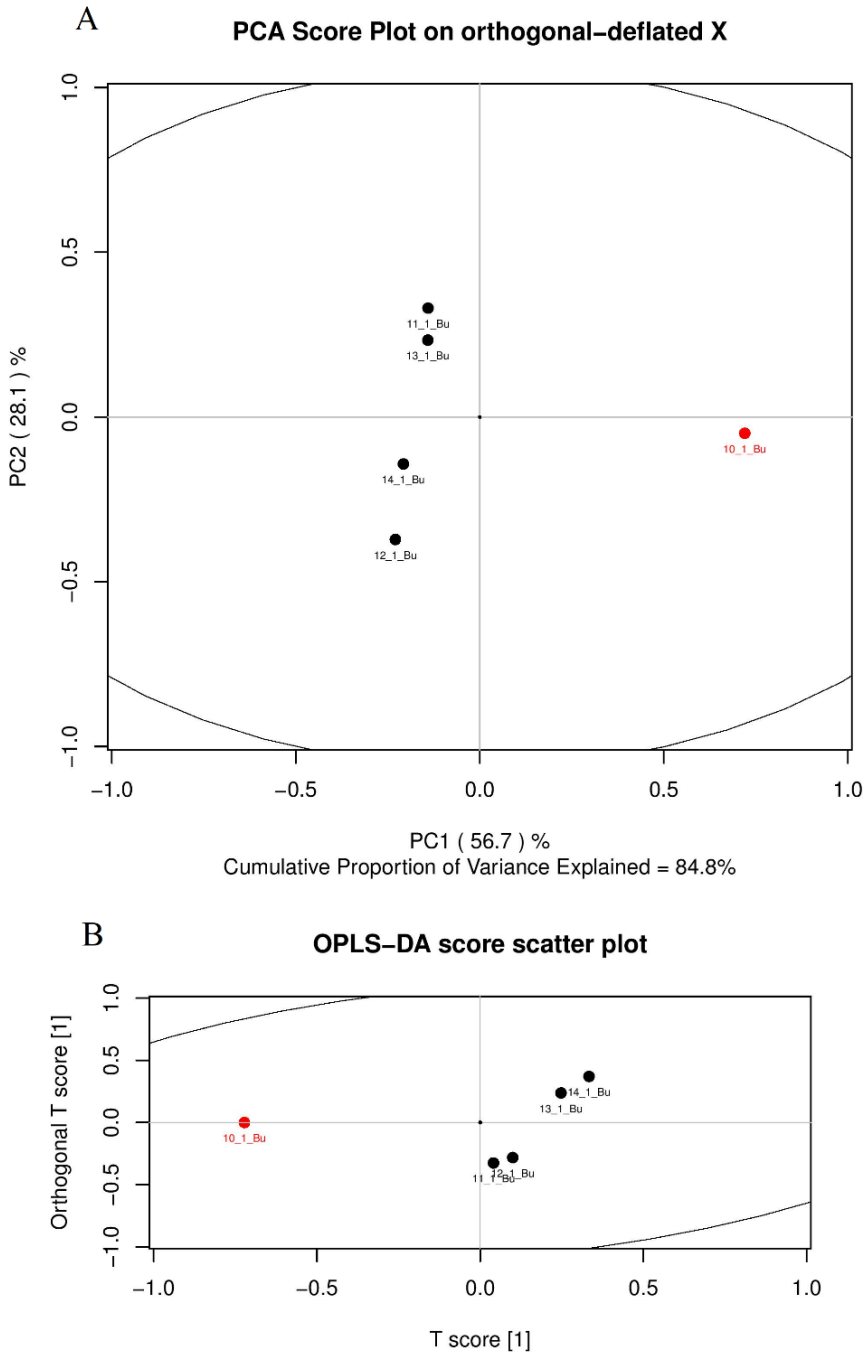


Figure 18. (A) PCA score plot (orthogonal-deflated X) of isolate 1 active butanol fractions (black) vs. inactive fraction (red). PC1 explains 57.6% variance while PC2 explains 28.1% with a cumulative variance of 84.4% explained. (B) An OPLS-DA score scatter plot of active vs inactive samples.

Figure 18 shows a clear separation between active and inactive samples based on PC1 thus variables with negative PC1 values were selected for further analysis. The OPLS-DA graph shown in image B shows clear separation between inactive and active samples but also shows within group variation between samples from batch 11 and 12 and 13 and 14. This variation may be due to time since batches 13 and 14 were fermented after batches 11 and 12.

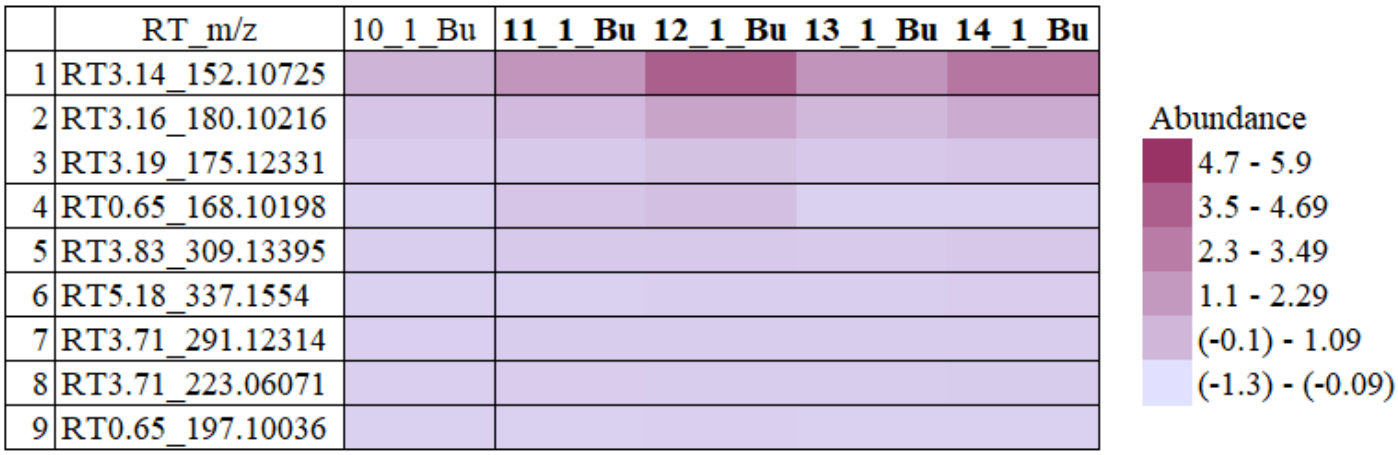


Figure 19. Heatmap of potential significant variables based on negative PC1 values.

Figure 19 shows metabolites occurring in higher abundance in the active samples at RT3.14_152.10725 and RT3.16_180.10216 as compared to the inactive sample. Further, a variation in the abundance between the 4 active samples is also observed for the two RT_m/z values mentioned previously. This variation may be the cause of the within group variation observed in the score plots present in Figure 18 and may be due to batch effect or the degradation of some metabolites during chemical extraction.

### 3.6.5 Isolate 6: Ethyl Acetate

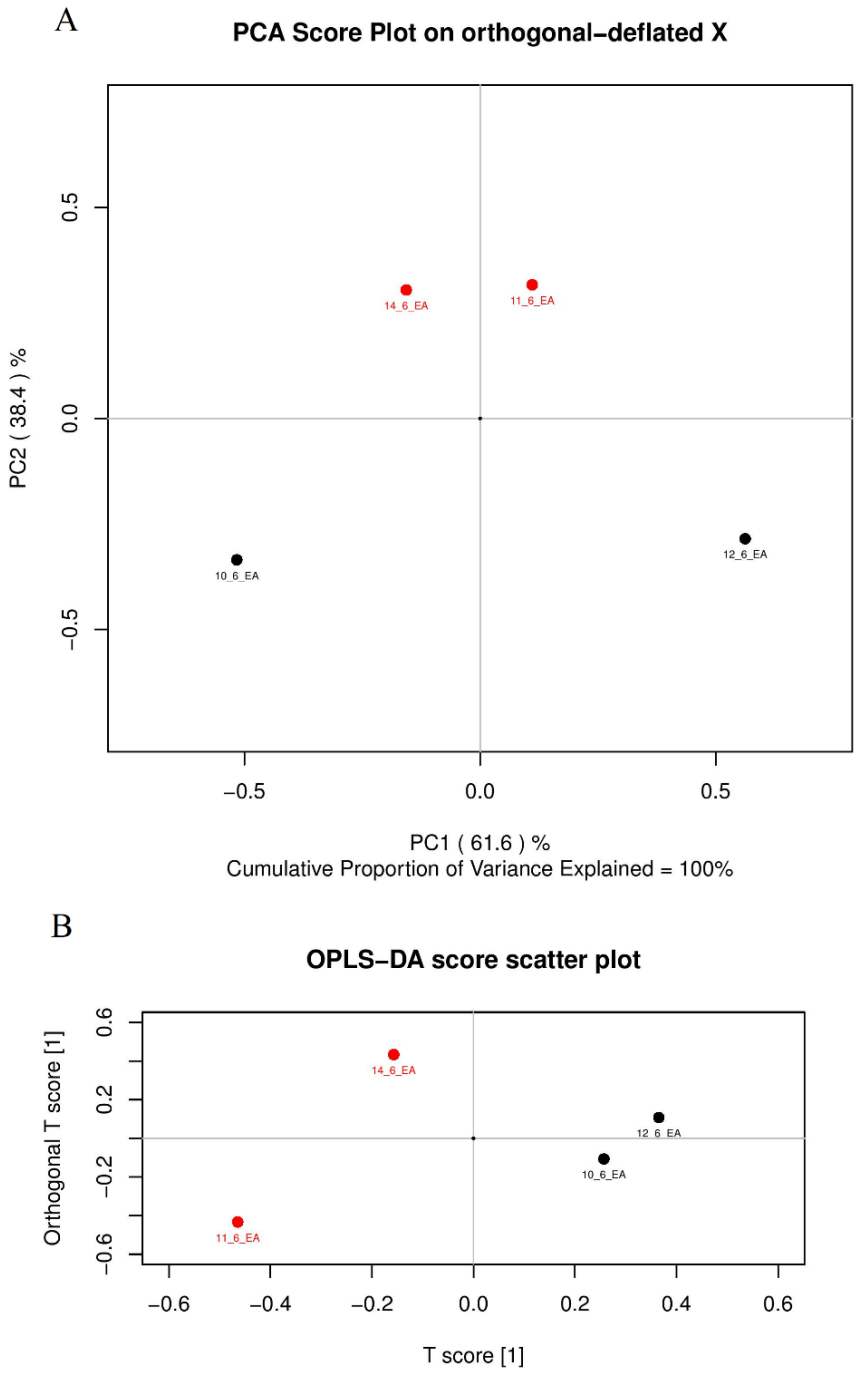


Figure 20. (A) PCA score plot (orthogonal-deflated X) of isolate 6 active ethyl acetate fractions (black) vs. inactive fractions (red). PC1 explains 61.6% variance while PC2 explains 38.4% with a cumulative variance of 100% explained. (B) An OPLS-DA score scatter plot of active vs inactive samples.

Figure 20 shows a clear separation of active and inactive samples based on PC2. The presence of active samples in the negative PC2 region indicates the metabolite(s) responsible may be found by highlighting variables with negative PC2 values. The OPLS-DA graph show in image B shows between group variance among active and inactive samples but also within group variation that may be caused by batch effect of time of fermentation of each batch or a mixture of both effects.

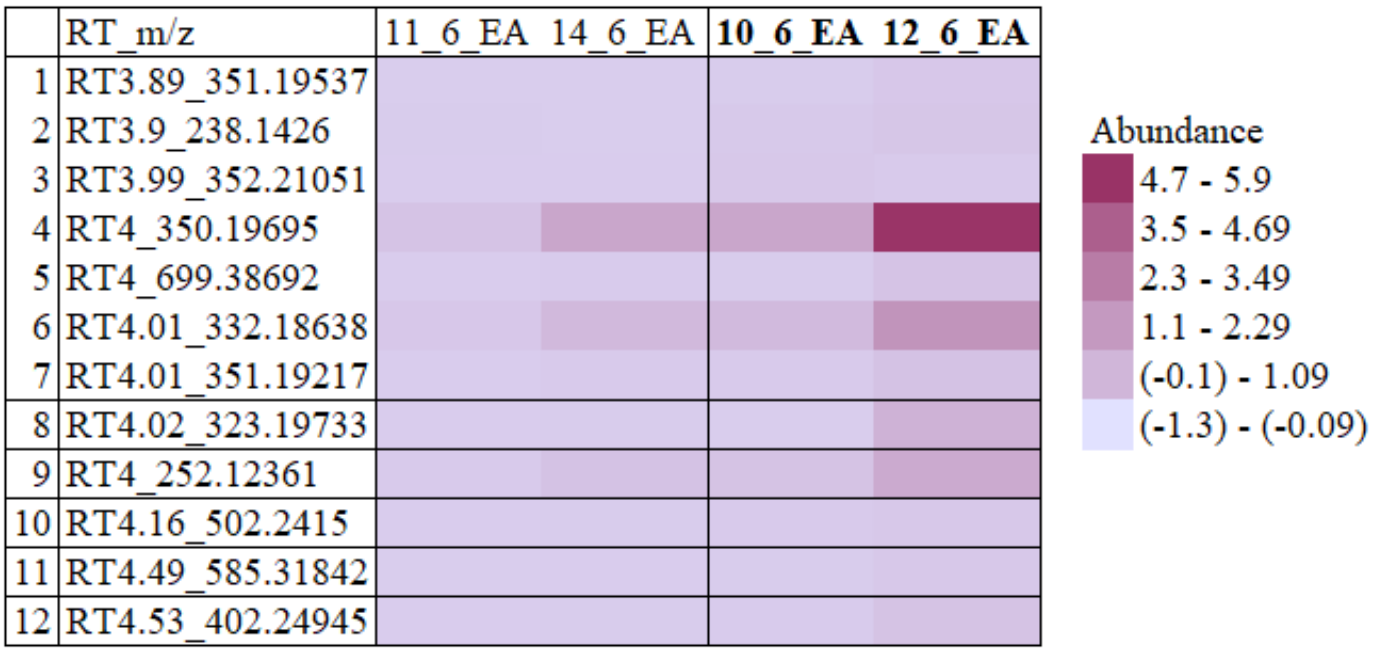


Figure 21. Heatmap of potential significant variables based on negative PC2 values.

Figure 21 shows the abundance of metabolites at potentially significant variables based on negative PC2 values. Overall, the abundance of metabolites observed reflects on both the within group and between groups variation seen in the score plots present in Figure 20. The difference in abundance among the active and inactive samples may be due to batch effect or the breakdown of metabolites during chemical extraction.

## 4. Discussion

The multipronged approach called Integrated Pest/Pathogen Management (IPM) system can be used to mitigate the effects of virulent pests and pathogens on economically important crops. One aspect of this approach involves the use of pesticides to prevent or eliminate such pests and pathogens. Over-reliance on pesticides triggered an evolutionary race between novel chemicals used as pesticides and the ability of pests and pathogens to develop resistance. The use of synthetic pesticides has also resulted in adverse effects on human health and off-target effects on the environment. Thus, pesticides that have novel mode-of-action(s) and are 'eco-friendly' are in high demand. Natural products are metabolites synthesized by organisms as a means of interacting with other organisms in their environment. Natural products can serve as a source of pesticides called biopesticides since they can have novel modes-of-action and are less likely to accumulate in the environment due to a shorter half-life.

The objectives of this thesis were to design and implement bioassays that would serve as effective and cost-efficient methods of identifying metabolites exhibiting toxicity towards fungi, insects, and/or snails. These organisms represent the three major pests or pathogens of the agriculture industry.

Fungi belonging to the *Trichoderma* genus are asexual organisms frequently found in soil in nearly all temperate and tropical regions. Further, ubiquitous strains are ecologically dominant able to grow in a wide array of environments including forests, deserts, marshes, and colder climate including Antarctic and tundra. The capabilities of these fungi to function as biocontrol agents against plant pathogens have been known since 1920s and thus the recent boom in fungicides in modern agriculture is due to the development of *Trichoderma*-based biofungicides. A few known metabolites produced by these fungi are polyketides, tricholin, peptaibols, antibiotics, viridin, glisoprenins, alamethicins, gliovirin, and derivatives of α-amino acids (Waghunde, Shelake, & Sabalpara, 2016; Begum *et al.,* 2018). These metabolites have a wide range of functions including plant growth promotion by enhancing plant immune response against pathogens in addition to biocontrol of pathogenic fungi and insect pests. For examples, Begum *et al.,* (2018) were able to isolate metabolite(s) exhibiting activity against pests and pathogens from reconstituted broth of *Trichoderma* species using ethyl acetate. Additionally, metabolite(s) produced by *Trichoderma harzianum* are currently being used as biopesticides

against soil borne plants pathogens including *Rhizoctonia, Pythium,* and *Fusarium (*Chandler *et al.,* 2011).

The literature on both *Penicillium virgatum* and *Ramularia vizellae* consists primarily on their taxonomic placement. Thus, this research is one of a first of its kind to test for active secondary metabolites of these species. Literature based on species of the same genera can be drawn upon to predict the type of metabolites present. For example, Petit *et al.,* (2009), identified three new novel naphthalenoids in ethyl acetate fraction of reconstituted broths of *Penicillium* sp. showing antimicrobial activity against *Candida albicans, Listeria monocitogenes,* and *Bacillus cereus.* However, the use of such studies to identify the chemical structures of the metabolites highlighted here is beyond the scope of this study.

Fermentation is one of the oldest techniques used to trigger the production of secondary metabolites in fungi in a cost-effective and efficient manner. To obtain previously unidentified metabolites, culture conditions can be manipulated. Manipulation of growth conditions can lead to the discovery of several metabolites from the same organism leading to the formation of the concept "OSMAC" – One Strain MAny Compounds (Scherlach & Hertweck, 2009). Thus, we set forth to elicit novel metabolite profile production in 10 different fungal isolates by fermenting in liquid media at the relatively low temperature of 13°C. The purpose of the fungal MIC served to determine the minimum inhibitory concentration of the isolates showing activity and to monitor consistent activity across different biological replicates of the fermented broths. Isolates 1, 6, and 9 exhibited relatively consistent anti-fungal activity across different biological replicates when compared to Isolates 2, 3, 4, 5, 7, 8, and 10, which, generally exhibited low or no anti-fungal activity.

Although the morphology of the mycelia masses was consistent between biological replicates, the inconsistent activity between biological replicates of Isolates 1, 6, and 9 may be attributed to variations such as the amount of inoculum added to the broths before fermentation (Gibbs, Seviour, & Schmid, 2000). Small inoculum amounts can lead to the production of target metabolite(s) at concentrations below the detection level thus leading to a false negative. Additional variations in the fermentation may lead to batch-to-batch differences including such differences as in conical flasks used between batches, temperature fluctuations, and nutrient concentration between different media batches (Gibbs, Seviour, & Schmid, 2000). Further, the

loss of activity in extracts can be caused by the degradation of the target metabolite(s) during chemical extraction or the separation of metabolite(s) that work synergistically to exert a toxic effect.

Based on my studies, anti-insect activity was observed in extracts of Isolates 1-7 and 9. The presence of metabolites which have a specific toxic effect on insects is evident based on the preliminary results that observed toxicity effects in flies exposed to extracts of Isolates 2-5 and 7. However, since the thesis design was based on using the fungal bioassay as a precursor for anti-insect and anti-mollusc activity, only Isolates 1 and 9 proceeded on for detailed investigation. Again, variation was observed between different batches of the same isolate. However, because my anti-insect assays were developed during the course of my studies, some of these batch variations may also be explained by the slight variations in the protocol itself. Further variations in the anti-insect assay may be attributed to differences in age among the parental generations transferred into experimental and control vials.

The auxotrophic nature of insects requires them to obtain many essential nutrients from their diet. In the case of *D.* melanogaster, procurement of these nutrients is possible by the establishment of a symbiotic relationship with yeast-like fungi which are consumed throughout the insect's life and are also present in the gut microbiome of the host (Boucias *et al.,* 2018). This relationship is crucial to various life traits of insects including the duration of larva-to-pupa development, pupal and larval survival, and decrease fertility in adults (Grangeteau *et al.,* 2018). Therefore, anti-fungal activity by extracts may impact fruit flies indirectly by affecting their fungal symbionts. The reconstituted broths may also contain metabolites which show toxicity specifically towards insects resulting in a dual toxic effect.

The preliminary snail bioassay results coupled with the results highlighted in the results section showed that the reconstituted broths from Isolates 2, 5-7, 9 and 10 had anti-mollusc activity. Based on these results, together with anti-fungal and anti-insect results, Isolates 2, 5, 7, and 10 appear to produce a metabolite(s) that specifically target molluscs. Extracts of Isolate 6 demonstrated inhibitory activity in both fungi and mollusc bioassays suggesting that either a single metabolite exhibits anti-fungal and anti-mollusc activity, or different toxic metabolites are produced - one of which exhibits anti-fungi activity and the other anti-mollusc activity. A similar observation is made with regards to inhibitory activity of the reconstituted broth of Isolate 9 –

either a single metabolite is responsible for anti-fungi, anti-insect, and anti-mollusc toxicity different target each organism specifically.

It should be noted that snail feeding behaviour, as a means of monitoring anti-mollusc activity, is quiet variable normally. Several possible explanations for variable feeding may be proposed including that the snails were in an environment that varied in temperature, and, like other animals, snails undergo fluctuations in feeding. Nevertheless, the use of relatively high concentrations of reconstituted broths enabled us to observe certain toxic effects in snails. For example, as concentration of the reconstituted broths of Isolate 9 increased, a decrease in food consumption was observed and snail mortality quickly ensued indicating acute toxicity. Similarly, a slight decrease in food consumption and slower growth rate was observed in snails exposed to increasing concentrations of reconstituted broths of Isolate 6 indicating developmental toxicity. A decrease in growth rate and a slight decrease in food intake may be caused by a reduction in nutrient uptake. However, further bioassays are to understand the mode of anti-mollusc activities. Further, to assess physiological damage to the digestive track, a follow-up histology analysis would be required after the bioassay.

Preliminary bioassay results of commercially available natural products suggest Tea Tree Oil and perhaps Grandevo as general toxins. Further, all products showed anti-mollusc activity while TTO, Venerate, Grandevo, and STK-53 showed anti-insect activity. Based on preliminary results of all the natural products tested in this study, the bioassays set forth can be used to identify inhibitory properties in addition to target specificity from other sources of natural products.

The successful implementation of the bioassays to detect inhibitory activity in a variety of natural product sources was only impeded by a few limitations. Although several batches of fungal isolates underwent fermentation, the incubator lacked sufficient space to obtain larger number of replicates of each broth. This limitation prevented thorough testing of all 10 fungal isolates against all 3 bioassays therefore we set forth to use the fungal bioassay as a guide to limit the number of isolates used. This lack of sufficient volumes of broths further hindered the study since a series of insect and mollusc bioassays could not be performed using a range of increasing volumes to construct a growth curve observing toxicity. In regard to the mollusc bioassay, the lack of sufficient volumes was not the only impeding factor – the environmental conditions were

not ideal for the snail colony to reproduce. Thus, there wasn't an adequate amount of snails available to perform a series of bioassays with increasing volumes.

Despite limited resources, the bioassay system set forth by this research are easy to set up and a cost-effective method of testing natural products to inhibitory activity against agriculture pests and pathogens. The 3 bioassays can be used individually or combined to get a more in-depth view at the natural product's target specificity. With a larger sample size, the bioassay system can successfully lead to the identification of target metabolites by carrying out a robust comparative metabolomic study. However, although the bioassay system can lead to the detection of novel secondary metabolites showing activity against pests and pathogens, it is unable to differentiate between beneficial and harmful organisms.

## 5. Conclusion

The overall design of the bioassay-guided identification of potentially novel toxic metabolites allowed for a preliminary metabolomic analysis of ethyl acetate, butanol, and water fractions of Isolates 1, 6 and 9. Additionally, insect and mollusc bioassays developed identified inhibitory activity despite the multivariate conditions surrounding the preliminary bioassays. The correlation graphs suggested the bioassays cannot predict inhibitory effects in each other however the 3 bioassays can be used concurrently to gain insights on the type of toxin present. Inhibitory activity in all 3 bioassays may indicate general toxicity to diverse biological systems, which would not be ideal since specificity is preferred to limit off-target effects of natural and synthetic pesticides alike. The groundwork created by this research can be expanded upon to create studies at a larger scale leading to the identification of target metabolite(s).

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# Appendices

## Appendix I: Preparation and Storage

### Fungal Bioassay – Reconstituted Broths

Yeast S288C strain was used as the model organism to determine minimum inhibitory concentration of each broth. S288C was grown on Yeast (Bioshop® Burlington, ON, Canada), Peptone (HIMEDIA), Dextrose (Bioshop® Burlington, ON, Canada) (YPD) and agar (0.75%; Bioshop® Burlington, ON, Canada) plates in the dark at 4°C. For the fungal bioassay, a single colony was transferred to 30ml of YPD broth in a 50ml Falcon tube and incubated at 30°C on a shaker (New Brunswick Scientific Innova® 40; RPM 201) for 4 hours. After incubation, 5µl of the yeast-YPD mixture was transferred to a haemocytometer (Petroff-Hausser Bacteria Counter). The CFU was calculated by counting cells in the 0.05x0.05mm square grids in the center of the haemocytometer. Yeast cells within the borders if 5 different squares were counted after which the average was calculated. The average number of cells was multiplied by $5X10^4$ to get the concentration in cells/mL. An example of the calculation is provided below:

Cell count per square: 10,10,5,8,2 = Σ35

$35 \times (5 \times 10^4) = 1.75x10^6$ cells/mL

The yeast stock solution was diluted by a factor of 2 before using the following formula to create a final concentration of 1,000 cells/mL and thus having around 150 cells/well:

$Concentration_1 \times Volume_1 = Concentration_2 \times Volume_2$

$C_1 = 1.75x10^6$ cells/mL

$V_1$ = X (Volume of cell stock needed to get a final volume of 20mL and 1,000 cells/mL)

$C_2$ = 1,000 cells/mL

$V_2$ = 20 mL

$$X = \frac{1{,}000\ \left(\frac{cells}{ml}\right) \times 20\ ml}{1.75 \times 10^6\ (\frac{cells}{ml})} = 0.011428\ ml\ or\ 11.43\ \mu l$$

To determine the MIC value of reconstituted broths showing anti-fungal activity the following formula was used:

Inhibition in well *n*:

$$X = \frac{Stock\ Concentration\ (\frac{mg}{\mu l})}{3^n} = MIC\ (\frac{mg}{\mu l})$$

**Fungal Bioassay – Pure Extracts**

The MIC bioassay principle was used to determine presence of activity in each fraction by resuspending the dried fractions in 1.25% Tween 80 to twice the MIC value of each broth determined through the crude MIC bioassays. An example of the calculations is provided below:

Batch 11 - broth 9 Ethyl Acetate Fraction (11.9.EA):

Crude MIC = 0.0087 mg/µl

Mass of dried EA fraction = 24.9 mg

$C_1V_1 = C_2V_2$

$V_1$ = 50 µl

$C_2$ = 0.0174 mg/ µl (2X MIC)

$V_2$ = 250 µl

$C_1$ = X

$$X = \frac{\left(0.0174 \frac{\text{mg}}{\mu\text{l}} * 250\ \mu\text{l}\right)}{50\ \mu\text{l}} = 0.087\ \text{mg}/\mu\text{l}$$

Volume required for resuspension:

Mass = Concentration x Volume

Volume = X

$$X = \frac{24.9\ \text{mg}}{0.087\ \text{mg}/\ \mu\text{l}} = 286.2\ \mu\text{l}$$

Samples 10.6.EA, 11.6.EA, 15.6.EA, 16.1.EA, 16.1.Bu, 16.6.EA, 16.9.EA, and 14C.EA had a resuspension volume less than the volume required for the MIC therefore all sample volumes were brought up to 50 µl, the least volume of extract required for MIC.

**Insect Bioassay**

*Drosophila melanogaster* strain used was established by combining 35 isofemale lines collected from London, Ontario, Canada (43°00'N, 81°15'W) and Niagara on the Lake, Ontario, Canada (43°04'N, 79°04'W). The fruit flies were reared on 2g of Instant Drosophila Medium which was rehydrated using 10ml cool tap water in Drosophila vials (Fisherbrand™ AS510; Pittsburg, PA 15275). After allowing the feed to set, a pinch (4-6 granules) of yeast granules were spread on top of the feed. Flies were knocked out using $CO_2$ and transferred to the conditioning vials for a 48-hour period in a 12-hour dark/light cycle at 24°C. After the conditioning period, flies were transferred, without knocking out, to experimental vials and placed back in the incubator. Vials were secured using foam stoppers.

**Snail Bioassay**

Golden Apple Snails, *Pomacea canaliculata,* were grown in 80L tanks containing de-chlorinated water (Nutrafin Aqua+Plus) placed in the greenhouse. The snails were fed romaine lettuce twice a week and the tanks were cleaned every other week. During the snail bioassays, the snails were fed 1 baby spinach leaf per day (Dole All-Natural Baby Spinach). The data generated from the bioassays was calculated as follows:

$$\text{Average Growth} = \left(\frac{\text{Final Size} - \text{Initial Size}}{\text{Final Size}}\right) * 100\%$$

$$\text{Amount Eaten} = \left(\frac{\text{Area of Leaf at 0 hrs} - \text{Area of Leaf at 24 hrs}}{\text{Area of Leaf at 0 hrs}}\right) * 100\%$$

**Liquid-Liquid Extraction Preparation**

Glass vials with plastic caps fitted with rubber inserts capable of holding 9ml volume were used in the extraction process. In a fume hood, the vials were solvent cleaned three times using HPLC grade Chloroform while the caps were immersed in HPLC grade 50% methanol for 15 minutes. The vials and caps were left in the fume hood overnight for the solvents to evaporate. Prior to extractions, an aliquot of each broth was freeze-dried then resuspended to 10X concentration. Each broth extraction procedure consisted of 4 glass vials labeled as follows:

Batch #. Isolate #. Solvent type ($H_2O$, H, EA, or Bu)

**Metabolomics – Data Manipulation**

The following formula was used to combine RT and m/z values:

=”RT”&B5&”_”&B2

**Appendix II: Batch Information**

Batch #1

- 4-week incubation period (October 10th – October 31st)
- 5% Tween 80 was used to resuspend broth intended for MIC

| No. | Strain | MIC (mg/µl) |
|---|---|---|
| **1** | S8I2ACS | - |
| **2** | RW3I1A | - |
| **3** | RW6A1P | - |
| **4** | RW3A2Pa | 0.0465 |
| **5** | PCA5P | - |
| **6** | OA1I5Mb | - |
| **7** | PCA20P | - |
| **8** | PCA22M | - |
| **9** | S3A2ACS | 0.0152 |
| **10** | S9A1R | - |
| **C** | Control (PDB) | - |

Batch #2

- 6-week incubation period (October 24th – December 5th)
- 5% Tween 80 was used to resuspend broths intended for MIC
- Fly Bioassay
    - 30% dry mass of each isolate was resuspended in 10ml tween: water suspension; 2g of feed was mixed with broth
    - 1.25% tween was used to resuspend broths

| No. | Strains | Anti-fungal MIC (mg/ml) | Anti-insect inhibitory concentration (mg/ml) |
|---|---|---|---|
| **1** | S8I2ACS | 11.6 | 3.484 |
| **2** | RW3I1A | - | 7.246 |
| **3** | RW6A1P | 5 | 1.514 |
| **4** | RW3A2Pa | 40 | 11.91 |
| **5** | PCA5P | - | 17.1 |
| **6** | OA1I5Mb | - | 9.402 |
| **7** | PCA20P | - | 11.01 |
| **8** | PCA22M | - | 18.37 |
| **9** | S3A2ACS | 35.5 | 10.64 |
| **10** | S9A1R | - | 7.83 |
| **C** | Control (PDB) | - | - |

Batch #3

- 6-week incubation period (December 16th – January 27th)
- MIC was not carried out
- Snail bioassay
    - 30% dried mass of each fungal isolate's reconstituted broth was used
    - Exposure and observation tanks contained 1L dechlorinated water
    - Exposure duration was 4 hours
    - Distance between object and camera was not standardized

| No. | Strains | Anti-mollusc concentration (mg/ml) |
|---|---|---|
| **1** | S8I2ACS | 0.0681 |
| **2** | RW3I1A | 0.259 |
| **3** | RW6A1P | 0.063 |
| **4** | RW3A2Pa | 0.1414 |
| **5** | PCA5P | 0.7311 |
| **6** | OA1I5Mb | 0.3951 |
| **7** | PCA20P | 0.4544 |
| **8** | PCA22M | 0.5048 |
| **9** | S3A2ACS | 0.1645 |
| **10** | S9A1R | 0.2031 |
| **C** | Control (PDB) | 0.5892 |

Batch #4

- 6-week incubation period (February 2nd – March 16th)
- Batch not used due to contaminations

Batch #5

- 7-week incubation period (February 14th – April 6th)
- Batch not used due to contaminations

Batch #6

- 6-week incubation period (May 18th – June 29th)
- 1.25% Tween 80 was used to resuspend broth intended for MIC
- Snail bioassay
  - 15ml broth in 1L de-chlorinated water for 6 hours (July 29th – August 12th)
  - 15ml broth in 500ml de-chlorinated water for 4 hours (August 4th – August 18th)
- Fly bioassay
  - 1× concentration
  - 15ml broth divided into 3 - 5ml replicates each in 1g of feed
  - 15ml broth divided into 2 - 5ml replicates each in 1g of feed

| **No.** | **Strains** | **Anti-fungal MIC (mg/ml)** | **Anti-insect concentration (mg/ml)** | | **Anti-mollusc concentration (mg/ml)** | |
|---|---|---|---|---|---|---|
| | | | Bioassay 1 Aug 3rd | Bioassay 2 Oct 7th | Bioassay 1 Aug 3rd | Bioassay 2 Aug 5th |
| **1** | S8I2ACS | 18.7 | 0.0050 | 0.0050 | 0.0745 | 0.149 |
| **2** | RW3I1A | - | 0.0101 | 0.0101 | 0.1522 | 0.3044 |
| **3** | RW6A1P | - | 0.0063 | 0.0063 | 0.0942 | 0.1884 |
| **4** | RW3A2Pa | 30.3 | 0.0147 | 0.0147 | 0.2201 | 0.4402 |
| **5** | PCA5P | - | 0.0264 | 0.0264 | 0.396 | 0.792 |
| **6** | OA1I5Mb | 14.6 | 0.0096 | 0.0096 | 0.144 | 0.288 |
| **7** | PCA20P | - | 0.005 | 0.005 | 0.0755 | 0.151 |
| **8** | PCA22M | - | 0.0152 | 0.0152 | 0.2275 | 0.455 |
| **9** | S3A2ACS | 19.3 | 0.0137 | 0.0137 | 0.2052 | 0.4104 |
| **10** | S9A1R | - | 0.0163 | 0.0163 | 0.2444 | 0.2888 |
| **C** | Control | - | 0.0280 | 0.0280 | 0.4201 | 0.8402 |

Batch #7

- 9-week incubation period (May 31st – August 2nd)
- 1.25% Tween 80 was used to resuspend broth intended for MIC
- 15 ml of broths were used for LLE
- Fly Bioassay
    - 2× concentration
    - Broths 1 and 9 were tested for activity (June 19th – July 5th)
    - 30 ml of broths were dried before resuspending to 15 ml using 1.25% Tween
    - The broths were divided into 2 x 7.5ml replicated before adding 1.5g of fly feed
    - Each replicate contained 8 flies with a 1:1 sex ratio
    - Incubated for 2 weeks at 24±0.11°C in 12-hour light/dark cycle

| No. | Strains | Anti-fungal MIC (mg/ml) | Anti-insect concentration (mg/ml) | Anti-mollusc concentration (mg/ml) |
|---|---|---|---|---|
| **1** | S8I2ACS | - | 4.413 | - |
| **6** | OA1I5Mb | 14.2 | - | - |
| **9** | S3A2ACS | - | 11.9 | - |
| **C** | Control | - | - | - |

Batch #8

- 6-week incubation period (July 9th – August 18th)
- 1.25% Tween 80 was used to resuspend broth intended for MIC
- Snail Bioassay
  - Broths 2, 5, 6, 9, and 10 were tested
  - 90ml of each broth was added to 510ml of de-chlorinated water for 6 hours
- Fly Bioassay
  - 2× concentration
  - Broths 1, 9, and 10 were tested for activity
  - 30ml of dried broths were resuspended to 15ml using 1.25% Tween 80
  - Broths were divided into 2 - 5ml replicates each mixed with 1g of fly feed
  - Each replicate contained 10 flies with a 1:1 sex ratio
  - Incubated for 2 weeks at 24±0.11°C in 12-hour light/dark cycle

| No. | Strains | Anti-fungal MIC (mg/ml) | Anti-insect concentration (mg/ml) | Anti-mollusc concentration (mg/ml) |
|---|---|---|---|---|
| **1** | S8I2ACS | 8 | 5.23 | - |
| **2** | RW3I1A | - | - | 6.432 |
| **5** | PCA5P | - | - | 25.39 |
| **6** | OA1I5Mb | 15 | - | 13.44 |
| **9** | S3A2ACS | 44.2 | 25.24 | 12.83 |
| **10** | S9A1R | - | 33.35 | 17.13 |
| **C** | Control | - | - | - |

Batch #9

- 12-week incubation period (July 27th – October 19th)
- 1.25% Tween 80 was used to resuspend broth intended for MIC
- 15 ml of broths were used for LLE
- Fly Bioassay
    - 2× concentration
    - 30 ml of broths were dried before resuspending to 15 ml using 1.25% Tween
    - The broths were divided into 2 - 7.5ml replicated before adding 1.5g of fly feed
    - Each replicate contained either 8 flies with a 1:1 sex ratio
    - Incubated for 2 weeks at 24±0.11°C in 12-hour light/dark cycle

| No. | Strains | Anti-fungal MIC (mg/ml) | Anti-insect concentration (mg/ml) | Anti-mollusc concentration (mg/ml) |
|---|---|---|---|---|
| **1** | S8I2ACS | - | 4.76 | - |
| **6** | OA1I5Mb | 24 | - | - |
| **9** | S3A2ACS | - | 5.57 | - |
| **C** | Control | - | - | - |

Batch #10

- 6-week incubation period (September 24th – November 5th)
- 1.25% Tween 80 was used to resuspend broth intended for MIC
- 30ml of broths were used for LLE

| No. | Strains | Anti-fungal MIC (mg/ml) | Anti-insect concentration (mg/ml) | Anti-mollusc concentration (mg/ml) |
|---|---|---|---|---|
| **1** | S8I2ACS | 1.2 | - | - |
| **6** | OA1I5Mb | - | - | - |
| **9** | S3A2ACS | 30.5 | - | - |
| **C** | Control | - | - | - |

Batch #11

- 6-week incubation period (November 5th – December 17th)
- 1.25% Tween 80 was used to resuspend broth intended for MIC
- 15 ml of broths were used for LLE

| No. | Strains | Anti-fungal MIC (mg/ml) | Anti-insect concentration (mg/ml) | Anti-mollusc concentration (mg/ml) |
|---|---|---|---|---|
| **1** | S8I2ACS | 4 | - | - |
| **6** | OA1I5Mb | - | - | - |
| **9** | S3A2ACS | - | - | - |
| **C** | Control | - | - | - |

Batch #12

- 6-week incubation period (December 5th – January 16th)
- 1.25% Tween 80 was used to resuspend broth intended for MIC
- 30 ml of broths were used for LLE
- Fly Bioassay
    - 2× concentration
    - 30 ml of broths were dried before resuspending to 15 ml using 1.25% Tween
    - The broths were divided into 2 - 7.5ml replicated before adding 1.5g of fly feed
    - Each replicate contained 10 flies with a 1:1 sex ratio
    - Incubated for 2 weeks at 24±0.11°C in 12-hour light/dark cycle

| No. | Strains | Anti-fungal MIC (mg/ml) | Anti-insect concentration (mg/ml) | Anti-mollusc concentration (mg/ml) |
|---|---|---|---|---|
| **1** | S8I2ACS | 8.9 | 5.31 | - |
| **6** | OA1I5Mb | 13.8 | - | |
| **9** | S3A2ACS | - | 17.23 | |
| **C** | Control | - | - | - |

Batch #13

- 6-week incubation period (December 20th – February 3rd)
- 1.25% Tween 80 was used to resuspend broths intended for MIC
- 15 ml of broths were used for LLE
- Fly Bioassay
    - 2× concentration
    - 30 ml of broths were dried before resuspending to 15 ml using 1.25% Tween
    - The broths were divided into 2 - 7.5ml replicated before adding 1.5g of fly feed
    - Each replicate contained 10 flies with a 1:1 sex ratio
    - Incubated for 2 weeks at 24±0.11°C in 12-hour light/dark cycle

| No. | Strains | Anti-fungal MIC (mg/µl) | Anti-insect concentration (mg/ml) | Anti-mollusc concentration (mg/ml) |
|---|---|---|---|---|
| **1** | S8I2ACS | - | 4.47 | - |
| **6** | OA1I5Mb | - | - | - |
| **9** | S3A2ACS | 11 | 7.26 | - |
| **C** | Control | - | - | - |

Batch #14

- 6-week incubation period (January 1st – February 12th)
- 1.25% Tween 80 was used to resuspend broths intended for MIC
- 15 ml of broths were used for LLE
- Fly Bioassay
  - 2× concentration
  - 30 ml of broths were dried before resuspending to 15 ml using 1.25% Tween
  - The broths were divided into 2 - 7.5ml replicated before adding 1.5g of fly feed
  - Each replicate contained 10 flies with a 1:1 sex ratio
  - Incubated for 2 weeks at 24±0.11°C in 12-hour light/dark cycle

| No. | Strains | Anti-fungal MIC (mg/ml) | Anti-insect concentration (mg/ml) | Anti-mollusc concentration (mg/ml) |
|---|---|---|---|---|
| **1** | S8I2ACS | - | 11.58 | - |
| **6** | OA1I5Mb | 18 | - | |
| **9** | S3A2ACS | 7 | 6.62 | |
| **C** | Control | - | - | - |

Batch #15

- 9-week incubation period (February 8th – April 13th)
- 1.25% Tween 80 was used to resuspend broths intended for MIC
- 15 ml of broths were used for LLE
- Fly Bioassay
    - 2× concentration
    - 30 ml of broths were dried before resuspending to15 ml using 1.25% Tween
    - The broths were divided into 2 - 7.5ml replicated before adding 1.5g of fly feed
    - Each replicate contained 8 flies with a 1:1 sex ratio
    - Incubated for 2 weeks at 24°C in 12-hour light/dark cycle

| **No.** | **Strains** | **Anti-fungal MIC (mg/ml)** | **Anti-insect concentration (mg/ml)** | **Anti-mollusc concentration (mg/ml)** |
|---|---|---|---|---|
| **1** | S8I2ACS | 4.2 | 5.093 | - |
| **6** | OA1I5Mb | - | - | |
| **9** | S3A2ACS | - | 16.39 | |
| **C** | Control | - | - | - |

Batch #16

- 12-week incubation period (January 21st – April 14th)
- 1.25% Tween 80 was used to resuspend broths intended for MIC
- 15 ml of broths were used for LLE
- Fly Bioassay
    - 2× concentration
    - 30 ml of broths were dried before resuspending to15 ml using 1.25% Tween
    - The broths were divided into 2 - 7.5ml replicated before adding 1.5g of fly feed
    - Each replicate contained 8 flies with a 1:1 sex ratio
    - Incubated for 2 weeks at 24°C in 12-hour light/dark cycle

| No. | Strains | Anti-fungal MIC (mg/ml) | Anti-insect concentration (mg/ml) | Anti-mollusc concentration (mg/ml) |
|---|---|---|---|---|
| **1** | S8I2ACS | 44 | 4.8 | - |
| **6** | OA1I5Mb | - | - | - |
| **9** | S3A2ACS | - | 6.693 | - |
| **C** | Control | - | - | - |

## Appendix III: Insect and Snail Protocols

### Insect Protocol

Equipment required

- *Drosophila melanogaster* colony
- Plastic vials with stoppers
- Dry fly feed
- Cool tap water
- Brewer's yeast granules
- Carbon dioxide tank and gas plate
- Microscope
- Filter-sterilized reconstituted broths (resuspended in Tween 80)

Protocol

1. Prepare vials for acclimatising by mixing dry feed with cool tap water and adding a pinch of yeast. Prepare the same number of these vials as the number of reconstituted broths to test
2. Knock out flies from colony vials and place on gas plate under the microscope. Create groups of flies with 1:1 sex ratio
3. Place each group of flies in a separate vial and incubate under conditions used for experiment (24°C with 24-hr light/dark cycle) for 2 days
4. Prepare experimental vials by mixing dry feed with the reconstituted broth in question. Label each vial accordingly
5. To transfer flies from acclimatising vial to experimental, tap the vial on a surface to disorient the flies and quickly turn vial over and on top of experimental vial. Gently tap the experimental vial (with the acclimatising vial inverted above) on the tabletop until all the flies have transferred. Quickly cover the experimental vial with a stopper.
6. Repeat with remaining vials before moving the vials into the incubator
7. Note the number of adults and pupae every 24 hrs for a 2-week period

**Snail Protocol**

Equipment required

- *Pomacea* sp. colony
- 1.5 – 2L plastic containers with lids (drill holes in lids to air exchange)
- API® Stress Coat+ (or any liquid used to dechlorinate water)
- Organic, washed baby spinach leaves
- Camera, ruler, labels for each day indicating eaten or fresh leaves
- Filter-sterilized reconstituted broths

Protocol

1. Label tanks as either "exposure" or "observation" for each broth being tested
2. Add 500ml of dechlorinated water to "exposure" tanks and add 1L of dechlorinated water to "observation" tanks
3. Select 2 snails roughly 1cm in length from the colony and place in "exposure" tanks for 24 hours
4. Once acclimatized, take snails out of their designated tanks, dry them using paper towels, and place on a grid sheet under their specified broth. Using a waterproof marker or liquid paper, tag one snail in each pair
5. Place a scale on the grid sheet and take a picture with the label "Day 0 – initial snail size (Date)"
6. Place snails back into their designated tanks
7. Add broth (if the volume of the reconstituted broth is more than 5ml then adjust the amount of dechlorinated water added to get a final volume of 500ml)
8. Monitor snail behaviour for the first 10-20 minutes of exposure. Assess their movement or lack of including antenna twitching and formation of a mucus layer around shell. Assess their behaviour 10-20 minutes before exposure ceases
9. Transfer snails from exposure tank to observation tank after 6 hours. Observe behaviour once transferred into clean water. Note: make sure the tanks are labelled properly so the snails exposed to broth of Isolate 1 are transferred to the observation tank labeled as "observation tank: Reconstituted Broth – Isolate 1"

10. Using the same grid sheet and scale, place a single spinach leaf under each broth name and take a picture designating that picture as “Day 1 Fresh leaves (Date)”. Note: use a tripod or a similar apparatus to eliminate the variability created by a difference in distance between camera and object
11. Place fresh leaf in observation tank and tank a picture 24 hours later along with tanking a picture of fresh leaves for day 2.
12. Repeat process for a 2-week period along with monitoring behaviour
13. On day 15, record final snail size
14. Use GIMP, a free software, to visually assess amount eaten and change in snail size
15. To decrease variability, use the frameshift average technique on the average amount eaten by snails every 4 days

**Appendix IV: Liquid-Liquid Extraction Sample Weights**

| No. | Sample name (batch#.broth#.extract type) | Bottle weight (g) | Bottle and dry mass weight (g) | Weight of dry mass (g) |
|---|---|---|---|---|
| **1** | 7.1.H | 10.0865 | 10.0943 | 0.0078 |
| **2** | 7.1.EA | 10.0475 | 10.0600 | 0.0125 |
| **3** | 7.1.Bu | 10.2352 | 10.2499 | 0.0147 |
| **4** | 7.1.$H_2O$ | 10.1546 | 10.1938 | 0.0392 |
| **5** | 7.6.H | 10.1217 | 10.1370 | 0.0153 |
| **6** | 7.6.EA | 10.2112 | 10.2294 | 0.0182 |
| **7** | 7.6.Bu | 10.1122 | 10.1215 | 0.0093 |
| **8** | 7.6.$H_2O$ | 10.1679 | 10.2054 | 0.0375 |
| **9** | 7.9.H | 10.1411 | 10.1486 | 0.0075 |
| **10** | 7.9.EA | 10.2653 | 10.2775 | 0.0122 |
| **11** | 7.9.Bu | 10.1916 | 10.2197 | 0.0281 |
| **12** | 7.9.$H_2O$ | 10.1712 | 10.2513 | 0.0801 |
| **13** | 9.1.H | 10.1721 | 10.889 | 0.7169 |
| **14** | 9.1.EA | 10.1933 | 10.2110 | 0.0177 |
| **15** | 9.1.Bu | 10.1271 | 10.1406 | 0.0135 |
| **16** | 9.1.$H_2O$ | 10.0845 | 10.1177 | 0.0332 |
| **17** | 9.6.H | 10.1579 | 10.1860 | 0.0281 |
| **18** | 9.6.EA | 10.1746 | 10.1853 | 0.0107 |
| **19** | 9.6.Bu | 10.0641 | 10.0811 | 0.017 |
| **20** | 9.6.$H_2O$ | 10.1073 | 10.1702 | 0.0629 |
| **21** | 9.9.H | 10.2150 | 10.2445 | 0.0295 |
| **22** | 9.9.EA | 10.1141 | 10.1312 | 0.0171 |
| **23** | 9.9.Bu | 10.1389 | 10.1804 | 0.0415 |
| **24** | 9.9.$H_2O$ | 10.3362 | 10.3973 | 0.0611 |
| **25** | 10.1.H | 10.1355 | 10.1419 | 0.0064 |
| **26** | 10.1.EA | 10.1356 | 10.1487 | 0.0131 |
| **27** | 10.1.Bu | 10.0923 | 10.1278 | 0.0355 |

| | | | | |
|---|---|---|---|---|
| **28** | 10.1.$H_2O$ | 10.1958 | 10.2590 | 0.0632 |
| **29** | 10.6.H | 9.9060 | 9.9646 | 0.0586 |
| **30** | 10.6.EA | 9.9563 | 9.9646 | 0.0083 |
| **31** | 10.6.Bu | 10.1940 | 10.306 | 0.112 |
| **32** | 10.6.$H_2O$ | 10.1577 | 10.6513 | 0.4936 |
| **33** | 11.1.H | 10.1640 | 10.1716 | 0.0076 |
| **34** | 11.1.EA | 10.2911 | 10.3012 | 0.0101 |
| **35** | 11.1.Bu | 10.1811 | 10.1976 | 0.0165 |
| **36** | 11.1.$H_2O$ | 10.1205 | 10.1604 | 0.0399 |
| **37** | 11.6.H | 10.1460 | 10.1601 | 0.0141 |
| **38** | 11.6.EA | 10.1381 | 10.1482 | 0.0101 |
| **39** | 11.6.Bu | 10.1072 | 10.1423 | 0.0351 |
| **40** | 11.6.$H_2O$ | 10.2541 | 10.3357 | 0.0816 |
| **41** | 11.9.H | 9.8523 | 9.8592 | 0.0069 |
| **42** | 11.9.EA | 10.1264 | 10.1513 | 0.0249 |
| **43** | 11.9.Bu | 10.1453 | 10.1890 | 0.0437 |
| **44** | 11.9.$H_2O$ | 10.1885 | 10.3628 | 0.1743 |
| **45** | 12.1.H | 10.3346 | 10.3503 | 0.0157 |
| **46** | 12.1.EA | 10.2087 | 10.2246 | 0.0159 |
| **47** | 12.1.Bu | 10.2159 | 10.2397 | 0.0238 |
| **48** | 12.1.$H_2O$ | 10.2837 | 10.3380 | 0.0543 |
| **49** | 12.6.H | 10.2988 | 10.3160 | 0.0172 |
| **50** | 12.6.EA | 10.2002 | 10.2198 | 0.0196 |
| **51** | 12.6.Bu | 10.2401 | 10.2955 | 0.0554 |
| **52** | 12.6.$H_2O$ | 10.1434 | 10.4352 | 0.2918 |
| **53** | 12.9.H | 9.9727 | 9.9797 | 0.007 |
| **54** | 12.9.EA | 10.1379 | 10.1618 | 0.0239 |
| **55** | 12.9.Bu | 10.2296 | 10.2706 | 0.041 |
| **56** | 12.9.$H_2O$ | 10.1324 | 10.3719 | 0.2395 |
| **57** | 13.C6.H | 10.1867 | 10.2019 | 0.0152 |

| | | | | |
|---|---|---|---|---|
| **58** | 13.C6.EA | 10.3391 | 10.3661 | 0.027 |
| **59** | 13.C6.Bu | 10.0860 | 10.1470 | 0.061 |
| **60** | 13.C6.$H_2O$ | 10.1615 | 10.4245 | 0.263 |
| **61** | 13.C6A.H | 9.9280 | 9.9498 | 0.0218 |
| **62** | 13.C6A.EA | 10.1135 | 10.1451 | 0.0316 |
| **63** | 13.C6A.Bu | 10.1497 | 10.2029 | 0.0532 |
| **64** | 13.C6A.$H_2O$ | 10.2295 | 10.5022 | 0.2727 |
| **65** | 13.1.H | 10.0872 | 10.0971 | 0.0099 |
| **66** | 13.1.EA | 10.0773 | 10.0852 | 0.0079 |
| **67** | 13.1.Bu | 10.1524 | 10.1674 | 0.015 |
| **68** | 13.1.$H_2O$ | 10.3654 | 10.4025 | 0.0371 |
| **69** | 13.9.H | 10.1871 | 10.1981 | 0.011 |
| **70** | 13.9.EA | 10.2279 | 10.2439 | 0.016 |
| **71** | 13.9.Bu | 9.7544 | 9.7731 | 0.0187 |
| **72** | 13.9.$H_2O$ | 9.7862 | 9.8684 | 0.0822 |
| **73** | 14.C6.H | 10.3417 | 10.3627 | 0.021 |
| **74** | 14.C6.EA | 10.1512 | 10.1696 | 0.0184 |
| **75** | 14.C6.Bu | 10.0950 | 10.1153 | 0.0203 |
| **76** | 14.C6.$H_2O$ | 10.1052 | 10.4220 | 0.3168 |
| **77** | 14.1.H | 10.0991 | 10.1096 | 0.0105 |
| **78** | 14.1.EA | 10.2047 | 10.2171 | 0.0124 |
| **79** | 14.1.Bu | 10.1625 | 10.1734 | 0.0109 |
| **80** | 14.1. $H_2O$ | 10.2212 | 10.2532 | 0.032 |
| **81** | 14.6.H | 10.3188 | 10.3297 | 0.0109 |
| **82** | 14.6.EA | 10.1652 | 10.1862 | 0.021 |
| **83** | 14.6.Bu | 10.1352 | 10.1683 | 0.0331 |
| **84** | 14.6.$H_2O$ | 10.1212 | 10.3666 | 0.2454 |
| **85** | 14.9.H | 10.2021 | 10.2122 | 0.0101 |
| **86** | 14.9.EA | 10.1133 | 10.1258 | 0.0125 |
| **87** | 14.9.Bu | 10.1747 | 10.1955 | 0.0208 |

| | | | | |
|---|---|---|---|---|
| **88** | 14.9.$H_2O$ | 10.1476 | 10.1792 | 0.0316 |
| **89** | 15.1.H | 10.1825 | 10.1856 | 0.0031 |
| **90** | 15.1.EA | 10.1423 | 10.1474 | 0.0051 |
| **91** | 15.1.Bu | 10.0393 | 10.0452 | 0.0059 |
| **92** | 15.1.$H_2O$ | 10.2451 | 10.3089 | 0.0638 |
| **93** | 15.6.H | 10.1638 | 10.1741 | 0.0103 |
| **94** | 15.6.EA | 10.1521 | 10.1655 | 0.0134 |
| **95** | 15.6.Bu | 10.0185 | 10.0830 | 0.0645 |
| **96** | 15.6.$H_2O$ | 10.1713 | 10.3880 | 0.2167 |
| **97** | 15.9.H | 10.0005 | 10.0055 | 0.005 |
| **98** | 15.9.EA | 10.1096 | 10.1153 | 0.0057 |
| **99** | 15.9.Bu | 10.2176 | 10.2418 | 0.0242 |
| **100** | 15.9.$H_2O$ | 10.0095 | 10.1136 | 0.1041 |
| **101** | 16.1.H | 10.2062 | 10.2095 | 0.0033 |
| **102** | 16.1.EA | 10.1586 | 10.1628 | 0.0042 |
| **103** | 16.1.Bu | 10.0699 | 10.0745 | 0.0046 |
| **104** | 16.1.$H_2O$ | 10.2372 | 10.2679 | 0.0307 |
| **105** | 16.6.H | 10.1848 | 10.1872 | 0.0024 |
| **106** | 16.6.EA | 10.1233 | 10.1334 | 0.0101 |
| **107** | 16.6.Bu | 10.0758 | 10.1318 | 0.056 |
| **108** | 16.6.$H_2O$ | 10.0372 | 10.2244 | 0.1872 |
| **109** | 16.9.H | 10.0803 | 10.0827 | 0.0024 |
| **110** | 16.9.EA | 10.0588 | 10.0666 | 0.0078 |
| **111** | 16.9.Bu | 10.0835 | 10.1119 | 0.0284 |
| **112** | 16.9.$H_2O$ | 10.1811 | 10.2239 | 0.0428 |

**Appendix V: Metabolomics Metadata**

| | | |
|---|---|---|
| **Experimental Design:** Isolates | Isolate 1: S8I2ACS | *Penicillium virgatum* |
| | Isolate 2: RW3I1a | *Cystofilobasidium capitatum* |
| | Isolate 3: RW6A1P | *Boeremia exigua* |
| | Isolate 4: RW3A2Pa | *Phacidium grevilleae* |
| | Isolate 5: PCA5P | *Phialemonium inflatum* |
| | Isolate 6: OA1I5Mb | *Ramularia vizellae* |
| | Isolate 7: PCA20P | *Oidiodendron* sp. |
| | Isolate 8: PCA22M | *Aspergillus caninus* |
| | Isolate 9: S3A2ACS | *Trichoderma* sp. |
| | Isolate 10: S9A1R | *Penicillium* sp. |
| Isolate storage | Media | Potato Dextrose Agar (PDA) |
| | Method | 1.5ml agar slants |
| | Temperature (°Celsius) | 13±0.2 |
| Growth conditions | Media | Potato Dextrose Broth (PDB) |
| | Volume of media | 50ml |
| | Incubation period (weeks) | 6 |
| | Temperature (°Celsius) | 13±0.2 |
| Harvest conditions | Samples | Isolate broths |
| | Mycelium removal | Cheesecloth |
| | Broth filtration | P5 filter paper (P8+P5 filter paper for isolates 2 and 4) |
| | Broth storage vials | 50ml blue cap falcon tubes |
| | Broth storage volume | 50ml |
| | Broth storage temperature (°Celsius) | -20 |
| Broth sterilization | Syringe filter disc | PES membrane |
| | Pore size (µm) | 0.22/0.45 |
| | Diameter (mm) | 30 |
| | Max. pressure | 4.5bars |
| | Broths sterilized | 7, 9, 13, 14, 15, & 16 |

| | | |
|---|---|---|
| **Liquid-Liquid Extraction** | Solvents (increasing polarity) | Hexane, ethyl acetate, 1-butanol, water |
| | control | Potato Dextrose Broth |
| | Volume of broths (ml) | 3/1.5 |
| | Volume ration of broth:solvent | 1.1 |
| | Number of washes with each solvent | 3 |
| | Solvent evaporation method | Nitrogen gas |
| | Solvent evaporation temp. (°Celsius) | 37.5 |
| | Sample extraction/storage | Broths were transferred into pre-weighed glass vials; washes were collected in their designated, pre-weighed glass vials |
| | Glass vials | Solvent cleaned with HPLC grade Chloroform |
| | Glass vial caps | Cleaned with 50% ethanol (diluted with $dH_2O$) |
| | Post extraction samples | Weighed to calculate amount of sample in each glass vial |
| | Sample resuspension liquid | Methanol |
| | Sample resuspension conc. (µg/ml) | 500 |
| **Metabolomic Comparison** | Incubation period (weeks): broths 10-14 | 6 |
| | Incubation period (weeks): broths 7 & 15 | 9 (not included in analysis) |
| | Incubation period (weeks) broths 9 & 16 | 12 (not included in analysis) |
| | Controls | PDB (13.C6, 13.C6A, 14.C6); MeOH |
| **Xcalibur** | Noise threshold | 5.8e4 |
| **MZmine2.4** | | |
| **Mass Detection** | Exact Mass | 5.8e4 |
| | Minimum time span (min) | 0.1 |

| | | |
|---|---|---|
| **Chromatogram Builder** | Min height | 5.8e4 |
| | m/z tolerance (m/z) | 0.005 or 5ppm |
| **Chromatogram Deconvolution: Local Minimum Search** | Chromatographic Threshold (%) | 95 |
| | Search min in RT range (min) | 0.1 |
| | Min relative height (%) | 35 |
| | Min absolute height | 5.8e4 |
| | Min ratio of peak top/edge | 1 |
| | m/z center calculation | auto |
| **Isotopic Peak Grouper** | m/z tolerance (m/z) | 0.005 or 5ppm |
| | Retention time tolerance (absolute (min)) | 0.1 |
| | Monotonic shape | Yes |
| | Maximum charge | 1 |
| | Representative isotope | Most intense |
| **Alignment: Join Aligner** | m/z tolerance (m/z) | 0.005 or 5ppm |
| | Weight for m/z | 20 |
| | Retention time tolerance (absolute (min)) | 0.1 |
| | Weight for RT | 10 |
| **Gap Filling: Peak Finder** | Intensity tolerance (%) | 5 |
| | m/z tolerance (m/z) | 0.005 or 5ppm |
| | Retention time tolerance (absolute (min)) | 0.1 |
| **Normalization: Linear Normalization** | Total raw signal | - |
| | Peak area | - |
| **Data Export** | Aligned list | Row ID, row m/z, row retention time, and peak height |
| | Gap-filled list | Row ID, row m/z, row retention time, and peak height |

| | | |
|---|---|---|
| | Normalized list | Row ID, row m/z, row retention time, and peak area |
| **R analysis – *muma*** | Scaling type | Pareto |

**Appendix VI: Script(s)**

***Muma* – Multivariate Analysis**

>setwd("C:/Users/Paradox/Desktop/2/muma/Active/bu1/bu1.csv")

>explore.data(file = "bu1.csv", scaling = "pareto")

>Plot.pca(pcx = 1, pcy = 2, scaling = "pareto")

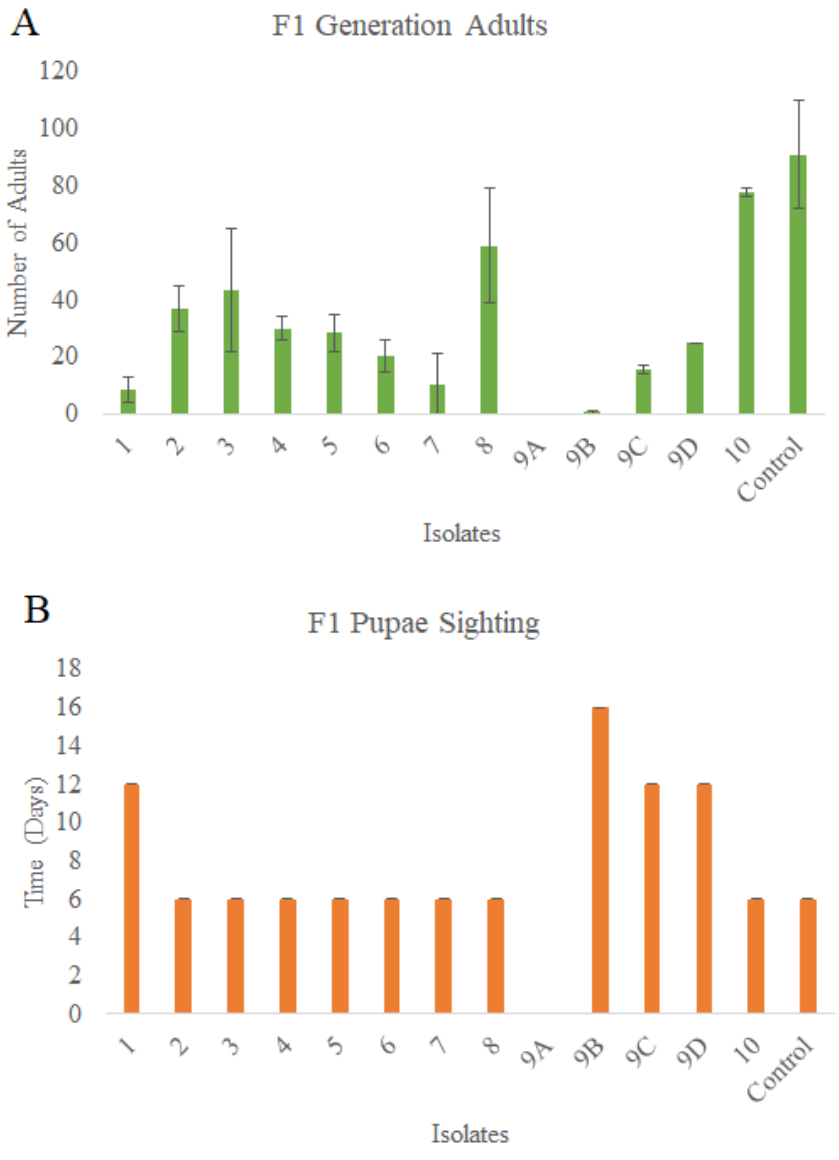
>Plot.pca(pcx = 1, pcy = 3, scaling = "pareto")

>Plot.pca(pcx = 2, pcy = 3, scaling = "pareto")

> oplsda(scaling=pareto)

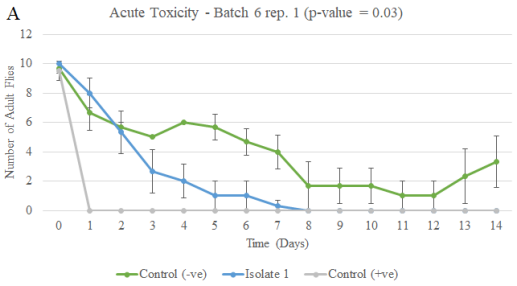
**Appendix VII: Preliminary Insect Results**

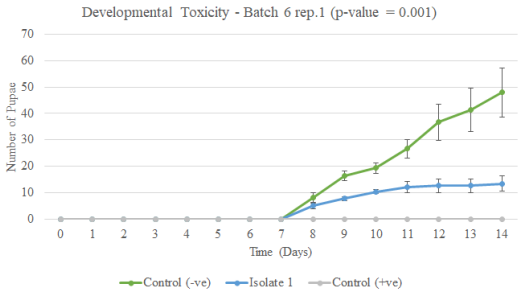
Batch 2 was used to carry out the preliminary bioassay using reconstituted broths from Isolates 1-10. The broths were dried from which 30% mass of each broth was mixed with 5 ml cool tap water, 1 g of dry fly food, and a pinch of yeast granules. Tap water mixed with 2.5% Tween was used as the carrier control. Flies were knocked-out using $CO_2$ and separated into groups of 10. Each group was placed in a vial and the number of F1 flies were observed daily for 14 days in ambient light and room temperature along with the time take for pupae sighting.



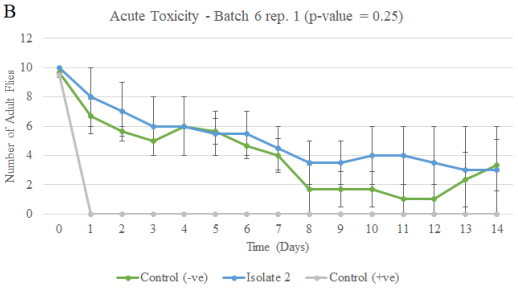
Two fly bioassays were carried out using reconstituted broths of isolates 1-10 including using PDB+Tween as the carrier control and 200 µl of Tea Tree Oil as the positive control. The first bioassay consisted of triplicates of each treatment from batch 6. 30ml of each broth was freeze-dried before resuspending to 30 ml in 1.25% Tween solution at a 1× concentration. For this bioassay, 3 × 5 ml of extract was mixed with 1g of dry fly food before adding a pinch of yeast granules. Flies were knocked out using $CO_2$ and grouped in numbers of 10 with a 1:1 sex ratio before transferring 1 group to each vial. The second bioassay used the same stock solution from batch 6 but was performed using duplicates thus each vial consisted of 7.5 ml extract, 1.5g of dry fly food, and a pinch of yeast granules. Flies were knocked out and groups in a similar manner before transferring into separate vials. Both bioassays were carried out in ambient light and room temperature. The number of adults and pupae were noted at 24-hour intervals for a 2-week period. The data was tabulated in Microsoft Excel where the standard deviation, standard error, and ANOVA test was performed on days 11-14.

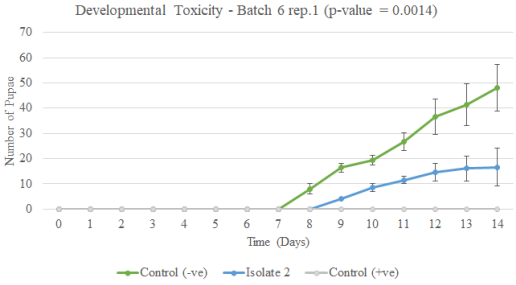
A


Acute Toxicity - Batch 6 rep. 1 (p-value = 0.03)
Number of Adult Flies
Time (Days)
Control (-ve)
Isolate 1
Control (+ve)

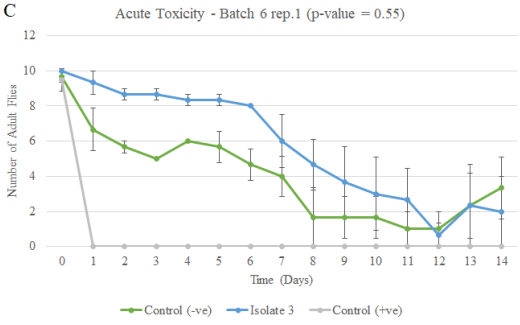

Developmental Toxicity - Batch 6 rep.1 (p-value = 0.001)
Number of Pupae
Time (Days)
Control (-ve)
Isolate 1
Control (+ve)

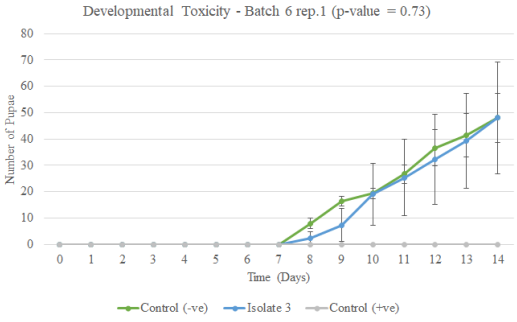
B


Acute Toxicity - Batch 6 rep. 1 (p-value = 0.25)
Number of Adult Flies
Time (Days)
Control (-ve)
Isolate 2
Control (+ve)

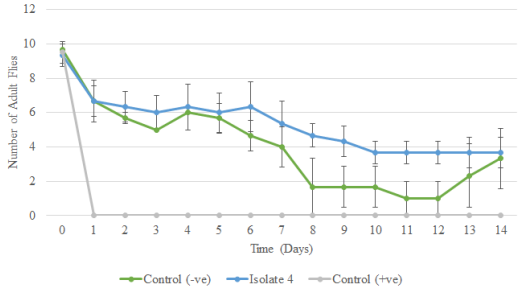

Developmental Toxicity - Batch 6 rep.1 (p-value = 0.0014)
Number of Pupae
Time (Days)
Control (-ve)
Isolate 2
Control (+ve)

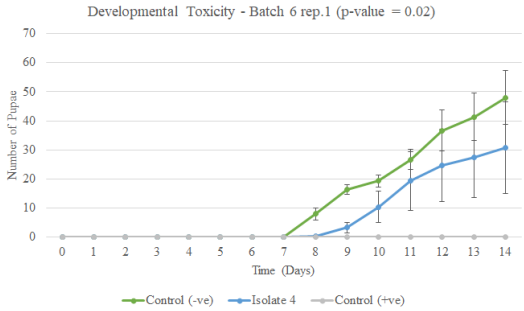
C


Acute Toxicity - Batch 6 rep.1 (p-value = 0.55)
Number of Adult Flies
Time (Days)
Control (-ve)
Isolate 3
Control (+ve)

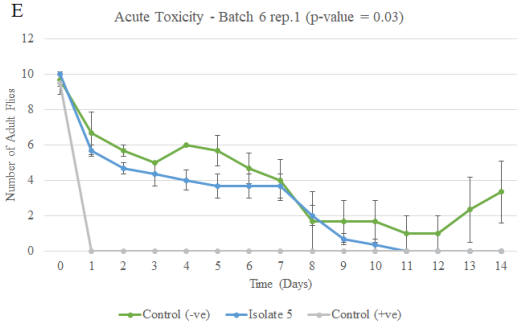

Developmental Toxicity - Batch 6 rep.1 (p-value = 0.73)
Number of Pupae
Time (Days)
Control (-ve)
Isolate 3
Control (+ve)

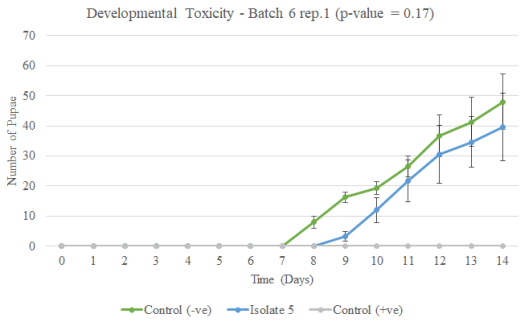
D


Acute Toxicity - Batch 6 rep.1 (p-value = 0.1)
Number of Adult Flies
Time (Days)
Control (-ve)
Isolate 4
Control (+ve)

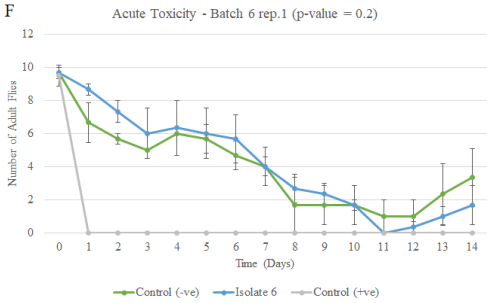

Developmental Toxicity - Batch 6 rep.1 (p-value = 0.02)
Number of Pupae
Time (Days)
Control (-ve)
Isolate 4
Control (+ve)

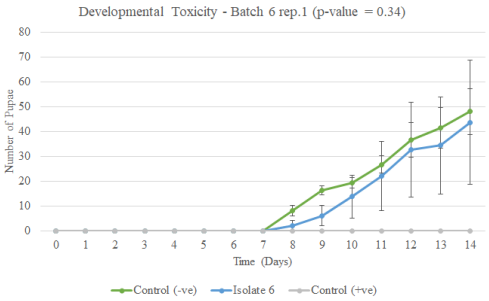
E


Acute Toxicity - Batch 6 rep.1 (p-value = 0.03)
Number of Adult Flies
Time (Days)
Control (-ve)
Isolate 5
Control (+ve)

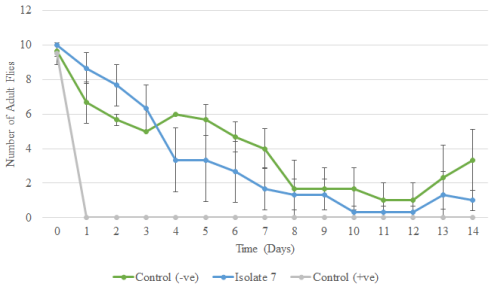

Developmental Toxicity - Batch 6 rep.1 (p-value = 0.17)
Number of Pupae
Time (Days)
Control (-ve)
Isolate 5
Control (+ve)

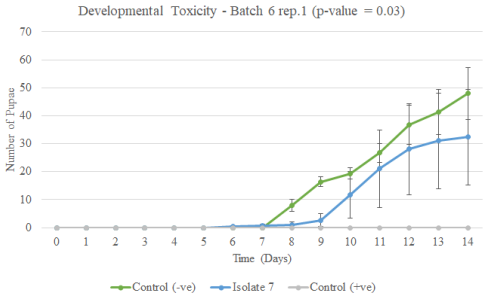
F


Acute Toxicity - Batch 6 rep.1 (p-value = 0.2)
Number of Adult Flies
Time (Days)
Control (-ve)
Isolate 6
Control (+ve)

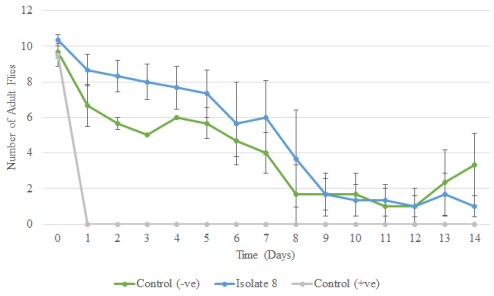

Developmental Toxicity - Batch 6 rep.1 (p-value = 0.34)
Number of Pupae
Time (Days)
Control (-ve)
Isolate 6
Control (+ve)

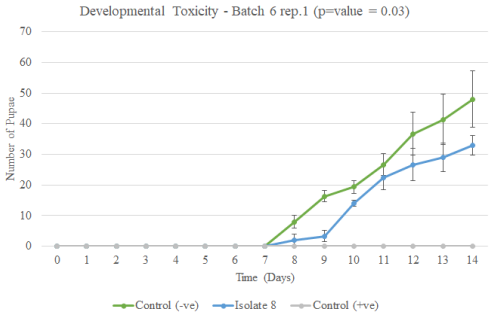
G


Acute Toxicity - Batch 6 rep.1 (p-value = 0.14)
Number of Adult Flies
Time (Days)
Control (-ve)
Isolate 7
Control (+ve)

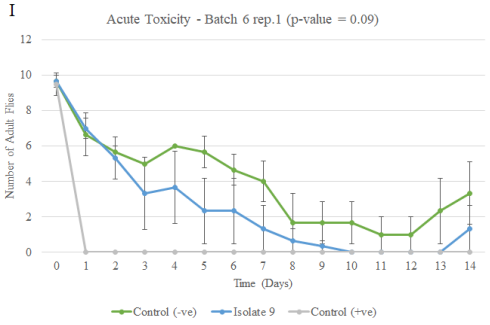

Developmental Toxicity - Batch 6 rep.1 (p-value = 0.03)
Number of Pupae
Time (Days)
Control (-ve)
Isolate 7
Control (+ve)

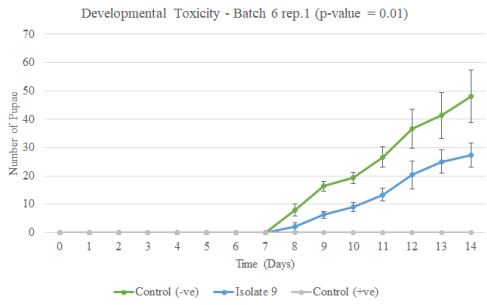
H


Acute Toxicity - Batch 6 rep.1 (p-value = 0.23)
Number of Adult Flies
Time (Days)
Control (-ve)
Isolate 8
Control (+ve)

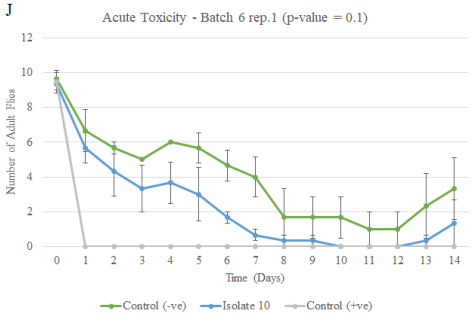

Developmental Toxicity - Batch 6 rep.1 (p=value = 0.03)
Number of Pupae
Time (Days)
Control (-ve)
Isolate 8
Control (+ve)

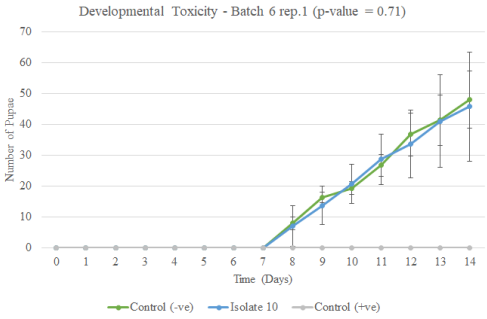
I


Acute Toxicity - Batch 6 rep.1 (p-value = 0.09)
Number of Adult Flies
Time (Days)
Control (-ve)
Isolate 9
Control (+ve)

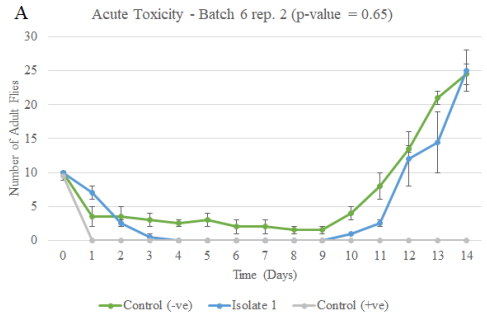

Developmental Toxicity - Batch 6 rep.1 (p-value = 0.01)
Number of Pupae
Time (Days)
Control (-ve)
Isolate 9
Control (+ve)

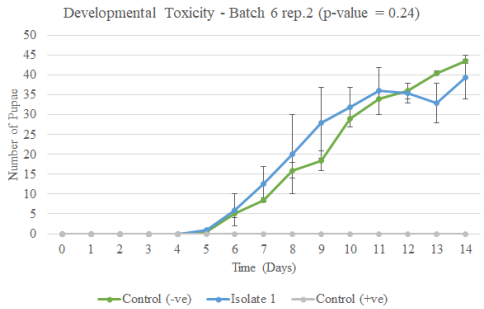
J


Acute Toxicity - Batch 6 rep.1 (p-value = 0.1)
Number of Adult Flies
Time (Days)
Control (-ve)
Isolate 10
Control (+ve)

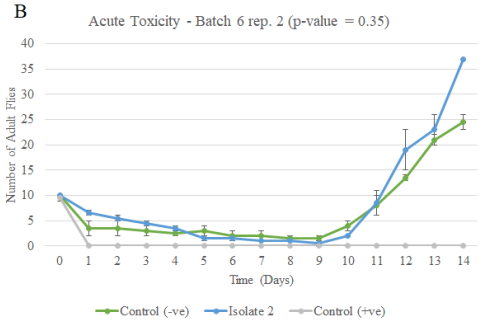

Developmental Toxicity - Batch 6 rep.1 (p-value = 0.71)
Number of Pupae
Time (Days)
Control (-ve)
Isolate 10
Control (+ve)

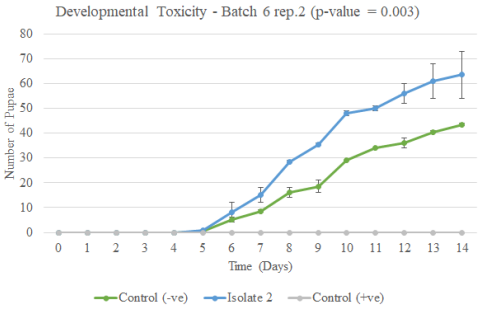
A


Acute Toxicity - Batch 6 rep. 2 (p-value = 0.65)
Number of Adult Flies
Time (Days)
Control (-ve)
Isolate 1
Control (+ve)

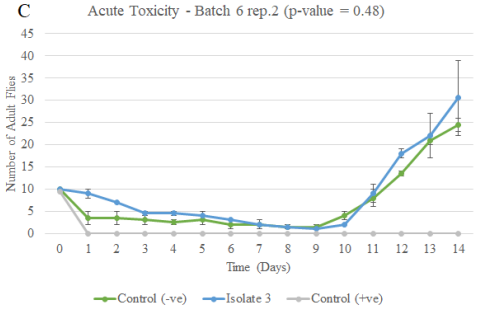

Developmental Toxicity - Batch 6 rep.2 (p-value = 0.24)
Number of Pupae
Time (Days)
Control (-ve)
Isolate 1
Control (+ve)

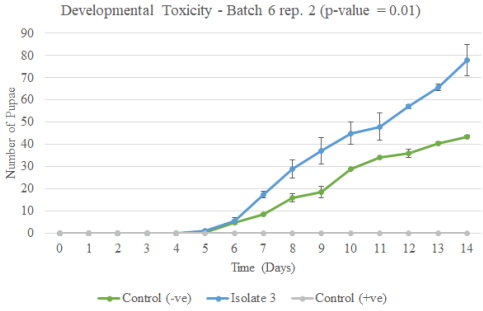
B


Acute Toxicity - Batch 6 rep. 2 (p-value = 0.35)
Number of Adult Flies
Time (Days)
Control (-ve)
Isolate 2
Control (+ve)

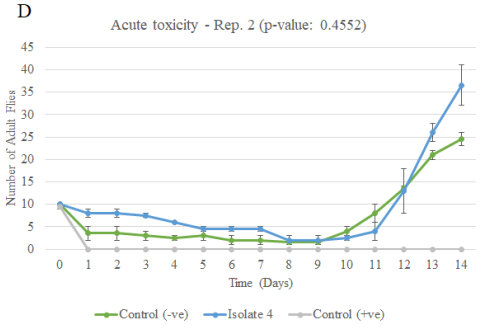

Developmental Toxicity - Batch 6 rep.2 (p-value = 0.003)
Number of Pupae
Time (Days)
Control (-ve)
Isolate 2
Control (+ve)

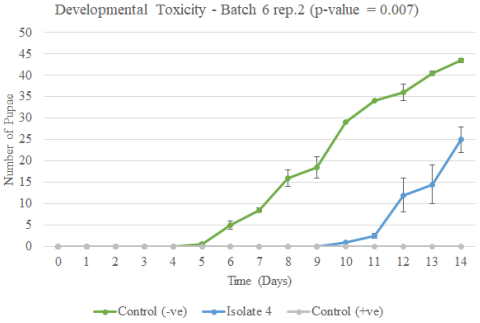
C


Acute Toxicity - Batch 6 rep.2 (p-value = 0.48)
Number of Adult Flies
Time (Days)
Control (-ve)
Isolate 3
Control (+ve)

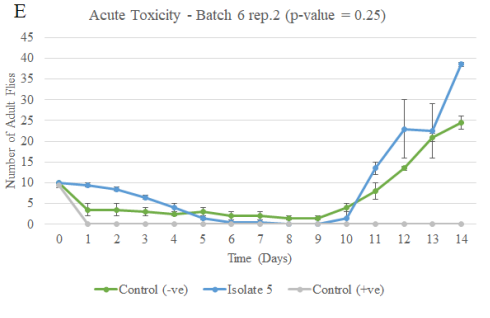

Developmental Toxicity - Batch 6 rep. 2 (p-value = 0.01)
Number of Pupae
Time (Days)
Control (-ve)
Isolate 3
Control (+ve)

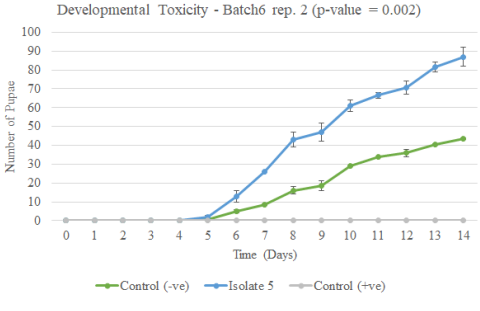
D


Acute toxicity - Rep. 2 (p-value: 0.4552)
Number of Adult Flies
Time (Days)
Control (-ve)
Isolate 4
Control (+ve)

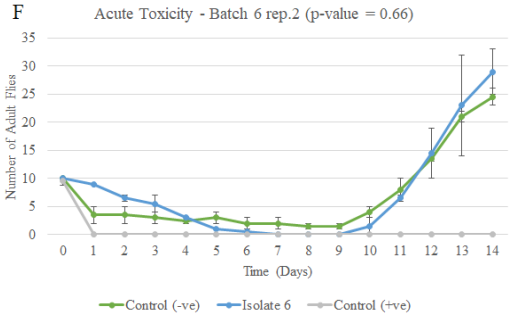

Developmental Toxicity - Batch 6 rep.2 (p-value = 0.007)
Number of Pupae
Time (Days)
Control (-ve)
Isolate 4
Control (+ve)

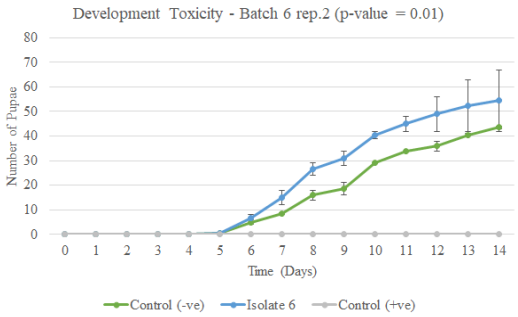
E


Acute Toxicity - Batch 6 rep.2 (p-value = 0.25)
Number of Adult Flies
Time (Days)
Control (-ve)
Isolate 5
Control (+ve)

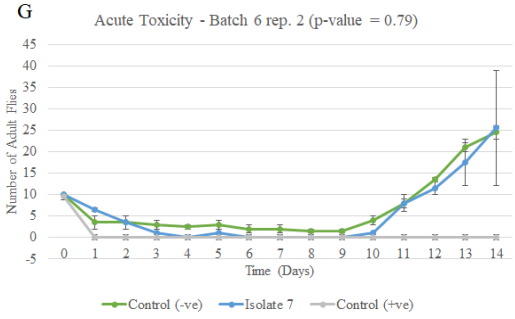

Developmental Toxicity - Batch6 rep. 2 (p-value = 0.002)
Number of Pupae
Time (Days)
Control (-ve)
Isolate 5
Control (+ve)

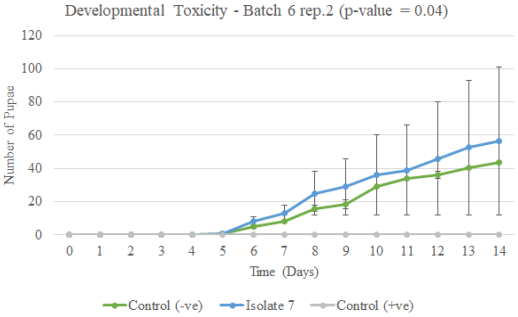
F


Acute Toxicity - Batch 6 rep.2 (p-value = 0.66)
Number of Adult Flies
Time (Days)
Control (-ve)
Isolate 6
Control (+ve)

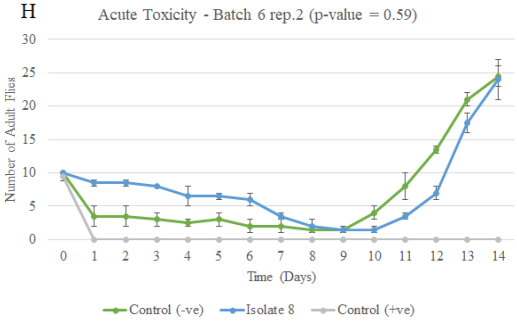

Development Toxicity - Batch 6 rep.2 (p-value = 0.01)
Number of Pupae
Time (Days)
Control (-ve)
Isolate 6
Control (+ve)

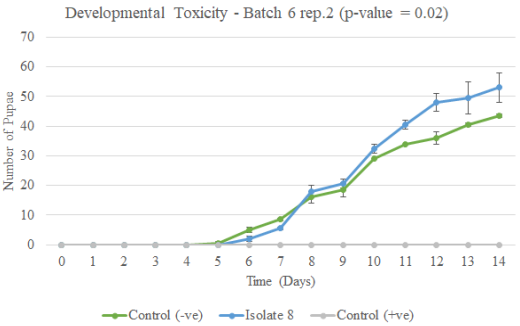
G


Acute Toxicity - Batch 6 rep. 2 (p-value = 0.79)
Number of Adult Flies
Time (Days)
Control (-ve)
Isolate 7
Control (+ve)

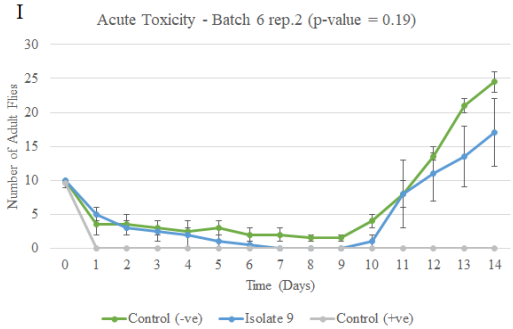

Developmental Toxicity - Batch 6 rep.2 (p-value = 0.04)
Number of Pupae
Time (Days)
Control (-ve)
Isolate 7
Control (+ve)

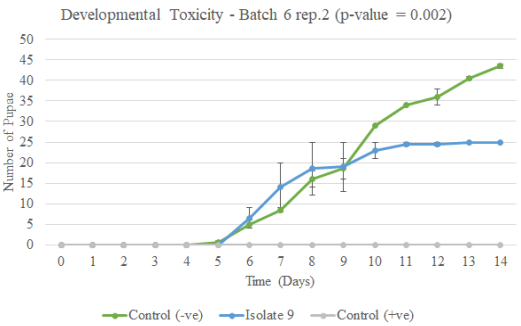
H


Acute Toxicity - Batch 6 rep.2 (p-value = 0.59)
Number of Adult Flies
Time (Days)
Control (-ve)
Isolate 8
Control (+ve)

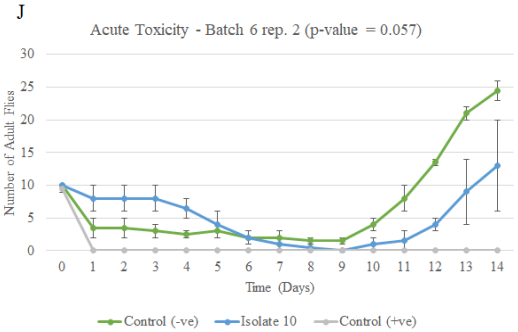

Developmental Toxicity - Batch 6 rep.2 (p-value = 0.02)
Number of Pupae
Time (Days)
Control (-ve)
Isolate 8
Control (+ve)

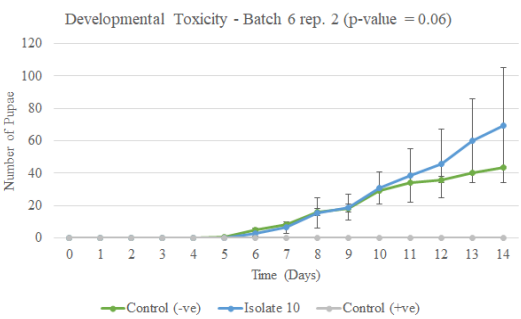
I


Acute Toxicity - Batch 6 rep.2 (p-value = 0.19)
Number of Adult Flies
Time (Days)
Control (-ve)
Isolate 9
Control (+ve)

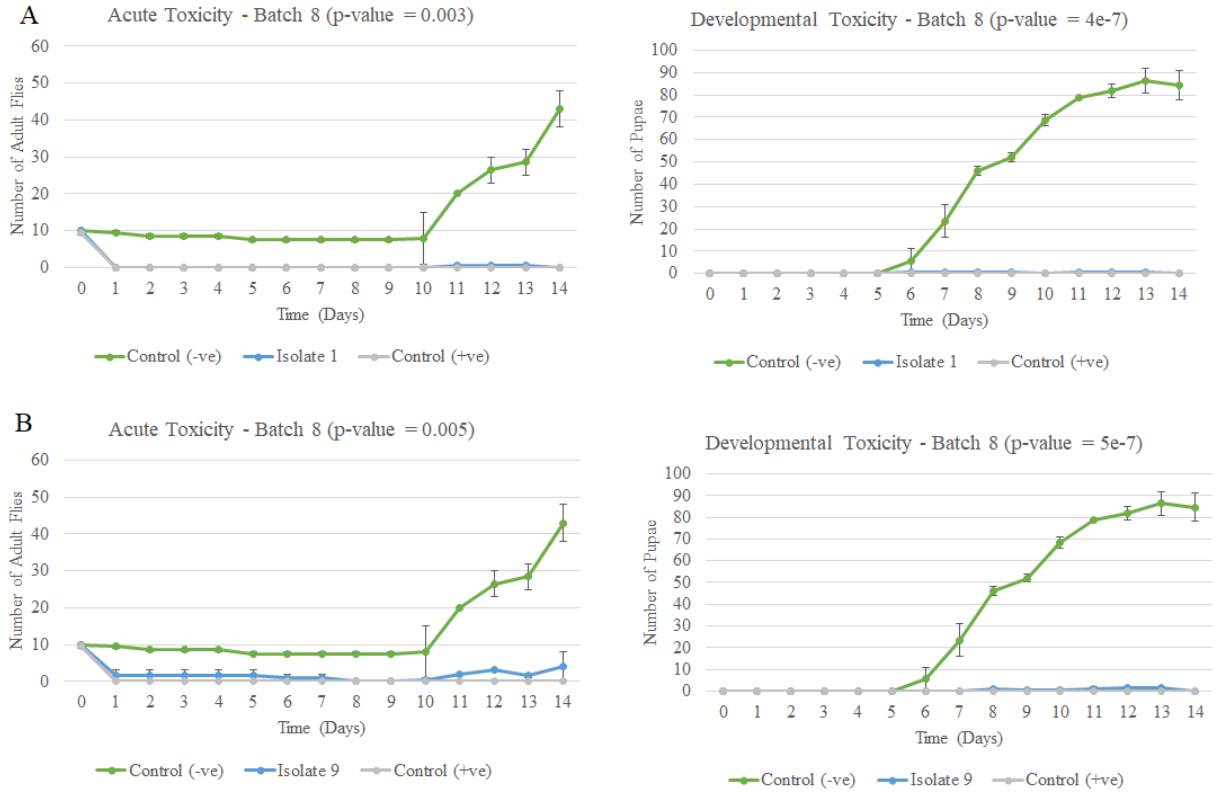

Developmental Toxicity - Batch 6 rep.2 (p-value = 0.002)
Number of Pupae
Time (Days)
Control (-ve)
Isolate 9
Control (+ve)

J


Acute Toxicity - Batch 6 rep. 2 (p-value = 0.057)
Number of Adult Flies
Time (Days)
Control (-ve)
Isolate 10
Control (+ve)

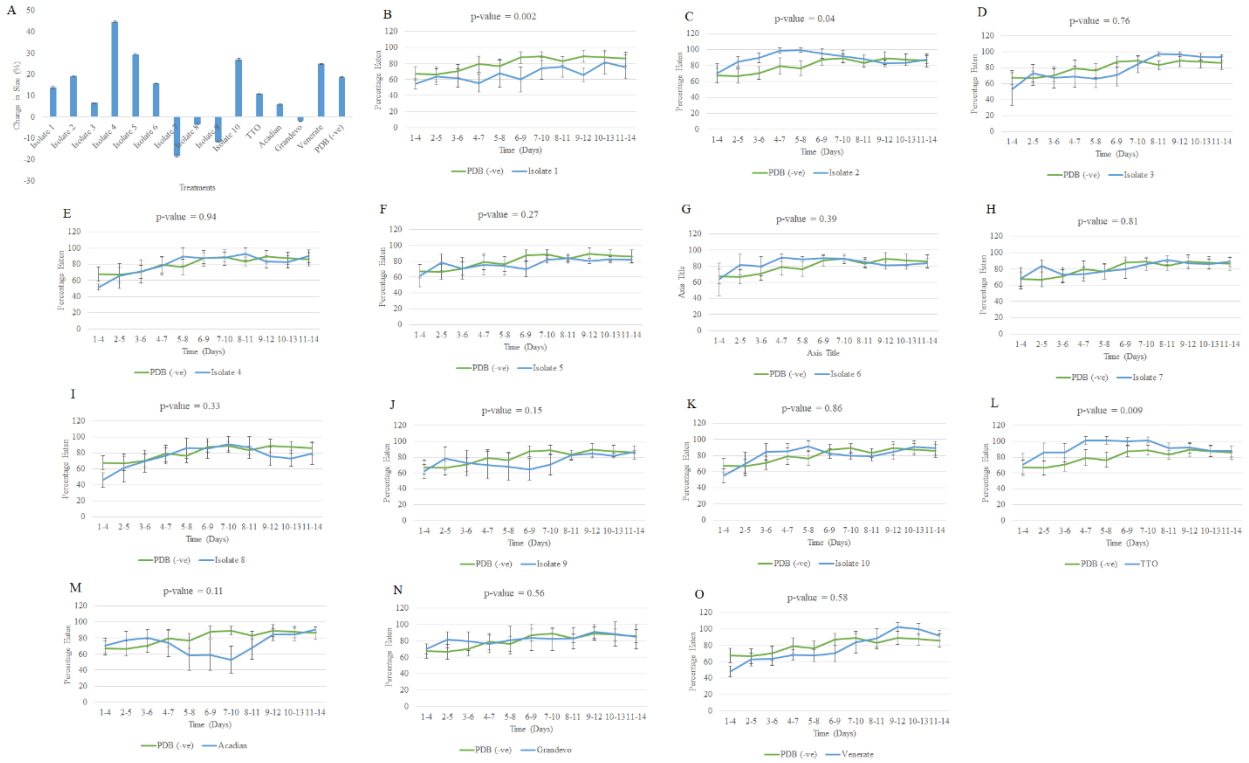

Developmental Toxicity - Batch 6 rep. 2 (p-value = 0.06)
Number of Pupae
Time (Days)
Control (-ve)
Isolate 10
Control (+ve)

Reconstituted broths of Isolate 1 and 9 from batch 8 were testing for inhibitory activity at 2× concentration. PDB+1.25% Tween was used as the carrier control while 200 µl of Tea Tree Oil was used as the positive control. 30 ml of each reconstituted broth was freeze-dried and then resuspended to 15ml using 1.25% Tween. 5 ml of extract was mixed with 1 g of dry fly food before adding yeast granules. Flies were knocked out using $CO_2$ and grouped in numbers of 10 with a 1:1 sex ratio before transferring each group to a vial. Duplicates were used for this bioassay. The vials were kept in an incubator at 24 ± 0.11 °C with a 12-hour light/dark cycle. The number of adults and pupae were noted at 24-hour intervals for a 2-week period. The data was tabulated in Microsoft Excel where the standard deviation, standard error, and ANOVA test was performed on days 11-14.



## Appendix VIII: Preliminary Snail Results

Batch 3 was used to carry out one of the preliminary snail bioassays using reconstituted broths from Isolates 1-10 including commercially available natural products – Tea Tree Oil, Venerate, Grandevo, and Acadian. 30% mass of freeze-dried broths of each Isolate was added to 1L of dechlorinated water in the 'experimental tank' containing 2 snails. Similarly, 1 ml of Tea Tree Oil, Venerate and Acadian and 1 g of Grandevo was added to their designated tanks. 30% dried mass of PDB was used as the carrier control. The exposure duration was 4 hours before transferring the snails in the 'observation' tanks. The size of each snail was measured prior to exposure and on day 15. Feeding behaviour was monitored every 24 hours by observing amount eaten for a 14-day period. A 4-day frame-shift average was calculated for the amount eaten to reduce variability before performing a 1-way ANOVA followed by a two-tail t-test to assess the significance of the data.

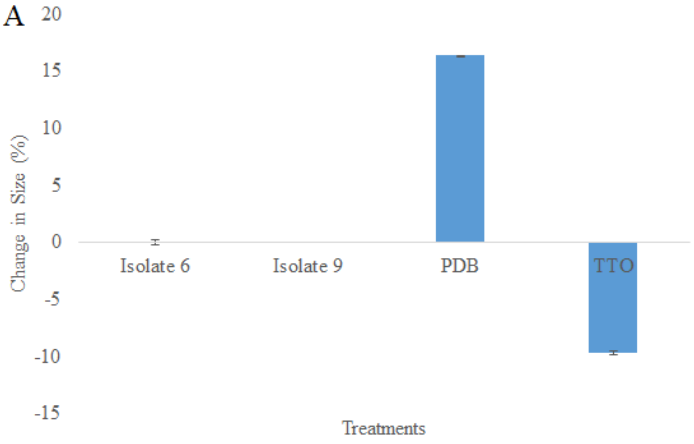


15 ml of reconstituted broths of Isolates 1-10 (batch 6) were used for the following bioassay along with testing the commercially available natural products including STK-53. PDB was used as the carrier control. This bioassay followed a similar protocol as described for batch 3 however the exposure duration was increased to 6 hours.

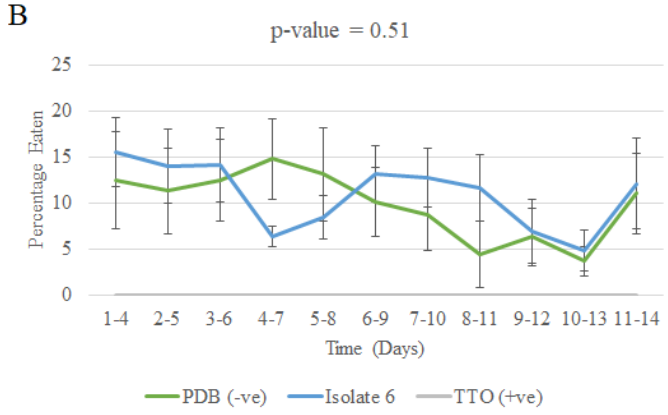
15 ml of reconstituted broths of Isolates 1-10 (batch 6) were used for the following bioassay along with testing the commercially available natural products. PDB was used as the carrier control. This bioassay followed a similar protocol as described above for batch 6 however the volume of dechlorinated water in the 'experimental' tank was reduced from 1 L to 500 ml.

30 ml of reconstituted broths of Isolates 6 and 9 (batch 8) were used for the following bioassay with PDB as the carrier control and TTO as the positive control. This bioassay included an exposure duration of 6 hours in 500 ml of dechlorinated water.

A




B




C