Development of a High-Throughput Screening System to Assess the Effect of Plant Extracts on Mammalian Cancer Cells

A Major Qualifying Project Report submitted to the Faculty of
WORCESTER POLYTECHNIC INSTITUTE
In partial fulfillment of the requirements for the Degree of Bachelor of Science in
CHEMICAL ENGINEERING

26 April 2018

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ABSTRACT

There is currently a heightened interest in the use of plants from pharmaceutical industries due to its genetic diversity and ability to make specialized compounds as a defense mechanism. More specifically, the National Cancer Institute previously performed a high throughput screening to identify over 88,000 extracts with anticancer properties. However, the use of whole plants to generate these specialized metabolites has numerous drawbacks, specifically the low and inconsistent production of compounds. This project aims to improve upon this screening system and develop a high-throughput screening system with the use of plant cell extracts to produce more reliable and consistent results, which can potentially lead to the discovery of novel specialized metabolites. By determining the optimal concentration of the solvent in which to extract plant metabolites as 0.01% (v/v) ethanol, the effects of the compounds being tested are further validated as it ensures there is very little interference or influence from the solvent. Additionally, the application of pure paclitaxel allowed the development of a standard response from each cancer cell line. Furthermore, the use of *Taxus* extracts on the screening system validated its use as the paclitaxel present in the extracts, specifically in 48-82A-2 and 1101-62A-5, resulted in consistent results in three different cancer cell lines as a decrease in cell viability.

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CHAPTER 1. INTRODUCTION

Plants have been historically utilized for medicinal purposes, and therefore, there is a significant research focus for drug discovery. In 1971, extracts obtained from the bark of *Taxus brevifolia* were found to have cytotoxic properties against various types of cancer cells, which led to the development of the first FDA-approved plant cell culture-based chemotherapy drug, Taxol™ (generic name paclitaxel)[1]. This project ultimately aims to search for other plant extracts (and compounds) that exhibit anticancer properties to potentially become new chemotherapy drug candidates.

1.1 Plants, the Environment, and their Pharmaceutical Promise

1.1.1 Specialized Metabolism

Historically, over 80% of the world has relied on plants for traditional medicine[2]. This can be attributed to the bioactive specialized (formerly known as secondary) metabolites that plants produce and secrete to defend or adapt to their environment. Fortunately, many of these compounds have been found to demonstrate therapeutic properties. Plants produce two broad classes of metabolites: conserved and specialized metabolites. Conserved metabolites are classified as compounds produced to sustain plant life and growth, such as cell division, photosynthesis, storage, and reproduction[3]. Specialized metabolites, on the other hand, are very low in abundance, and stored away in specific organelles of the cell[3]. Specialized metabolites play a significant role in a plant's adaptation to the environment as they are specific compounds that allow the plant to better integrate within the ecosystem[3] (Bourgaud, 2001). For instance, to combat stressful situations in the environment, phytoalexins are secreted to protect against pathogens or alleopathy to protect from other plants[4]. Additionally, UV absorbing compounds can be secreted to protect the leaves from radiation[4]. Overall, different classes of specialized metabolites harnessed from medicinal plants provide a valuable platform for drug development in the pharmaceutical industry.

1.1.2 Impact in the Pharmaceutical Industry

For decades, the mission of the pharmaceutical industry has been to "discover and develop new drugs for the treatment of disease"[5]. This mission still holds true today as drug development continually evolves to meet consumers' needs and demands. Drug development primarily relied on metabolites produced by microorganisms[5]. However, from 1930 to 1970, pharmaceutical

companies, such as Merck and Bristol-Meyers Squibb, focused on their efforts on manufacturing natural products from plants. Pharmaceutical manufacturers have taken a liking to plant and other natural products because of their genetic diversity and ability to make unique compounds. During 1990 to 2000, or more commonly known as the Green Rush, pharmaceutical companies worldwide increased their research efforts to discover novel natural products[6]. With technologies such as the High-Performance Liquid Chromatography (HPLC), High Resolution Mass Spectrometry (HRMS), and High Field Nuclear Magnetic Resonance Spectrometry (NMR), it was made possible to characterize these natural products. Although natural products were highly beneficial, harvesting the plants posed many problems. For instance, there are high harvesting costs as many plant species flourish in specific environments and depletion of species lead to endangerment[7] **(citation).** Despite these setbacks, plants have continuously contributed to the discovery and development natural product-based medicine.

1.1.3 Impact in Research

Plants have gained momentum in cancer research as at least 250,000 plant species have been reported to contain significant anticancer properties[8]. This finding led to the creation of the Developmental Therapeutics Program (DTP) by the National Cancer Institute (NCI) in 1955 in order to discover and develop anticancer agents as potential therapeutic treatments for cancer patients[8]. Since its creation, the DTP has screened over 88,000 different plant extracts against 60 human tumor cell lines, including lung, brain, colon, ovarian, and breast cancer[8]. This high-throughput screening from the DTP sparked multiple screenings of different plant extracts on tumor cell lines done at various institutions. The anti-proliferative properties of grape extracts were assessed against liver (HepG2) and cervical (HeLa) cancer cell growth, as grapes have been known for their positive effects on human health[9]. Plant extracts can exhibit more than one biological activity. Lemon balm, or *Melissa officinalis*, is commonly used for sleep and gastrointestinal disorders; however, more recently it was found to inhibit the viability of various tumor cell lines, such as human colon cancer (HCT 116) and breast cancer (MCF-7), in a concentration-dependent manner[10]. These findings show promise in the use of plant extracts as chemotherapy drugs.

1.1.4 Paclitaxel

Paclitaxel (Taxol^{TM}) was discovered in the 1960's from the extensive screening performed the NCI. Taxanes are a family of molecules that all exhibit a similar molecular structure and some serve as microtubule inhibitors. As a member of the taxane class, the primary mechanism of

paclitaxel is the "suppression of microtubule spindle dynamics"[11]. This inability to transition from metaphase to anaphase leads to the inhibition of mitosis, which ultimately causes the cell to undergo apoptosis. The unique characteristic of paclitaxel that makes it very successful as a chemotherapy drug is its ability to stabilize microtubules to prevent tubulin disassembly, especially against rapidly dividing cells such as those creating tumors[12].

Originally extracted from the Pacific yew tree, or *Taxus breviforia*, paclitaxel showed unique anti-proliferative properties that propelled it to become the first FDA approved plant cell culture-based chemotherapy drug[11]. As a natural product therapy, it gained tremendous success due to its high response rates against a variety of tumors, such as breast, ovarian, lung, and bladder cancer.

1.2 Plant Cell Culture

Plant tissue culture has played a more crucial role in the production of small molecules and recombinant pharmaceuticals[13] (Xu, 2015). Plant cell culture is a technique where de-differentiated cells, tissues, and organs of plants are introduced into a sterile artificial environment with appropriate conditions for the plant cells to thrive[13]. Because plant cell culture is an *in vitro* technique, conditions can be changed to optimize synthesis and yields of specialized metabolites[14]. Cell growth is mainly supported by a basic medium, composed of water, macro- and micro-nutrients, and a carbohydrate source, to replace the carbon obtained through the atmosphere by photosynthesis[7]. The cells usually form a callus – an undifferentiated mass of cells which can give rise to a functional part of a plant due to its totipotency – capable of growing, dividing, and giving rise to any cell type[15]. Suspension cultures arises when the callus cells are allowed to proliferate in a liquid medium in order to break up the cell aggregate of the callus for faster proliferation. Figure 1 demonstrates the development of a plant cell culture.

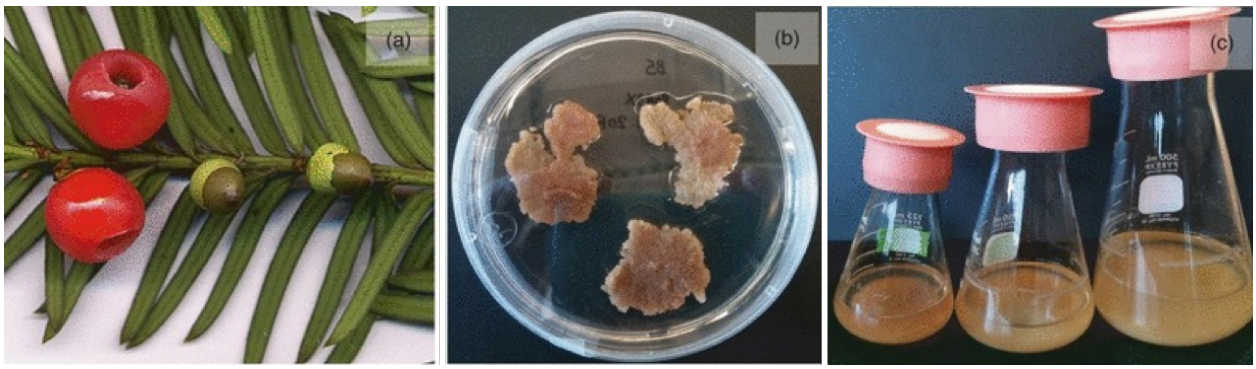

Figure 1. Development of a plant tissue culture. A) sterile plant; B) callus culture; C) suspension culture. (Recent advances towards development and commercialization of plant cell cultures processes for the synthesis of biomolecules. (Adapted from Wilson, 2012)

1.2.1 Using Whole Plants for Drug Discovery

One of the main gaps in the medicinal assessments of plants is the inconsistent production of specialized metabolites and loss of original plant collections[1]. Some plants have relatively short lifespans, therefore, it is possible that once the specialized metabolites have been characterized, the primary plant source has been depleted. The metabolite may only be produced by a specific species or genus, and may only be activated during a specific developmental stage or under a particular environmental condition[16]. The entire plant may be unable to produce the specialized metabolite, only a specific section, or a different compound profile may be present. Different samples of the sample plant can contain various amounts of metabolic compounds, as the metabolites are produced and stored in different plant organs and/or cells[6]. Furthermore, harnessing these specialized metabolites from whole plants can be very challenging and expensive, may miss identification of potent compounds, and may only be able to collect and produce very low yields[16].These challenges of using whole plants for drug discovery can be overcome by assessing cells as plant cell culture, a generic cell that can be accounted for the whole plant under different conditions.

1.2.2 Benefits of Plant Cell Cultures

Plants cells grown *in vitro*, however, have a more controlled, sterile environment that allows for precise and consistent control over cell growth and protein production[13] (Xu, 2015). With plant cell cultures, the continuous production of specialized metabolites in large-scale bioreactors can be used to scale up the production of the plant cell cultures[16,17]. Plant cell cultures are less prone to the possibility of contamination with agrochemicals and fertilizers, pests and diseases, different cultivation conditions, such as soil quality, microclimate, and other local differences[7]. Furthermore, plant cell culture allows for the conservation of endangered plant species for future generations[16]. These benefits of plant cell cultures therefore make them more advantageous compared to whole plants.

1.2.1 UMASS Plant Cell Culture Library

The University of Massachusetts–Amherst contains one of the largest living plant cell culture collections, from over 1,000 diverse plant species worldwide[18]. This library is accessible for the discovery and identification of novel specialized metabolites that can potentially be harnessed for

medicinal purposes[18]. Additionally, the plant cell culture library (PCCL) contains a database of medicinal properties from peer-reviewed literature based on the cultures in the collection, such as antivirial, antifungal, and anticancer. Through the PCCL, it is possible to re-create and improve upon the screening performed by the NCI. This provides the opportunity to use of plant cell cultures rather than whole plants for controlled specialized metabolite synthesis (e.g., under stress conditions).

1.3 Elicitation

As previously mentioned, plants have the ability to produced specialized metabolites in response to pathogenic attacks, such as from bacteria and fungi. As plant cell cultures are utilized, elicitation arises as a promising method to stimulate and enhance the production of specialized metabolites in plant cells *in vitro*[4]. Elicitation is defined as the stimulation of stress conditions that occur in the natural environment[19]. Elicitors can be classified into two broad categories: abiotic and biotic elicitors (Figure 2).

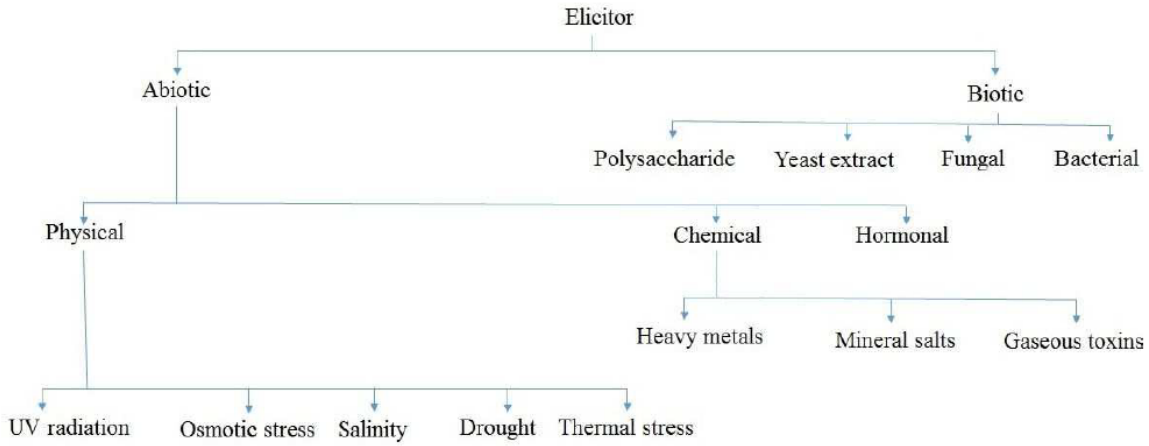

Figure 2. Classifications of elicitors. (Adapted from Naik & Al-Khayri, 2015)

1.3.1 Abiotic Elicitors

Abiotic elicitors can be classified as physical factors and chemical compounds that increase flux to the production of specialized metabolites[19]. Examples of inorganic elicitors are salts and heavy metal ions (e.g., copper, cadmium, and calcium)[4]. Physical stressors can be considered as UV light, windfall, freezing and thawing cycles, high pH, or high pressure[4,16]. Additionally, chemical stressors such as pesticides and aerosols can disrupt the plant cell membranes and elicit specialized metabolite synthesis[19].

1.3.2 Biotic Elicitors

Biotic elicitors can be classified as any compound of biological origin such as yeast, pathogens, and bacteria[16,20]. Pathogens trigger a defense response by inducing a signal cascade in the plant leading to alterations in gene expression. Yeast is a biotic elicitor that can be used in many applications. For example, it was used to elicit the production of ethylene in a tomato[19]. In addition, other fungi can be used to elicit specialized metabolites in medicinal plant cell cultures. Fungal spores have been shown to increase the amount of codeine and morphine in the plant species *Papaver somniferum*[19]. *Taxus chinensis* cells have also been treated with an endophytic fungus that increased cellular production of paclitaxel. Scientists believe that biotic elicitors function by binding to the membrane receptor of the plant that signals reactive oxygen species (ROS) to be produced which are converted into specialized metabolites[19].

1.3.2.1 Methyl Jasmonate

Jasmonates, more specifically methyl jasmonate (MeJa), are classified as biotic elicitors as they are commonly secreted by plants when attacked or wounded to signal stress to other cells in the organism[16]. MeJa plays a significant role in the regulation of plant defense genes through signal transduction pathways leading to the production of specialized defense compounds. Because of this property, the application of MeJa to plant cell cultures can induce production of various specialized metabolites, such as terpenoids, flavonoids, and alkaloids[4]. In regards to the production of paclitaxel, MeJa was reported to be one of the most effective methods in enhancing paclitaxel production from *Taxus* cell cultures[16]. MeJa has been found broadly to influence most plant species; for example, it was beneficial for vinblastine, an anticancer compound extracted from *Catharantus roseus* hair roots. With an optimal concentration of MeJa, it is possible to enhance production of a variety of specialized metabolites, many with anticancer properties.

1.3.2.2 Endophytic Fungi and Yeast Extracts

As previously mentioned, elicitation plays a key role in plant production of specialized metabolites, which can be utilized for pharmaceutical purposes. Though the yield of specialized metabolites within plant cell cultures is often low in comparison to that of whole plants, there is a wide range of bioactive compounds detected within the plant cell cultures[4]. The use of fungal cell wall fragments of *Trichoderma viride*, *Aspergillus niger*, and *Fusarium moniliforme* was used to elicit *Catharanthus roseus* cell cultures to enhance the levels of specialized metabolites, such as ajmalicine. In these studies, higher concentration of the fungal fragments increased the production

levels of ajmalicine[4]. Biotic elicitors, such as endophytic microbes and yeast, show significant promise for increasing the yield of specialized metabolites in plants as they have been shown to have coevolved with similar pathways to produce natural products[21].

Endophytic fungi are of particular interest as they have demonstrated an established relationship with the plant host through parasitic or mutualistic methods without causing negative effects to the plant[22]. The synergistic relationship between endophytic fungi and plants suggest endophytes as a promising source of elicitors of specialized metabolites as it is utilized for the facilitation of the fungi's innate connection to the plant and the protection from invading pathogens[23]. Because of this synergistic relationship, both endophytic fungi and their respective plant host share similar metabolic pathways that allow the transfer of information and production of similar specialized metabolites[22]. Plants with medicinal properties are often hosts to endophytic fungi which provides the additional production of novel and pharmaceutically promising metabolites[22]. For instance, a study performed by Wu, et. al. (2015) demonstrated how endophyte-derived secondary metabolites can play a role in the anticancer properties of fruit. Based on their study, they determined three of the leaf-derived endophytic fungi inhibited the growth of lung, prostate, and breast, all of which are human carcinoma cell lines[22].

To attain the elicited plant extracts for this project, *Saccharomyces cerevisiae* (common baker's yeast), a thoroughly researched fungus, was used as the fungal elicitor. Because yeast, fungi, and mammals share the same highly conserved basic signaling pathways and cellular machinery, *S. cerevisiae* is an excellent model for the identification of anticancer and antifungal compounds[24]. Qaddouri reported to identify "14 plant extracts and 8 natural product molecules with clear growth-inhibitory effects" with *S. cerevisiae* (2011). This study focused on the effects of a plant-derived natural alkaloid product, Lyc, on yeast growth and it was found that Lyc heavily inhibited the growth of yeast[25]. This finding using *S. cerevisiae* suggests yeast extract as a promising model for the identification of natural products with anti-proliferative properties and potential therapeutic value[25].

Drug discovery has been hindered due to the similarities between the targeted fungal cell and the infected mammalian cell as they share similar structural and functional cellular properties[25]. The use of *S. cerevisae* as a biotic elicitor may allow the discovery of plant-derived compounds with

not only antifungal properties but also anticancer properties. Due to the evolutionary similarity between mammalian and fungal cells, fungal species used for drug studies have human homologs and share the similar drug non-selectivity[26]. For instance, rapamycin, a natural product with antimicrobial and immunosuppresant activities, is found to be toxic to numerous tumor cell lines and is a potent antifungal agent[25]. Rapamycin holds antifungal and anticancer properties as its production is derived from a highly conserved binding protein between yeast and mammalian cells, the peptide propel isomerase, FKBP12[25]. As *S. cerevisae* is a highly researched species, its chemical-genetic profile greatly contributes to the assessment of the mode of action by bioactive compounds in human cells[25].

1.4. Mammalian Cell Cultures

Various experiments in this work utilized the following mammalian cancer cell lines, HeLa, A549, and HCT 116, to assess the potential medicinal properties of plant cell extracts. The properties of the plant extracts can be quantified using cell based assays, such as an MTT assay. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium) assay is a reduction assay. The ability of a viable cell to metabolize and covert MTT into formazan crystals by reducing NADH to NAD^+, signified by a purple color, determines mitochondrial activity of the cell line which quantifies its viability (**van Meerlo, NCBI**)

1.4.1.1 HeLa

The cervical cancer cell line, HeLa, originated from a 31-year-old African American cervical cancer patient, Henrietta Lacks **(Baker, 2011**). It is hypertriploid and has a modal chromosome number of 82, with four copies of chromosome 12 and three copies of chromosomes 6, 8, and 17; therefore, it has an abnormal amount of chromosomes (**ATCC)**. HeLa was considered to be the first immortal cell line to be successfully cultured in the lab, which enabled numerous scientific and medical breakthroughs **(Lucey, 2009**). Various types of plant extracts with medicinal properties, ranging from grape extracts to Turkish medicinal plants, have been tested against HeLa to assess anticancer, antiproliferative, and overall cytotoxic properties (Bozkurt-Guzel, 2017; Reddy, 2013).

1.4.1.2 HCT 116

HCT 116 is a male colon cancer cell line with a near diploid stem line chromosome and modal number at 45 (62%) and polyploids occurring at 6.8% (**ATCC**). Numerous plant extracts have

been tested against HCT 116, such as *Melissa officinalis*, an herbal tea extract, and rice suspension cultures. With HCT 116, *M. officinalis* reported to have antiproliferative properties and the rice cultures were able to inhibit the growth of the cancer cell line without affecting the growth of the normal cell lines[27].

1.4.1.3 A549

The lung cancer cell line, A549, was obtained from a 58-year-old Caucasian male lung cancer patient. A549 is a hypotriploid human cell line with the modal chromosome number of 66 occurring in 24% of the cells (**ATCC**). A549 is a commonly used cell line against numerous plant extracts, such as *Ebenus boissieri*, to test for immunomodulatory effects, and cytotoxic and antiproliferative properties[28].

1.5 Method Development

The overall aim of this project is to validate the use of a high-throughput screening system of plant cell lines using a model medicinal species, *Taxus*, as it is known to produce paclitaxel. For this project, we developed three objectives to enable the validation this unique screening system, which can allow the potential discovery of novel metabolites with anticancer compounds for drug development.

1.5.1 Objective 1. Method Development: Selecting an optimal solvent to test extracts against cancer cell lines.

As the first objective was to select an optimal solvent for the screening platform, our goal was to identify a solvent with minimal effect on cell viability of the three cancer cell lines. The solvents being assessed were dimethyl sulfoxide (DMSO) and ethanol. Choosing an optimal solvent is important as it is used to dissolve the plant cell extracts and paclitaxel.

1.5.2 Objective 2. Method Development: Screening of Known Paclitaxel Concentrations.

The second objective was to perform an initial test to verify the cytotoxic effects of paclitaxel on the three cancer cell lines: HeLa, A549, and HCT 116, by testing known paclitaxel concentrations. In order to achieve this goal, serial dilutions of paclitaxel were used to produce different concentrations ranging from 5 μM to 500 μM, based on values previously reported in the literature. From these studies, it was possible to determine the most potent paclitaxel concentration for each cell line.

1.5.3 Objective 3. Method Validation: Use of Taxus Cell Cultures.

The final objective was to validate the effectiveness of the screening platform by using *Taxus* extracts as they are found to produce paclitaxel. Through the use of this platform, we are also able to assess if these *Taxus* extracts being tested are producing paclitaxel and validate their effects against the three cancer cell lines. The *Taxus* cell cultures obtained from the Roberts' lab are known to produce paclitaxel and it is expected that when elicited with methyl jasmonate, there will be an enhanced production of paclitaxel, while when elicited with ethanol, there will be minimal or no production of paclitaxel.

CHAPTER 2. METHODOLOGY

2.1 Maintenance of Taxus Cell Cultures

Taxus cultures utilized for these experiments (i.e., 48-82A-2, 48-82A-3, 48-82A-4, 48-82A-32, 1101-62A-5) originated from *Taxus chinensis*. Cells were cultured in suspension in aqueous medium containing 20 g/L sucrose, 3.21 g/L Gamborg's B5, 8.3×10^{-3} M 6-benzylaminopurine (BA), 1.03×10^{-3} M 1-naphthaleneacetic acid supplemented with antioxidants which are 1.42×10^{-3} M ascorbic acid, 1.3×10^{-3} M citric acid, and 9.99×10^{-3} M L-glutamine. Cells were maintained in the dark at 25°C on an orbital shaker at 125 rpm and cultured every 14 days. For elicitation purposes, the two 50 mL flasks of cells were elicited with either sterile 200 μM methyl jasmonate (+MeJa) or ethanol (-MeJa) on day 7.

2.2 Preparation of Taxus Extracts

Elicited *Taxus* culture samples (1 mL total culture collected in a microfuge tube) were collected on day 14 and evaporated overnight for approximately 9 hours in an evaporative centrifuge. The plant cells were then extracted with 1 mL acidified methanol (0.01% (v/v) acetic acid) at room temperature with a combination of homogenization procedures. Such homogenization steps included periodic vortexing, sonication in an ultrasonic bath for 20 min, and manual homogenization using a spatula. The combined extracts were centrifuged at 20,000 rpm for 20 minutes to remove and collect the supernatant liquid. The supernatants from each cell line under either +MeJa or –MeJa were then pooled together and vortexed. The combined supernatant was then again evaporated using an evaporative centrifuge for 90 minutes at 10,000 rpm. To avoid paclitaxel degradation, the *Taxus* extracts were stored in an -80°C freezer until they were ready to be used. Prior to treating the mammalian cancer cells with the *Taxus* extracts, the final plant extract was dissolved with 100% ethanol in sterile conditions.

2.3 Cell Cultures

HCT116 cells, A549 cells, and HeLa cells were cultured in McCoy's, or RPMI medium, respectively, with 10% FBS and 1% penicillin-streptomycin (Gibco). The monolayer cultures were maintained in 75 cm^2 tissue culture flasks at 37°C in a humidified 5% CO_2 incubator. To treat the cells, 1 mL of ethanol was used to dissolve paclitaxel and *Taxus* extracts; 0.01% (v/v) ethanol and

compound solution were used to treat each well in a 96-well plate, amounting to 2 μL per well. A hemocytometer was used to quantify the number of viable cells used for the MTT experiments.

2.4 Cytotoxicity Assay

The cytotoxicity levels of the *Taxus* extracts, solvents, and paclitaxel on HeLa, A549, and HCT 116 cell was determined using 3-(4,5-dimethylthiazol-2-yl)-2, diphenyltetrazolium bromide (MTT) (Thermo Fisher Scientific). For the MTT assay, 5000 tumor cells/well were seeded into 96-well tissue culture plates. Cells were treated with 2 μL either solvent, paclitaxel, or *Taxus* extract after a 24-hour incubation period at 37°C in a humidified 5% CO_2 incubator. The cells were given fresh RPMI medium without phenol red after 72 hours with the treatment in addition to 10 μL MTT (5 mg/mL; Vybrant) into each well. After 4 hours of incubation, the MTT was aspirated off and cells were lysed with 100 μL of dimethylsulfoxide (DMSO). Metabolically viable cells were assessed based on their ability to metabolize and convert MTT into formazan crystals. Based on the absorbance recorded through Multiskan GO 96-well microtiter plate reader at 549 nm (ThermoFisher Scientific). The levels of cell viability were calculated using the following formula: % viability = $[(A - B)/A] \times 100\%$, where A is denoted as the average absorbance of the control cells and B is denoted as the absorbance from each individual treatment.

2.5 Statistical Analysis

Results are reported as mean ± standard deviation. The data obtained from the experiments were tested for statistical significance using a repeated-measures one-way ANOVA for tests determining the optimal concentration of a solvent and experiments testing known paclitaxel concentrations. A two-way ANOVA using Tukey's test was used in tests comparing effects of DMSO and ethanol, and *Taxus* extracts between +MeJa and –MeJa. The GraphPad Prism 7 software was used for these analyses. A value of $p < 0.05$ was considered to denote statistical significance.

CHAPTER 3. RESULTS & DISCUSSION

3.1 Method Development: Selecting an Optimal Solvent

Varying concentrations of DMSO and ethanol were tested from 0.01% to 0.1 % (v/v) in cell culture medium to assess which solvent combination produced approximately or closest to 100% cell viability. This was done to ensure little to no interference in the results when assessing compounds. These experiments were performed in triplicate for all three cell lines.

3.1.1 Identifying Optimal Solvent for Use in Future Experiments

Initially, the cytotoxic effects between DMSO and ethanol were compared to each other to determine the optimal solvent. The results show that ethanol produced a higher cell viability across all three cancer cell lines from 0.01 to 0.1% (v/v) compared to DMSO. With the exception of HeLa, ethanol consistently decreased cell viability in A549 and HCT 116 cells, moreso as the concentration of ethanol increased (Figure 1a; 1c). The significant decrease in cell viability induced by DMSO correlated with the activity found in the literature as it was reported that higher concentrations of DMSO, such as 5% DMSO reduces cell viability by more than 50% **(Wang, 2012)**.

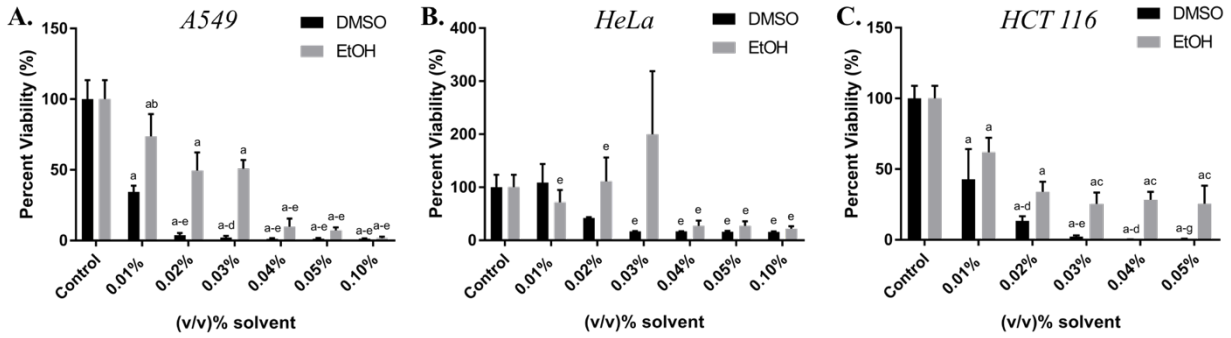

Figure 1. Cytotoxic effects of DMSO versus ethanol (EtOH) on mammalian cancer cells after 3 days of treatment as determined by MTT assay. A) A549; B) HeLa; C) HCT 116. *Note:* Letters denote significance of $p < 0.05$ from a = control, b = 0.01% DMSO, c = 0.01% EtOH, d = 0.02% EtOH, e = 0.03% EtOH, f = 0.04% EtOH, g = 0.05% EtOH.

3.1.2 Optimization of Percentage Concentration of Solvent

As the optimal solvent was identified, a wider concentration range of ethanol concentrations was tested from 0.005% to 0.1% (v/v) to determine the optimal solvent concentration. Based on the results from all three cancer cell lines, it was found that the concentration ranges from 0.005% to 0.015% (v/v) ethanol decreased cell viability by only 25%, whereas higher concentrations

decreased viability by as much as 75% (Figure 2). From this finding, 0.01% (v/v) ethanol proved to be an optimal solvent and was used in the following experiments and preparation of paclitaxel and *Taxus* extracts.

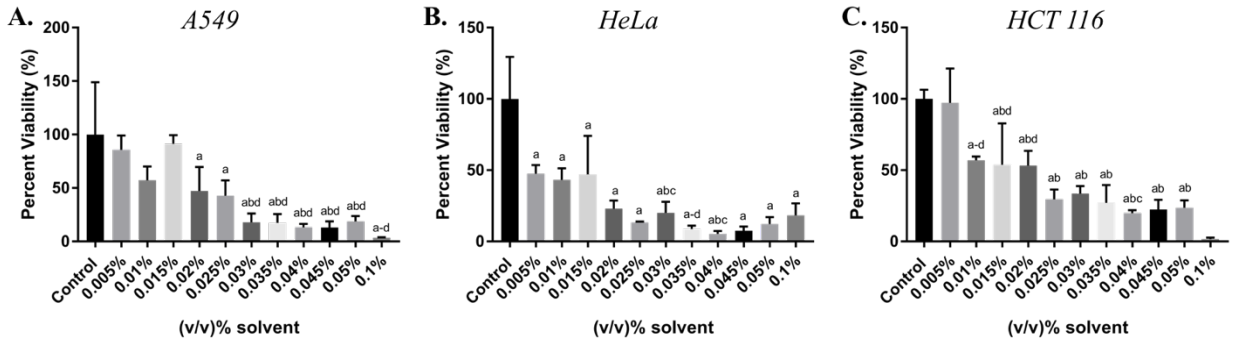

Figure 2. Cytotoxicity effects of varying percent concentrations (v/v) of EtOH on mammalian cancer cells after 3 days of treatment. A) A549; B) HeLa; C) HCT 116. *Note:* Letters denote significance of $p < 0.05$ from a = control, b = 0.005%, c = 0.01%, d = 0.015% (A549 & HCT 116) or 0.01% (HeLa).

3.2 Methods Development: Screening of Known Paclitaxel Concentrations

Different concentrations of paclitaxel ranging from 5 μM to 500 μM were tested against HeLa, A549, and HCT 116 to determine the extent to which paclitaxel reduced cell viability. Three trials of the experiments were performed in triplicate for all three cell lines.

3.2.1 Paclitaxel Effects on HeLa

Figure 3 illustrates the three different trials for HeLa, cervical cancer cell line. Each paclitaxel concentration from 0 μM to 500 μM reduced cell viability in all trials and were found to be statistically significant from the healthy, untreated cells. In comparison to the control, HeLa cells generally were found to be 10-40% viable based on the overall paclitaxel concentrations. These results indicate that paclitaxel had a cytotoxic effect to over 50% of the cells. Although the cell viabilities between the trials differed, there was no statistical significance amongst the paclitaxel concentrations within each trial. Furthermore, trial 1 (Figure 3a) showed the least reduction in cell viability in comparison to trials 2 and 3 (Figures 3b and c), which showed the greatest reduction in cell viability.

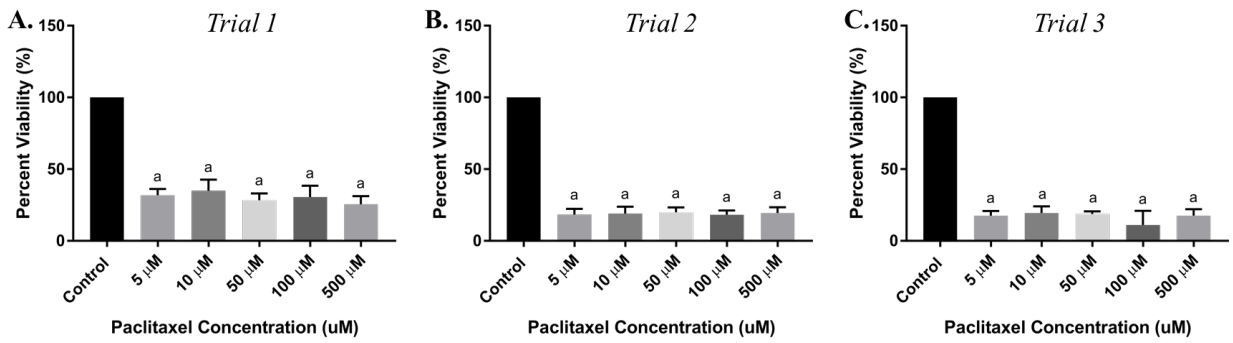

Figure 3. Cytotoxicity of paclitaxel concentrations from 5 μM to 500 μM against HeLa cells after three days of treatment with three separate experimental trials. A) Trial 1; B) Trial 2; C) Trial 3. *Note:* Letters denote significance of $p < 0.05$ from a = control.

3.2.2 Paclitaxel Effects on A549

Figures 4 shows the three different trials for A549, lung cancer cell line. All paclitaxel concentrations were found to reduce cell viability and were statistically significant from the healthy, untreated cells. As observed in trials 1 and 2 (Figures 4a; 4b), small concentrations of paclitaxel (5-10 μM) reduced the overall cell viability compared to high concentrations of 100 and 500 μM paclitaxel. The paclitaxel concentrations were not statistically significant amongst each other in all trials. Additionally, there was no clear correlation between decreased cell viability and increasing paclitaxel concentration. However, 500 μM paclitaxel, the highest concentration of paclitaxel tested, was consistently the least effective in reducing cell viability in all three trials.

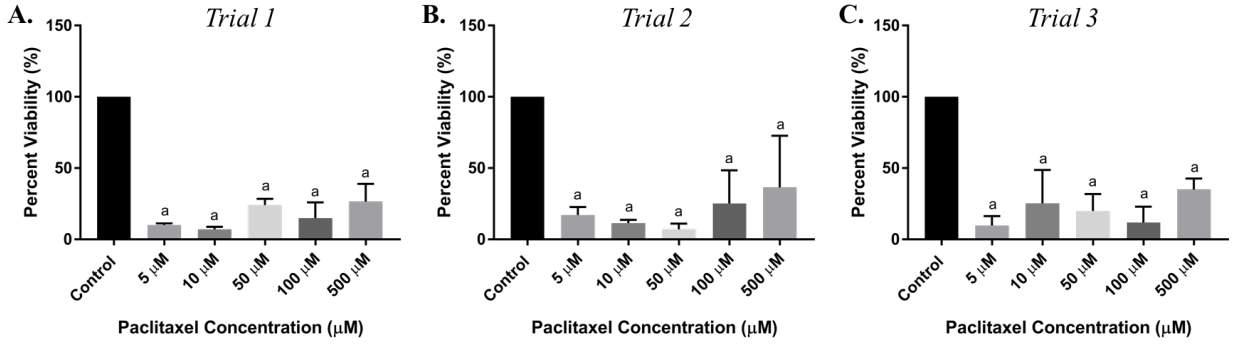

Figure 4. Cytotoxicity of paclitaxel concentrations from 5 μM to 500 μM against A549 cells after three days of treatment with three separate experimental trials. A) Trial 1; B) Trial 2; C) Trial 3. *Note:* Letters denote significance of $p < 0.05$ from a = control.

3.2.3 Paclitaxel Effects on HCT 116

Figures 5 shows the three different trials for HCT 116, colon cancer cell line. All paclitaxel concentrations were found to reduce cell viability and were statistically significant from the healthy, untreated cells; in-depth statistical analyses can be found in Appendix C. Trials 2 and 3

(Figure 5b; 5c) show additional statistical significance amongst various paclitaxel concentrations in reducing cell viability. For instance, in trial 2 (Figure 5b) paclitaxel at 500 μM is statistically significant from the control, 5 μM, and 10 μM. For all three trials, paclitaxel at 100 μM consistently had the greatest effect in reducing cell viability. Additionally, there was a clear response trend against various concentrations of paclitaxel. Between 5 - 100 μM, the cell viability steadily decreases with 100 μM producing the least number of viable cells. However, at 500 μM the cells respond positively to the paclitaxel and produce comparatively equal number of viable cells as 5-10 μM.

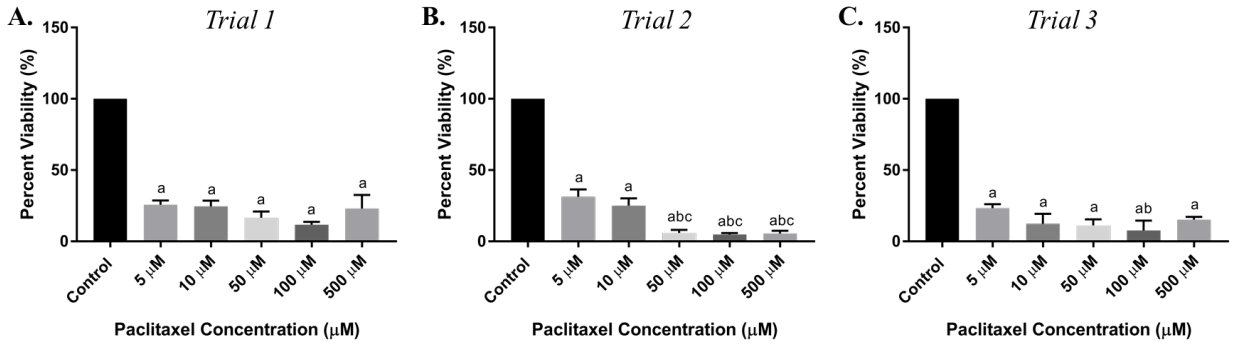

Figure 5. Cytotoxicity of paclitaxel concentrations from 5 μM to 500 μM against HCT 116 cells after three days of treatment with three separate experimental trials. A) Trial 1; B) Trial 2; C) Trial 3. *Note:* Letters denote significance of $p < 0.05$ from a = control, b = 5 μM, c = 10 μM.

3.2.3 Comparison of Paclitaxel Effects from Three Cell Lines

Although each paclitaxel concentrations induced death in HeLa cells (Figure 6), 100 μM paclitaxel showed the most potent effect in decreasing cell viability. 10 μM paclitaxel, in comparison, produced the least effect in HeLa cell viability and also had a higher variance compared to other paclitaxel concentrations. In the case of A549 (Figure 6a), paclitaxel at 50 μM was found to have the most effect in decreasing cell viability while 500 μM paclitaxel had the least reductive effect. These results corresponded with the literature as it was reported that higher concentrations of paclitaxel have a less potent effect on A549 due to an increased resistance from the higher exposure to paclitaxel[12]. In comparison, paclitaxel at 100 μM was found to have the greatest effect in decreasing the overall cell viability of HCT 116 (Figure 6c).

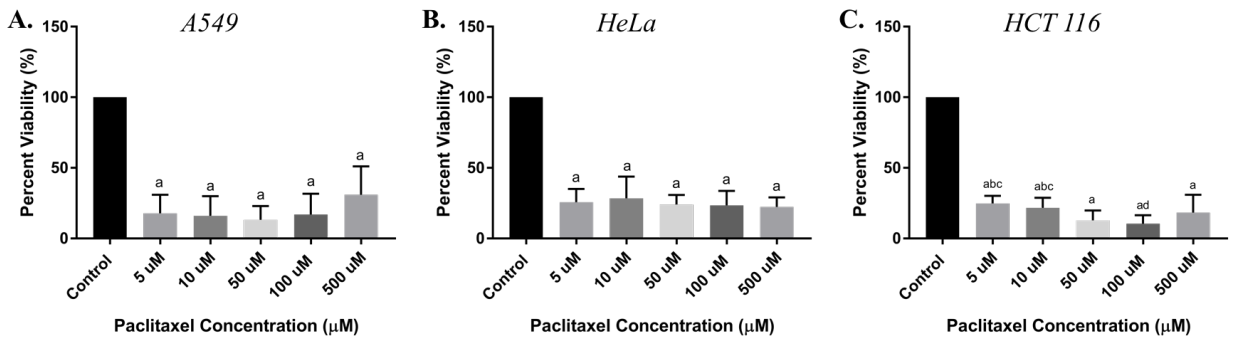

Figure 6. Comparison of cytotoxicity effects of paclitaxel concentrations from 5 μM to 500 μM from all three cancer cell lines after three days of treatment with results average from separate experimental trials. A) A549; B) HeLa; C) HCT 116. *Note:* Letters denote significance of $p < 0.05$ from a = control, b = 5 μM, c = 10 μM.

Overall, paclitaxel was found to significantly reduce viability in all three cell lines. Although the concentrations tested during these experiments were higher than used in literature, it is important to note that paclitaxel, even at lower doses (< 10 nM) has been found to cause mitotic arrest and induce apoptosis in HeLa and HCT 116 cancer cell lines[29,30]. In the case of A549, low concentrations of paclitaxel were found to cause abnormal division of cells and prolong the mitotic stage, thereby leading to the post-mitotic arrest of the cells[12]. An increase in paclitaxel concentration, however, inhibits the pathway that causes a prolonged mitotic stage, leading to a mitotic slippage and a greater chance of cells surviving[12]. For the case of method development, it was not necessary to test comparatively low concentrations of paclitaxel we devised standard dose-responses that can serve as a baseline for the *Taxus* extracts.

3.3 Method Validation: Testing Taxus Cell Cultures

Extracts from *Taxus* cell lines: 48-82A-2, 48-82A-3, 48-82A-4, 48-82A-32, and 1101-62A-5, were obtained based on the elicitation and extraction procedure described in Chapter 2. 48-82A-2, 48-82A-3, 48-82A-4, and 48-82A-32 are from *Taxus chinesis*, commonly known as Chinese yew (Harvard Arboretum). Plant cell line, 1101-62A-5, is a hybrid of *Taxus baccata* and *Taxus cuspidate* (Harvard Arboretum). Each plant cell line produced two extracts elicited with either MeJa, which was expected to induce specialized metabolite synthesis, or ethanol, which was expected to produce little to no paclitaxel (or general specialized metabolites) in the cells. Ten plant extracts were tested against HeLa, A549, and HCT 116 to validate the anticancer properties of *Taxus* cultures and verify the effectiveness of the high throughput screening system on plant

cell culture extracts. Three trials of the experiments were performed in triplicate for all three cell lines.

3.3.1 Taxus Effects on HeLa

The addition of all plant extracts found to decrease the cell viability for all three trials (Figure 7). Each extract tested was found to be statistically significant with each other at $p < 0.05$. In-depth statistical analyses can be found in Appendix D. For each trial, 48-82A-2 –MeJa had a greater effect in reducing cell viability in comparison to its +MeJa counterpart. With regards to 48-82A-3 and 48-82A-32, +MeJa produced cell viabilities below 20% and was significantly less than –MeJa. For some cell lines, there were no consistent responses between the MeJA-elicited and mock-elicited extracts from all three trials. For instance, 48-82A-4 +MeJa in trial 1 produced a comparatively lower cell viability than –MeJa, however, for trials 2 and 3, the behaviors were switched. This was also be observed in 1101-62A-5.

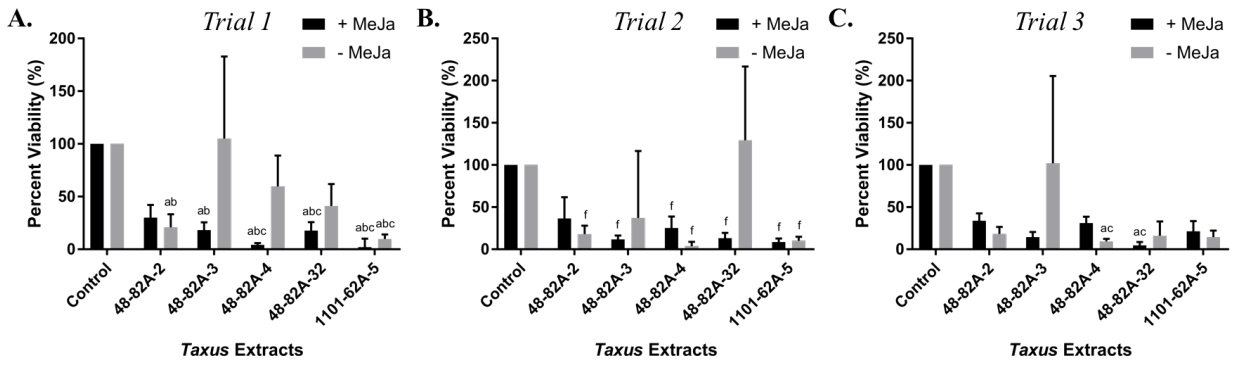

Figure 7. Viability of *Taxus* cell extracts both methyl jasmonate elicited and ethanol (mock) elicited against HeLa cells after three days of treatment with three separate experimental trials. A) Trial 1; B) Trial 2; C) Trial 3. *Note:* Letters denote significance of $p < 0.05$ from a = control, b = 48-82A-2 (+MeJa), c = 48-82A-3 (-MeJa), d = 48-82A-3 (+MeJa), e = 48-82A-4 (-MeJa), f = 48-82A-32 (-MeJa), g = 1101-62A-5 (+MeJa), h = 1101-62A-5 (-MeJa), i = 48-82A-2 (-MeJa).

3.3.1 Taxus Effects on A549

The addition of all plant extracts decreased the cell viability of A549 for all three trials. Each extract was found to be statistically significant amongst each other at $p < 0.05$. In-depth statistical analyses can be found in Appendix D. Based on Figure 8, each extract generated relatively similar responses on A549 for all three trials. Plant cell extracts +MeJa and –MeJa from 48-82A-2 appeared to produce similar cell viabilities in all three trials. Although in trials 2 and 3 –MeJa had comparatively lower cell viabilities, the standard deviations from both trials were large and

therefore statistically the cell viabilities from both types of extracts were similar to each other. Plant extracts, 48-82A-3, 48-82A-4 and 1101-62A-5 +MeJa, followed our initial hypothesis as +MeJa extracts decreased the cell viability significantly more than –MeJa in all trials, producing cell viabilities less than 20% (Figure 8). Extracts from 48-82A-32, however, responded in an opposite manner with –MeJa extracts decreasing the viability to approximately 40%, which is significantly lower than that of +MeJa.

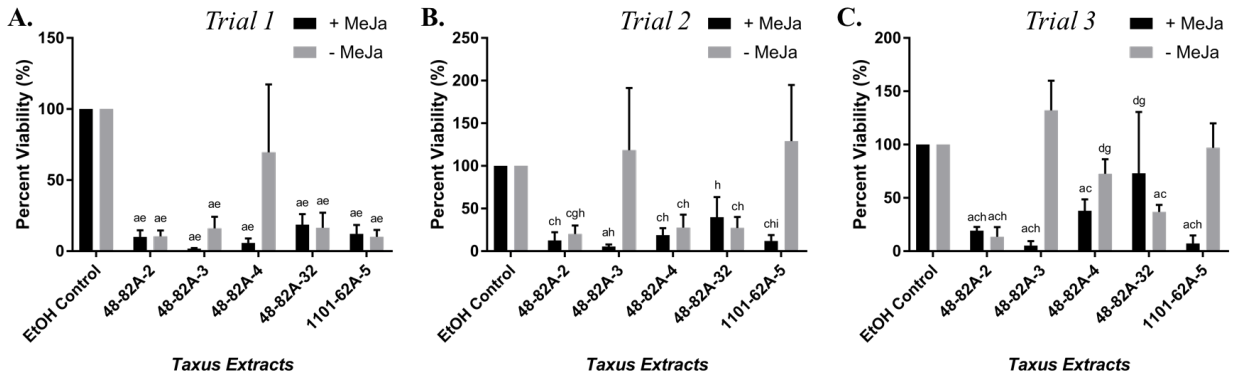

Figure 8. Viability of *Taxus* cell extracts both methyl jasmonate elicited and ethanol (mock) elicited against A549 cells after three days of treatment with three separate experimental trials. A) Trial 1; B) Trial 2; C) Trial 3. *Note:* Letters denote significance of $p < 0.05$ from a = control, b = 48-82A-2 (+MeJa), c = 48-82A-3 (-MeJa), d = 48-82A-3 (+MeJa), e = 48-82A-4 (-MeJa), f = 48-82A-32 (-MeJa), g = 1101-62A-5 (+MeJa), h = 1101-62A-5 (-MeJa), i = 48-82A-2 (-MeJa).

3.3.1 Taxus Effects on HCT 116

The addition of all plant extracts decreased the cell viability of HCT 116 for all three trials. Each extract was found to be statistically significant amongst each other at $p < 0.05$; in-depth statistical analyses can be found in Appendix D. Based on Figure 9, extracts from 48-82A-2, 48-82A-3, and 48-82A-4 generated relatively similar responses as HCT 116 in all three trials. The results obtained from plant cell extracts, 48-82A-3 and 48-82A-4 +MeJa, reaffirmed our hypothesis as +MeJa extracts produced relatively low cell viabilities of less than 30% and were significantly different than –MeJa extracts. Extracts from 48-82A-2 produced an opposite effect as –MeJa caused 0 - 10% cell viability but were still similar to the effects from +MeJa. Although 48-82A-32 and 1101-62A-5 produced inconsistent results when considering all three trials, two out of the three trials demonstrated that –MeJa had a more potent effect on HCT 116 than +MeJa extracts.

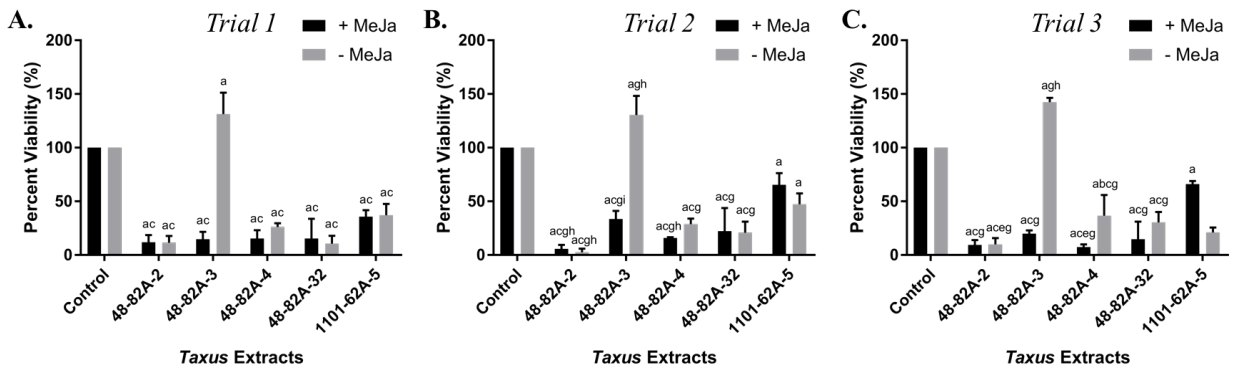

Figure 9. Viability of *Taxus* cell extracts both methyl jasmonate elicited and ethanol (mock) elicited against HCT 116 cells after three days of treatment with three separate experimental trials. A) Trial 1; B) Trial 2; C) Trial 3. *Note:* Letters denote significance of $p < 0.05$ from a = control, b = 48-82A-2 (+MeJa), c = 48-82A-3 (-MeJa), d = 48-82A-3 (+MeJa), e = 48-82A-4 (-MeJa), f = 48-82A-32 (-MeJa), g = 1101-62A-5 (+MeJa), h = 1101-62A-5 (-MeJa), i = 48-82A-2 (-MeJa).

3.3.1 Comparison of Taxus Effects from Three Cell Lines

Generally, the addition of plant extracts decreased the viability of all three mammalian cancer cell lines. It was found that 48-82A-3 and 1101-62A-5 showed significant differences amongst extracts that were MeJa-elicited and mock-elicited, and also had consistent responses for all three mammalian cell lines. 1101-62A-5 significantly decreased the cell viability in HCT 116 and A549 by approximately 80% with +MeJa, whereas 48-82A-3 demonstrated consistent behavior in all three mammalian cell lines producing cell viabilities of 20-30% with +MeJa.

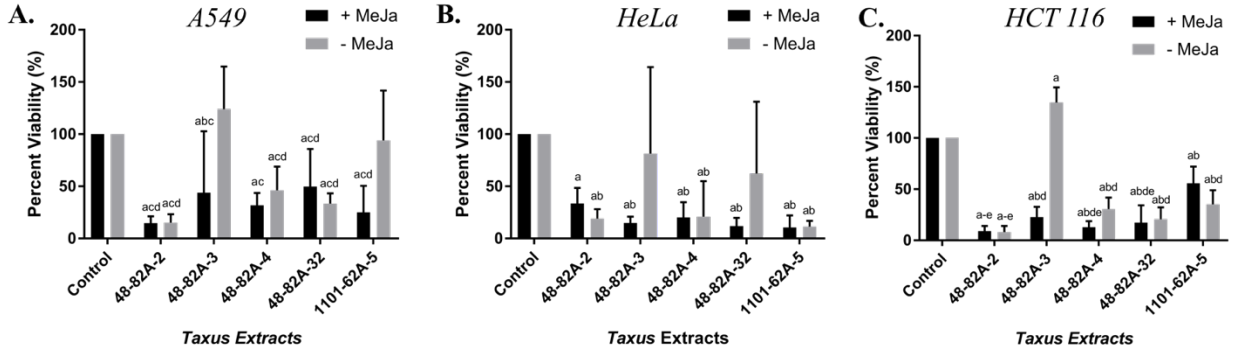

Figure 10. Comparison of cytotoxic effects of *Taxus* cell extracts both methyl jasmonate elicited and ethanol (mock) elicited on all three cell lines after three days of treatment with averaged results from separate experimental trials. A) A549; B) HeLa; C) HCT 116. *Note:* Letters denote significance of $p < 0.05$ from a = control, b = 48-82A-2 (+MeJa), c = 48-82A-3 (-MeJa), d = 48-82A-3 (+MeJa), e = 48-82A-4 (-MeJa), f = 48-82A-32 (-MeJa), g = 1101-62A-5 (+MeJa), h = 1101-62A-5 (-MeJa), i = 48-82A-2 (-MeJa).

CHAPTER 4. CONCLUSIONS

From method development to validation, we were able to successfully create a high-throughput screening system using plant cell cultures to identify and harness secondary metabolites with medicinal properties. Based on the screening platform, we determined 0.01% (v/v) ethanol in medium had the least effect in affecting mammalian cell viability. Additionally, by testing known concentrations of paclitaxel on the three different cancer lines, we were able to elucidate a general dose-response which enabled the estimation of the concentrations of plant cells extracts to add by comparing their cytotoxic effects. And discuss how you tested plant extracts and how some of the lines had lower viability in the MeJA+ cells. Furthermore, we determined that both MeJa-elicited and EtOH-elicited plant cell extracts generally lowered mammalian cancer cell viability with MeJa-elicited cells as the more potent of the two, which indicates the possible presence of other compounds in the extracts in addition to paclitaxel.

CHAPTER 5. FUTURE DIRECTIONS

To further improve upon the high throughput system developed for this project, we would like to expand upon three main areas. As this project optimized the solvent in use, future work can focus on the optimization of the screening platform. This can be done by utilizing hydrogels to develop three-dimensional (3D) cancer cell cultures of each mammalian cancer line as it mimics the *in vivo* conditions of the cell, thereby allowing for more sensitivity when treated with various plant extracts. Additionally, to further validate the effectiveness of the high throughput system in detecting the effects of anticancer compounds of plant extracts, we recommend testing plant extracts with no known anticancer properties reported in literature to act as a control.

Another area of future work can be performed to further analyze the compounds present in the tested *Taxus* extracts. More specifically, the concentration of paclitaxel present from the five *Taxus* cell lines can be quantified using UPLC analysis. Additionally, as certain cell cultures elicited with ethanol produced a lower cell viability in mammalian cancer cells, such as HeLa, we recommend performing LC-MS analysis to determine the compounds present to assess any synergistic effects responsible for decreasing the cell viability.

Finally, we recommend applying the high throughput system with various plant cell cultures that have reported anticancer or medicinal properties in the literature. This can be obtained from the collaboration with University of Massachusetts Savinov Lab and the Plant Cell Culture Library. The cells can the elicited with both yeast and MeJa to discover novel metabolites, which can later be applied to new natural product drug development platform developed here. The co-culture of these elicitors, such as endophytic fungi and yeast, would promote synergistic relationship which can increase the production of the specialized metabolites. The criteria that was used to which plant cell cultures were to be tested was based on mainly their anticancer property, high growth activity, and availability for use from the PCCL. The list of plant cell cultures chosen and elicitation/extraction methods can be found in Appendix I.

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APPENDICES

Appendix A. Solvent Optimization P-charts

Table 1. Statistical analysis of experiments comparing cytotoxic effects of DMSO and EtOH in HeLa cells. Note: D = DMSO; E = EtOH; ns = not significant.

	Control	0.01% D	0.02% D	0.03% D	0.04% D	0.05% D	0.1% D	0.01% E	0.02% E	0.03% E	0.04% E	0.05% E	0.1% E
Control	NS												
0.01% D		NS											
0.02% D			NS										
0.03% D				NS						***			
0.04% D					NS					***			
0.05% D						NS				***			
0.1% D							NS			***			
0.01% E								NS		*			
0.02% E									NS	*			
0.03% E				***	***	***	***	*	*	NS	***	***	***
0.04% E										***	NS		
0.05% E										***		NS	
0.1% E										***			NS

Table 2. Statistical analysis of experiments comparing cytotoxic effects of DMSO and EtOH in A549 cells. Note: D = DMSO; E = EtOH; ns = not significant

	Control	0.01% D	0.02% D	0.03% D	0.04% D	0.05% D	0.1% D	0.01% E	0.02% E	0.03% E	0.04% E	0.05% E	0.1% E
Control	NS	****	****	****	****	****	****	*	****	****	****	****	****
0.01% D	****	NS	**	**	**	**	**	***			*	*	**
0.02% D	****	**	NS					****	****	****			
0.03% D	****	**		NS				****					
0.04% D	****	**			NS			****	****	****			
0.05% D	****	**				NS		****	****	****			
0.1% D	****	**					NS	****	****	****			
0.01% E	*	***	****	****	****	****	****	NS	*		****	****	****
0.02% E	****		****	****	****	****	****	*	NS		****	****	****
0.03% E	****		****		****	****	****			NS	****	****	****
0.04% E	****	*						****	****	****	NS		
0.05% E	****	*						****	****	****		NS	
0.1% E	****	**						****	****	****			NS

Table 3. Statistical analysis of experiments comparing cytotoxic effects of DMSO and EtOH in HCT 116 cells. Note: D = DMSO; E = EtOH; ns = not significant

	Control	0.01% D	0.02% D	0.03% D	0.04% D	0.05% D	0.1% D	0.01% E	0.02% E	0.03% E	0.04% E	0.05% E	0.1% E
Control	NS	****	****	****	****	****	****	****	****	****	****	****	****
0.01% D	****	NS	***	****	***	****							
0.02% D	****	***	NS					****	*				
0.03% D	****	****		NS				****	**		*		
0.04% D	****	***			NS			****	**				
0.05% D	****	****				NS		****	**	*	*	*	
0.1% D	****						NS						
0.01% E	****		****	****	****	****		NS	**	****	****	****	****
0.02% E	****		*	**	**	**		**	NS				
0.03% E	****					*		****		NS			
0.04% E	****			*		*		****			NS		
0.05% E	****					*		****				NS	
0.1% E	****							****					NS

Appendix B. Solvent Concentration Optimization P-charts

Table 4. Statistical analysis of experiments determining optimal EtOH concentration in HeLa cells.

	Control	0.005%	0.01%	0.015%	0.02%	0.025%	0.03%	0.035%	0.04%	0.045%	0.05%	0.1%
Control	NS	**	***	**	****	****	****	****	****	****	****	****
0.005%	**	NS						*	*	*		
0.01%	***		NS						*			
0.015%	**			NS				*	*	*		
0.02%	****				NS							
0.025%	****					NS						
0.3%	****						NS					
0.035%	****							NS				
0.04%	****	*		*					NS			
0.045%	****	*	*	*						NS		
0.05%	****	*		*							NS	
0.1%	****											NS

Table 5. Statistical analysis of experiments determining optimal EtOH concentration in A549 cells.

	Control	0.005%	0.01%	0.015%	0.02%	0.025%	0.03%	0.035%	0.04%	0.045%	0.05%	0.1%
Control	NS				*	*	***	***	***	***	***	****
0.005%		NS					**	**	**	**	**	***
0.01%			NS									*
0.015%				NS			**	**	***	***	**	***
0.02%	*				NS							
0.025%	*					NS						
0.3%	***	**		**			NS					
0.035%	***	**		**				NS				
0.04%	***	**		***					NS			
0.045%	***	**		***						NS		
0.05%	***	**		**							NS	
0.1%	****	***	*	***								NS

Table 6. Statistical analysis of experiments determining optimal EtOH concentration in HCT116 cells.

	Control	0.005%	0.01%	0.015%	0.02%	0.025%	0.03%	0.035%	0.04%	0.045%	0.05%	0.1%
Control	NS		*	**	**	****	****	****	****	****	****	****
0.005%		NS	*	*	*	****	***	****	****	****	****	****
0.01%	*	*	NS						*			***
0.015%	**	*		NS								**
0.02%	**	*			NS							**
0.025%	****	****				NS						
0.3%	****	***					NS					
0.035%	****	****						NS				
0.04%	****	****	*						NS			
0.045%	****	****								NS		
0.05%	****	****									NS	
0.1%	****	****	***	**	**							NS

Appendix C. Paclitaxel Experiments P-charts

C.i A549 cells

Table 7. Statistical analysis of A549 cells viability when treated with paclitaxel from a concentration range of 5 μM to 500 μM under three trials.

	Trial 1	Trial 2	Trial 3
Control	****	****	****
5 μM	****	****	****
10 μM	****	****	****
50 μM	****	****	****
100 μM	****	****	****
500 μM	****	****	****

Table 8. Statistical analysis of A549 cells viability when treated with paclitaxel from a concentration range of 5 μM to 500 μM averaged from three trials.

	Control	5 μM	10 μM	50 μM	100 μM	500 μM
Control	NS	****	****	****	****	****
5 μM	****	NS				
10 μM	****		NS			
50 μM	****			NS		
100 μM	****				NS	
500 μM	****					NS

C.ii HeLa cells

Table 9. Statistical analysis of HeLa cells viability when treated with paclitaxel from a concentration range of 5 μM to 500 μM under three trials.

	Trial 1	Trial 2	Trial 3
Control	****	****	****
5 μM	****	****	****
10 μM	****	****	****
50 μM	****	****	****
100 μM	****	****	****
500 μM	****	****	****

Table 10. Statistical analysis of HeLa cells viability when treated with paclitaxel from a concentration range of 5 μM to 500 μM averaged from three trials.

	Control	5 μM	10 μM	50 μM	100 μM	500 μM
Control	NS	****	****	****	****	****
5 μM	****	NS				
10 μM	****		NS			
50 μM	****			NS		
100 μM	****				NS	
500 μM	****					NS

C.iii HeLa cells

Table 11. Statistical analysis of HCT116 cells viability when treated with paclitaxel from a concentration range of 5 µM to 500 µM for trial 1.

	Trial 1
Control	****
5 µM	****
10 µM	****
50 µM	****
100 µM	****
500 µM	****

Table 12. Statistical analysis of HCT116 cells viability when treated with paclitaxel from a concentration range of 5 µM to 500 µM for trial 2. Note: NS = not significant.

	Control	5 µM	10 µM	50 µM	100 µM	500 µM
Control	NS	****	****	****	****	****
5 µM	****	NS		****	****	****
10 µM	****		NS	***	****	****
50 µM	****	****	***	NS		
100 µM	****	****	****		NS	
500 µM	****	****	****			NS

Table 13. Statistical analysis of HCT116 cells viability when treated with paclitaxel from a concentration range of 5 µM to 500 µM for trial 3. Note: NS = not significant.

	Control	5 µM	10 µM	50 µM	100 µM	500 µM
Control	NS	****	****	****	****	****
5 µM	****	NS			*	
10 µM	****		NS			
50 µM	****			NS		
100 µM	****	*			NS	
500 µM	****					NS

Table 14. Statistical analysis of HCT116 cells viability when treated with paclitaxel from a concentration range of 5 µM to 500 µM averaged from three trials. Note: NS = not significant.

	Control	5 µM	10 µM	50 µM	100 µM	500 µM
Control	NS	****	****	****	****	****
5 µM	****	NS		****	****	
10 µM	****		NS	**	***	
50 µM	****	****	**	NS		
100 µM	****	****	***		NS	*
500 µM	****				*	NS

Appendix D. Taxus Experiments P-charts

D.i A549 cells

Table 15. Statistical analysis of A549 cells viability when treated with Taxus extracts elicited with +MeJa or -MeJa for trial 1. Note: NS = not significant, + denotes +MeJa, - denotes –MeJa, 2 = 48-82A-2, 3 = 48-82A-3, 4 = 48-82A-4, 32 = 48-82A-32, 5 = 1101-62A-5.

	Control	2+	2-	3+	3-	4+	4-	32+	32-	5+	5-
Control	NS	****	****	****	****	****		****	****	****	****
2+	****	NS					**				
2-	****		NS				**				
3+	****			NS			***				
3-	****				NS		**				
4+	****					NS	**				
4-		**	**	***	**	**	NS	*	**	**	**
32+	****						*	NS			
32-	****						**		NS		
5+	****						**			NS	
5-	****						**				NS

Table 16. Statistical analysis of A549 cells viability when treated with Taxus extracts elicited with +MeJa or -MeJa for trial 2. Note: NS = not significant, + denotes +MeJa, - denotes –MeJa, 2 = 48-82A-2, 3 = 48-82A-3, 4 = 48-82A-4, 32 = 48-82A-32, 5 = 1101-62A-5.

	Control	2+	2-	3+	3-	4+	4-	32+	32-	5+	5-
Control	NS			*							
2+		NS			*						**
2-			NS		*					**	**
3+	*			NS							**
3-		*	*		NS	*	*		*	**	
4+					*	NS					**
4-					*		NS				*
32+								NS			*
32-					*				NS		*
5+			**		**					NS	**
5-		**	**	**		**	*	*	*	**	NS

Table 17. Statistical analysis of A549 cells viability when treated with Taxus extracts elicited with +MeJa or -MeJa for trial 3. Note: NS = not significant, + denotes +MeJa, - denotes –MeJa, 2 = 48-82A-2, 3 = 48-82A-3, 4 = 48-82A-4, 32 = 48-82A-32, 5 = 1101-62A-5.

	Control	2+	2-	3+	3-	4+	4-	32+	32-	5+	5-
Control	NS	**	**	***		*			*	***	
2+	**	NS			****						**
2-	**		NS		****						**
3+	***			NS	****		*	*			***
3-		****	****	****	NS	***			***	****	
4+	*				***	NS					
4-				*			NS				
32+				*				NS			
32-	*				***				NS		
5+	***				****		*	*		NS	***
5-		**	**	***						***	NS

Table 18. Statistical analysis of A549 cells viability when treated with Taxus extracts elicited with +MeJa or -MeJa averaged from 3 trials. Note: NS = not significant, + denotes +MeJa, - denotes –MeJa, 2 = 48-82A-2, 3 = 48-82A-3, 4 = 48-82A-4, 32 = 48-82A-32, 5 = 1101-62A-5.

	Control	2+	2-	3+	3-	4+	4-	32+	32-	5+	5-
Control	NS	****	****	**		***	**	*	***	****	
2+	****	NS			****						****
2-	****		NS		****						****
3+	**		****	NS	****						*
3-		****	****	****	NS	****	****	****	****	****	****
4+	***				****	NS					***
4-	**				****		NS				*
32+	*				****			NS			
32-	***				****				NS		**
5+	****				****					NS	***
5-		****	****	*	****	***	*		**	***	NS

D.ii HeLa cells

Table 19. Statistical analysis of HeLa cells viability when treated with Taxus extracts elicited with +MeJa or -MeJa for trial 1. Note: NS = not significant, + denotes +MeJa, - denotes –MeJa, 2 = 48-82A-2, 3 = 48-82A-3, 4 = 48-82A-4, 32 = 48-82A-32, 5 = 1101-62A-5.

	Control	2+	2-	3+	3-	4+	4-	32+	32-	5+	5-
Control	NS		*	*		**		*		**	*
2+		NS	*	*		**		*		**	*
2-	*	*	NS		*						
3+	*	*		NS	*						
3-			*	*	NS	**		*		**	**
4+	**	**			**	NS					
4-							NS				
32+	*	*			*			NS			
32-									NS		
5+	**	**			**					NS	
5-	*	*			**						NS

Table 20. Statistical analysis of HeLa cells viability when treated with Taxus extracts elicited with +MeJa or -MeJa for trial 2. Note: NS = not significant, + denotes +MeJa, - denotes –MeJa, 2 = 48-82A-2, 3 = 48-82A-3, 4 = 48-82A-4, 32 = 48-82A-32, 5 = 1101-62A-5.

	Control	2+	2-	3+	3-	4+	4-	32+	32-	5+	5-
Control	NS										
2+		NS									
2-			NS						*		
3+				NS					*		
3-					NS						
4+						NS			*		
4-							NS		**		
32+								NS	*		
32-			*	*		*	**	*	NS	*	*
5+									*	NS	
5-									*		NS

Table 21. Statistical analysis of HeLa cells viability when treated with Taxus extracts elicited with +MeJa or -MeJa for trial 3. Note: NS = not significant, + denotes +MeJa, - denotes –MeJa, 2 = 48-82A-2, 3 = 48-82A-3, 4 = 48-82A-4, 32 = 48-82A-32, 5 = 1101-62A-5.

	Control	2+	2-	3+	3-	4+	4-	32+	32-	5+	5-
Control	NS						*	*			
2+		NS									
2-			NS								
3+				NS							
3-					NS		*	*			
4+						NS					
4-	*				*		NS				
32+	*				*			NS			
32-									NS		
5+										NS	
5-											NS

Table 22. Statistical analysis of HeLa cells viability when treated with Taxus extracts elicited with +MeJa or -MeJa averaged from 3 trials. Note: NS = not significant, + denotes +MeJa, - denotes –MeJa, 2 = 48-82A-2, 3 = 48-82A-3, 4 = 48-82A-4, 32 = 48-82A-32, 5 = 1101-62A-5.

	Control	2+	2-	3+	3-	4+	4-	32+	32-	5+	5-
Control	NS	**	***	****		***	***	****		****	****
2+	**	NS									
2-	***		NS		**						
3+	****			NS	**						
3-			**	**	NS	*	*	**		**	**
4+	***				*	NS					
4-	***				*		NS				
32+	****				**			NS			
32-									NS		
5+	****				**					NS	
5-	****				**						NS

D.iii HCT116 cells

Table 23. Statistical analysis of HCT116 cells viability when treated with Taxus extracts elicited with +MeJa or -MeJa for trial 1. Note: NS = not significant, + denotes +MeJa, - denotes –MeJa, 2 = 48-82A-2, 3 = 48-82A-3, 4 = 48-82A-4, 32 = 48-82A-32, 5 = 1101-62A-5.

	Control	2+	2-	3+	3-	4+	4-	32+	32-	5+	5-
Control	NS	****	****	****	*	****	****	****	****	****	****
2+	****	NS			****						
2-	****		NS		****						
3+	****			NS	****						
3-	*	****	****	****	NS	****	****	****	****	****	****
4+	****				****	NS					
4-	****				****		NS				
32+	****				****			NS			
32-	****				****				NS		
5+	****				****					NS	
5-	****				****						NS

Table 24. Statistical analysis of HCT116 cells viability when treated with Taxus extracts elicited with +MeJa or -MeJa for trial 2. Note: NS = not significant, + denotes +MeJa, - denotes –MeJa, 2 = 48-82A-2, 3 = 48-82A-3, 4 = 48-82A-4, 32 = 48-82A-32, 5 = 1101-62A-5.

	Control	2+	2-	3+	3-	4+	4-	32+	32-	5+	5-
Control	NS	****	****	****	*	****	****	****	****	*	****
2+	****	NS			****					****	**
2-	****		NS	*	****					****	***
3+	****		*	NS	****					*	
3-	*	****	****	****	NS	****	****	****	****	****	****
4+	****				****	NS				***	*
4-	****				****		NS			**	
32+	****				****			NS		**	
32-	****				****				NS	***	
5+	*	****	****	*	****	***	**	**	***	NS	
5-	****	**	***		****	*					NS

Table 25. Statistical analysis of HCT116 cells viability when treated with Taxus extracts elicited with +MeJa or -MeJa for trial 3. Note: NS = not significant, + denotes +MeJa, - denotes –MeJa, 2 = 48-82A-2, 3 = 48-82A-3, 4 = 48-82A-4, 32 = 48-82A-32, 5 = 1101-62A-5.

	Control	2+	2-	3+	3-	4+	4-	32+	32-	5+	5-
Control	NS	****	****	****	***	****	****	****	****	**	****
2+	****	NS			****		*			****	
2-	****		NS		****		*			****	
3+	****			NS	****					****	
3-	***	****	****	****	NS	****	****	****	****	****	****
4+	****				****	NS	*			****	
4-	****	*	*		****	*	NS			*	
32+	****				****			NS		****	
32-	****				****				NS	**	
5+	**	****	****	****	****	****	*	****	**	NS	****
5-	****				****					****	NS

Table 26. Statistical analysis of HCT116 cells viability when treated with Taxus extracts elicited with +MeJa or -MeJa averaged from 3 trials. Note: NS = not significant, + denotes +MeJa, - denotes –MeJa, 2 = 48-82A-2, 3 = 48-82A-3, 4 = 48-82A-4, 32 = 48-82A-32, 5 = 1101-62A-5.

	Control	2+	2-	3+	3-	4+	4-	32+	32-	5+	5-
Control	NS	****	****	****	****	****	****	****	****	****	****
2+	****	NS			****		**			****	***
2-	****		NS		****		**			****	****
3+	****			NS	****					****	
3-	****	****	****	****	NS	****	****	****	****	****	****
4+	****				****	NS				****	**
4-	****	**	**		****		NS			***	
32+	****				****			NS		****	*
32-	****				****				NS	****	
5+	****	****	****	****	****	****	***	****	****	NS	**
5-	****	***	****		****	**		*		**	NS

Appendix E. Solvent Optimization Raw Data

Table 27. Raw data of A549 cells viability when treated with DMSO and EtOH from a concentration range of 0.01% to 0.1% (v/v) under three trials.

	Trial 1	Trial 2	Trial 3
Control	84.48	108.16	107.36
0.01% DMSO	31.12	39.26	32.72
0.02% DMSO	5.52	2.72	3.12
0.03% DMSO	3.36	2.16	1.04
0.04% DMSO	1.52	1.36	0.56
0.05% DMSO	1.84	0.8	1.2
0.1% DMSO	1.6	1.28	0.88
0.01% EtOH	71.76	58.88	90.32
0.02% EtOH	60	35.44	53.28
0.03% EtOH	44.72	57.94	50.56
0.04% EtOH	3.28	13.68	12.56
0.05% EtOH	8.08	4.8	8.72
0.1% EtOH	1.36	2.8	1.04

Table 28. Raw data of HeLa cells viability when treated with DMSO and EtOH from a concentration range of 0.01% to 0.1% (v/v) under three trials.

	Trial 1	Trial 2	Trial 3
Control	82.11	91.06	126.83
0.01% DMSO	100.81	78.46	147.15
0.02% DMSO	42.28	43.50	40.65
0.03% DMSO	15.85	17.48	17.073
0.04% DMSO	16.26	16.67	17.073
0.05% DMSO	15.45	18.09	14.63
0.1% DMSO	15.04	16.26	16.67
0.01% EtOH	51.22	65.85	97.15
0.02% EtOH	161.38	97.97	74.39
0.03% EtOH	192.28	84.96	322.36
0.04% EtOH	36.59	28.46	16.26
0.05% EtOH	23.98	20.33	36.99
0.1% EtOH	23.58	16.67	25.20

Table 29. Raw data of HCT116 cells viability when treated with DMSO and EtOH from a concentration range of 0.01% to 0.1% (v/v) under three trials.

	Trial 1	Trial 2	Trial 3
Control	98.01	91.07	110.86
0.01% DMSO	55.43	57.26	50.71
0.02% DMSO	9.89	18.12	13.09
0.03% DMSO	3.35	1.83	1.68
0.04% DMSO	0	0.30	0
0.05% DMSO	0	0.15	0
0.1% DMSO	0	0	0
0.01% EtOH	72.94	57.26	67.31
0.02% EtOH	46.14	34.42	35.63
0.03% EtOH	28.02	29.69	37.16
0.04% EtOH	30.61	27.56	31.07
0.05% EtOH	30.61	27.56	31.07
0.1% EtOH	42.33	38.22	28.32

Appendix F. Solvent Concentration Optimization Raw Data

Table 30. Raw data of A549 cells viability when treated with EtOH from a concentration range of 0.005% to 0.1% (v/v) under three trials.

	Trial 1	Trial 2	Trial 3
Control	44.89	138.29	116.81
0.005%	101.06	79.79	76.17
0.01%	42.77	61.49	67.45
0.015%	92.55	82.55	98.72
0.02%	64.68	55.11	22.13
0.025%	27.02	19.78	55.11
0.03%	9.79	18.09	25.96
0.035%	26.59	11.06	14.68
0.04%	9.57	14.26	15.74
0.045%	12.34	7.87	19.15
0.05%	23.19	19.79	13.40
0.1%	2.77	3.62	3.83

Table 31. Raw data of HeLa cells viability when treated with EtOH from a concentration range of 0.005% to 0.1% (v/v) under three trials.

	Trial 1	Trial 2	Trial 3
Control	132.28	93.04	74.68
0.005%	53.79	46.83	41.77
0.01%	41.77	36.08	51.89
0.015%	77.85	36.08	27.22
0.02%	17.72	29.11	22.15
0.025%	12.66	13.92	13.29
0.03%	26.58	11.39	22.15
0.035%	6.96	11.39	7.59
0.04%	7.59	4.43	3.79
0.045%	5.06	10.76	6.96
0.05%	8.86	17.72	10.13
0.1%	17.09	27.22	10.76

Table 32. Raw data of HCT116 cells viability when treated with EtOH from a concentration range of 0.005% to 0.1% (v/v) under three trials.

	Trial 1	Trial 2	Trial 3
Control	107.09	94.47	98.44
0.005%	102.41	118.32	70.85
0.01%	54.95	56.12	59.86
0.015%	38.11	36.01	87.22
0.02%	52.61	63.83	43.02
0.025%	32.74	21.75	34.37
0.03%	28.99	39.28	32.50
0.035%	40.92	16.61	23.85
0.04%	22.21	18.23	18.94
0.045%	14.96	24.55	27.83
0.05%	26.89	26.42	17.77
0.1%	2.81	1.40	0.93

Appendix G. Paclitaxel Experiments P-charts

Table 33. Raw data of A549 cells viability when treated with paclitaxel from a concentration range of 5 μM to 500 μM averaged from three experimental trials.

	Trial 1	Trial 2	Trial 3
Control	100	100	100
5 μM	10.03	9.49	18.39
10 μM	7.55	15.19	10.96
50 μM	25.89	17.59	7.19
100 μM	8.93	6.39	11.68
500 μM	27.79	30.66	19.18

Table 34. Raw data of HeLa cells viability when treated with paclitaxel from a concentration range of 5 μM to 500 μM averaged from three experimental trials.

	Trial 1	Trial 2	Trial 3
Control	100	100	100
5 μM	30.56	17.79	17.15
10 μM	33.33	20.06	21.68
50 μM	27.78	20.06	19.19
100 μM	33.33	17.79	14.49
500 μM	26.67	20.39	18.12

Table 35. Raw data of HCT116 cells viability when treated with paclitaxel from a concentration range of 5 μM to 500 μM averaged from three experimental trials.

	Trial 1	Trial 2	Trial 3
Control	100	100	100
5 μM	27.18	32.99	24.78
10 μM	25.90	22.99	15.57
50 μM	18.07	5.03	9.02
100 μM	12.65	4.91	4.50
500 μM	20.09	5.94	15.78

Appendix H. Taxus Experiments Raw Data

Table 36. Raw data of A549 cells viability when treated with Taxus extracts elicited with +MeJa or –MeJa averaged from three experimental trials.

	Trial 1	Trial 2	Trial 3
Control	100	100	100
2+	7.81	7.69	19.67
2-	9.05	15.38	12.50
3+	1.96	5.13	3.28
3-	16.05	76.32	100
4+	7.19	21.05	37.70
4-	81.69	35.90	70.49
32+	21.24	48.72	50.82
32-	12.09	25	107.05
5+	12.96	12.82	6.56
5-	11.11	89.99	70.49

Table 37. Raw data of HeLa cells viability when treated with Taxus extracts elicited with +MeJa or –MeJa averaged from three experimental trials.

	Trial 1	Trial 2	Trial 3
Control	100	100	100
2+	35.85	30.19	38.10
2-	20.75	16.98	18.87
3+	16.98	9.52	16.98
3-	111.90	121.42	73.58
4+	4.81	20.76	32.08
4-	73.81	9.52	7.55
32+	20.75	16.67	6.73
32-	45.28	76.19	7.14
5+	7.55	7.69	26.19
5-	14.29	11.32	20.75

Table 38. Raw data of HCT116 cells viability when treated with Taxus extracts elicited with +MeJa or –MeJa averaged from three experimental trials.

	Trial 1	Trial 2	Trial 3
Control	100	100	100
2+	12	5	11
2-	15	4	10
3+	13	38	17
3-	100	100	100
4+	13	16	6
4-	27	27	27
32+	5	16	7
32-	7	26	36
5+	33	68	66
5-	42	47	21

Appendix H. Mammalian Cell Imaging

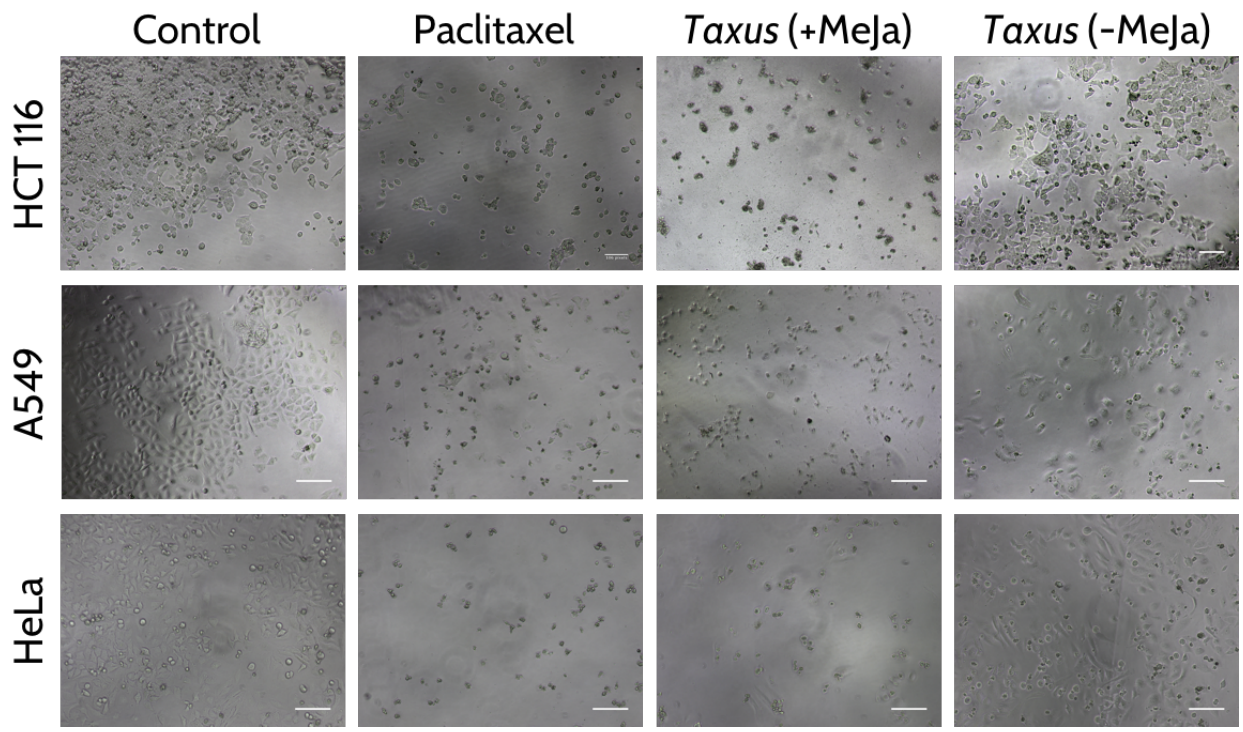

Figure 11. Representative images of HCT116, A549, and HeLa under various conditions such as 5 µM paclitaxel, MeJa-elicited *Taxus* extracts, and mock-elicited *Taxus* extracts after 3 days of treatment. Images taken with brightfield microscopy. Scale bar = 100 µm.

Appendix I. PCCL Plant Cell Extracts

I.i Excel Spreadsheet of Plant Cell Cultures

Accepted Name	Legacy Name	Genus	Family	Order	AV	AB	AF	TC	AH	IC	AC	ecies Co	vity Co	Cell Line ID	Cryo Status	Callus Status
Aloe ferox	Aloe candelabrum	Aloe	Xanthorrhoeaceae	Asparagales	Gn	Sp	Gn			Gn	Sp	1	2	AC-13589		High Growth
Argemone mexicana	Argemone mexicana	Argemone	Papaveraceae	Ranunculales		Sp	Sp	Sp		Sp	Sp	1	5	AM2831-L1		High Growth
Cascabela thevetia	Thevetia peruviana	Cascabela	Apocynaceae	Gentianales							Sp	1	1	TP-02961		High Growth
Centella asiatica	Hydrocotyle asiatica	Centella	Apiaceae	Apiales	Sp	Sp			Sp	Sp	Sp	1	5	HA-09891-R1	Cryopreserved	High Growth
Cleome gynandra	Gynandropsis gynandra	Cleome	Cleomaceae	Brassicales				Sp	Sp	Sp	Sp	1	4	GG-01114-E1		High Growth
Cucumis melo	Cucumis callosus	Cucumis	Cucurbitaceae	Cucurbitales							Sp	1	1	CC-12810-R1		High Growth
Cyanus montanus	Centaurea montana	Cyanus	Compositae	Asterales							Sp	1	1	CM1209-R2		High Growth
Datura innoxia	Datura innoxia	Datura	Solanaceae	Solanales		Gn	Gn		Gn	Gn	Sp	1	1	DI-07051		High Growth
Dictamnus albus	Dictamnus albus	Dictamnus	Rutaceae	Sapindales			Sp	Sp		Sp	Sp	1	4	DA-00132-R0		High Growth
Dioscorea composita	Dioscorea composita	Dioscorea	Dioscoreaceae	Dioscoreales		Gn	Gn			Gn	Sp	1	1	DC3484		High Growth
Gleditsia sinensis	Gleditsia sinensis	Gleditsia	Leguminosae	Fabales	Gn						Sp	1	1	GS-12410		High Growth
Gloriosa superba	Gloriosa rothschildiana	Gloriosa	Colchicaceae	Liliales		Sp				Sp	Sp	1	3	GR-12915-R2		High Growth
Glycyrrhiza glabra	Glycyrrhiza glabra	Glycyrrhiza	Leguminosae	Fabales	Sp	Sp	Sp			Sp	Sp	1	5	GG-12529-R1		High Growth
Gossypium hirsutum	Gossypium hirsutum	Gossypium	Malvaceae	Malvales	Sp	Gn				Sp	Sp	1	3	GH-00171-T4-G5		High Growth
Hordeum vulgare	Hordeum vulgare	Hordeum	Poaceae	Poales			Sp				Sp	1	2	HV-11779		High Growth
Humulus lupulus	Humulus lupulus	Humulus	Cannabaceae	Rosales		Sp	Sp			Sp	Sp	1	4	HL-00210-R0	Cryopreserved	High Growth
Indigofera tinctoria	Indigofera tinctoria	Indigofera	Leguminosae	Fabales		Gn					Sp	1	1	IT1943-R0		High Growth
Lavandula angustifolia	Lavandula spica	Lavandula	Lamiaceae	Lamiales		Sp	Sp	Gn		Sp	Sp	1	4	LS-02407-G11		High Growth
Lonicera japonica	Lonicera japonica	Lonicera	Caprifoliaceae	Dipsacales	Sp	Sp	Sp				Sp	1	4	LJ-00477-T4-L2	Cryopreserved	High Growth
Lycopersicon esculentum	Lycopersicon esculentum	Lycopersicon	Solanaceae	Solanales			Sp			Sp	Sp	1	3	LE-00138-R0		High Growth
Maclura pomifera	Maclura pomifera	Maclura	Moraceae	Rosales	Gn		Gn				Sp	1	1	MP-10828		High Growth
Melissa officinalis	Melissa officinalis	Melissa	Lamiaceae	Lamiales	Sp	Sp	Sp			Sp	Sp	1	5	MO748-T4-L1		High Growth
Mentha spicata	Mentha x spicata	Mentha	Lamiaceae	Lamiales	Gn	Sp	Sp			Sp	Sp	1	4	MX-14478		High Growth
Morinda citrifolia	Morinda citrifolia	Morinda	Rubiaceae	Gentianales	Sp	Sp		Gn		Sp	Sp	1	4	MC-00256-R0		High Growth
Morus nigra	Morus nigra	Morus	Moraceae	Rosales	Gn	Gn	Gn			Gn	Sp	1	1	MN-07928		High Growth
Nicotiana tabacum	Nicotiana tabacum	Nicotiana	Solanaceae	Solanales			Sp		Sp	Sp	Sp	1	4	NT-12392	Cryopreserved	High Growth
Ocimum basilicum	Ocimum basilicum	Ocimum	Lamiaceae	Lamiales	Sp	Sp	Sp	Gn	Gn	Sp	Sp	1	5	OB543-R0	Cryopreserved	High Growth
Oroxylum indicum	Oroxylum indicum	Oroxylum	Bignoniaceae	Lamiales							Sp	1	1	OI-00794-R0	Cryopreserved	High Growth
Oryza sativa	Oryza sativa	Oryza	Poaceae	Poales			Sp				Sp	1	2	OS-12658-R1	Cryopreserved	High Growth
Phyllanthus emblica	Emblica officinalis	Phyllanthus	Phyllanthaceae	Malpighiales	Sp	Sp	Gn			Sp	Sp	1	4	EO-11611	Cryopreserved	High Growth
Pittosporum viridiflorum	Pittosporum vividiflorum	Pittosporum	Pittosporaceae	Apiales							Sp	1	1	PV-13684		High Growth
Platycodon grandiflorus	Platycodon grandiflorus	Platycodon	Campanulaceae	Asterales							Sp	1	1	PG-12767		High Growth
Psidium cattleianum	Psidium cattleianum	Psidium	Myrtaceae	Myrtales	Gn	Gn	Gn	Gn		Gn	Sp	1	1	PC-12680	Cryopreserved	High Growth
Rosmarinus officinalis	Rosmarinus officinalis	Rosmarinus	Lamiaceae	Lamiales		Sp	Sp			Sp	Sp	1	4	RO-14482		High Growth
Ruta graveolens	Ruta graveolens	Ruta	Rutaceae	Sapindales			Sp	Sp	Sp	Sp	Sp	1	5	RG-03757		High Growth
Schisandra chinensis	Schisandra chinensis	Schisandra	Schisandraceae	Austrobaileyales	Sp						Sp	1	2	SC-13378		High Growth
Senna petersiana	Senna petersii	Senna	Leguminosae	Fabales	Gn	Gn	Gn		Gn	Gn	Sp	1	1	SP-09233		High Growth
Silybum marianum	Silybum marianum	Silybum	Compositae	Asterales	Sp						Sp	1	2	SM2989-R1		High Growth
Sophora tomentosa	Sophora tomentosa	Sophora	Leguminosae	Fabales	Gn			Gn			Sp	1	1	ST-14424		High Growth
Withania somnifera	Withania somniferum	Withania	Solanaceae	Solanales	Sp	Sp	Sp			Sp	Sp	1	5	WS1967-T4-G5		High Growth

I.ii Cell Cultures

These cultures were prepared and elicited by undergraduates, Matthew Tauras and Zain Chaudry, under the guidance of Professor Sergey Savinov.

+											-
+	1) CP-13934	2) DA-00132-R0	3) EK-02128-R1	4)EO-11611	5) GG-01114-E1	6) CP-13934	7) DA-00132-R0	8)EK-02128-R1	9) EO-11611	10) GG-01114-E1	-
+	11)HL-00210-R0	12) IT1943-R0	13) MP-10828	14) OV-13911	15) SA-02878-G4	16)HL-00210-R0	17) IT1943-R0	18) MP-10828	19) OV-13911	20) SA-02878-G4	-
+	21) SP-09233	22) TD944-R0	23)VR-10031-R1	24) CA3068-G5	25) EP-12317	26) SP-09233	27) TD944-R0	28)VR-10031-R1	29) CA3068-G5	30)EP-12317	-
+	31) RS-07801	32) HA-09891-R1	33) PC-11506-R1	34) RO-00231-L1	35) HF2244	36)RS-07801	37) HA-09891-R1	38) PC-11506-R1	39)RO-00231-L1	40) HF2244	-
+	41) DS-01138	42) GD-13212 C1	43) HP-02360	44) PV-13684 C1	45)MO748-T4-L1	46)DS-01138	47) GD-13212 C1	48) HP-02360	49)PV-13684 C1	50) MO748-T4-L1	-
+	51) RI-00691	52) CC-04646	53) EM-10045	54) DI-07051	55) HI2192-G4	56) RI-00691	57) CC-04646	58) EM-10045	59) DI-07051	60)HI2192-G4	-
+	61) NS-05192	62) SA-02880-R0	63) SM2989-R1	64) AH279-G15	65) CJ-11831	66) NS-05192	67) SA-02880-R0	68)SM2989-R1	69) AH279-G15	70) CJ-11831	-
+	71)PV-12067 C1	72) CS1952-R1	73) RO-14482-C2	74) AL-01796-L1	75) CP-10715-R1	76) PV-12067 C1	77)CS1952-R1	78) RO-14482-C2	79)AL-01796-L1	80)CP-10715-R1	-

1 and 6) CP-13934
2 and 7) DA-00132-R0
3 and 8) EK-02128-R1
4 and 9) EO-11611
5 and 10)GG-01114-E1
11 and 16) HL-00210-R0
12 and 17) IT1943-R0
13 and 18) MP-10828
14 and 19) OV-13911
15 and 20) SA-02878-G4

21 and 26) SP-09233
22 and 27) TD944-R0
23 and 28) VR-10031-R1
24 and 29) CA3068-G5
25 and 30) EP-12317
31 and 36) RS-07801
32 and 37) HA-09891-R1
33 and 38) PC-11506-R1
34 and 39) RO-00231-L1
35 and 40) HF2244
41 and 46) DS-01138
42 and 47) GD-13212 C1
43 and 48) HP-02360
44 and 49) PV-13684 C1
45 and 50) MO748-T4-L1
51 and 56) RI-00691
52 and 57) CC-04646
53 and 58) EM-10045
54 and 59) DI-07051
55 and 60) HI2192-G4
61 and 66) NS-05192
62 and 67) SA-02880-R0
63 and 68) SM2989-R1
64 and 69) AH279-G15
65 and 70) CJ-11831
71 and 76) PV-12067 C1
72 and 77) CS1952-R1
73 and 78) RO-14482-C2
74 and 79) AL-01796-L1
75 and 80) CP-10715-R1\

I.ii Protocols

Preparing Yeast

Media we will be using for yeast will be **TSB**. Will ask antifungal team to help us out with recipes and general culture preparation. We will do a test run with a yeast culture before running the actual experiment to optimize the growth yeast. We want to optimize the yeast to get **10^6** CFU (colony forming unit). The yeast by itself will be the negative control.

Once optimized and ready for the experiment,we will fill every well with yeast overnight and dilute with TSB, til we get 10^6 CFU.

Elicitation with Yeast

When aliquotes are in the wells, we then start the elicitation experiment. Add yeast @ 10^6 CFU in TSB. Shake the co-culture overnight @ room temperature. (not over stress, look into RPM for liquid suspension experiment approx 100) Spin down plate using regular centrifuge to pellet the cellular debris at the bottom of wells. After this, use multichannel pipette to transfer the supernatents to new 96-well plate. We then spin down the plate in evaporative centrifuge (@ 30 degrees C for both settings) until yellowing brownish residue remains in the plate. If there is still residual water, we will use NaSO4 to soak up. (Not needed if evap centrifuge works correctly)We will then add 100µL of 100% ethanol to wells in the new plate and proceed to sonicate with ice added to the sonicator for 30 minutes until the residue is yellowish-brown in the liquid phase. After sonication, we spin down using regular centrifuge. Transfer the liquid to new 96 well plate with conical bottom (this will be the third plate we will be using. With the new 3rd plate, we then vacuum centrifuge (or gentle air flow) to evaporate volatiles. Once dry, seal with parafilm and put into freezer.

Elicitation with MeJe

MeJe is used in the well (approximately 1mM per well). We will use autoclaved liquid MS media with vitamins and sugar for this experiment to reduce the stress on the plant cells. We will add together 1mM MJ in 100mL liquid MS.(add 0.01 mmol of MJ -> 2.24 mg -> 2.18 uL). These two will be together before pipetting into 96 well, so we just have to transfer once to plate using a multichannel pipette and a reservoir. Incubate with this media overnight in a shaker at RT and 100RPM. Pipet liquid at the bottom of the culture to leave just the cells on the plate. Wash with MS ¼ strength (25% MS 75% sterile water).<---- potentially skip if we take a sufficient amount of water out. Add methanol to lyse plant cells and then place on shaker for a couple of hours. Sonicate if pigmented cells do not release their pigments. Spin down using

regular centrifuge at max rpm and rt . Transfer liquid supernatant to 96 well storage plate with a conical bottom. Evaporate to dryness via vacuum centrifuge or gentle air flow. Once dry, seal with parafilm and put into freezer.

In Lab Procedure-
Done on 3/24/18

Yeast

- Took plates out of the shaker
- Prior to centrifugation
    - Filled an empty plate with water to balance
    - Balanced plates with the right amount of water
    - Put in centrifuge --> Ran at max rpm (4000)for **15 mins**
    - Applied parafilm to plates to prevent bumping out of wells
- 1st Centrifugation
    - Spun at 4 degree for 15 mins
    - Spun again at same conditions for 15 min for a total of 30 mins
- Supernatant Transfer
    - Transferred to a new 96 well plate
- Evap Centrifuge
    - At 30 degrees for 2 hours initially
    - Balanced with another empty plate
    - Ethanol to be added post centrifugation at room temp
- Ethanol added at 1:39
- Sonicated for 30 mins
- Spun in regular centrifuge for 20 mins at 4 degree at 4000 rpm
- Transferred to new plate
- Evap centrifuge for 45 mins

MeJe

- Wash plant cells with MS (25%) and H20 (75%) sterile
    - Prepped 60 mLs of this media and added to reservoir
- Cells were washed and the solution in wells was discarded
- Methanol is added to the wells now to aid in lysing the cells
- Methanol added and cells were placed on shaker at room temp (started at 11:00 rpm of 120 for 2 hours)
- At 1:30, removed from shaker
- Placed in bath sonicator at 1:39 for 30 minutes
- Placed in centrifuge with the yeast sample. Spun for 20 mins at 4 degree at 4000 rpm.
- Transferred to new plate

- Evap centrifuge for 45 mins