Hudson, Nancy M., Establishment of plant cell culture methods to investigate wound healing properties of various plant cell lines. Internship Practicum Report, Biotechnology, May 2010, 118 pp., 2 tables, 17 figures, 5 graphs.

The investigation of plant cell lines often reveals a plethora of novel compounds with medicinal potential. The goal of this internship thesis was that after development of effective plant cell culture methods, the extracts obtained from plant cell culture cells will possess wound healing properties. The specific aims for this internship were: to establish plant cell culture methods, establish extraction methods of plant biomass, and develop bioassays to screen biomass extracts for wound healing applications. A fully operational plant cell research facility was established in available industrial space. Effective methods for the investigation of three plant cell lines were developed, and data was collected that indicate these cell lines produce medicinal compounds of significance within the various phases of wound healing.

ESTABLISHMENT OF PLANT CELL CULTURE METHODS TO INVESTIGATE WOUND - HEALING PROPERTIES OF VARIOUS PLANT CELL LINES

INTERNSHIP PRACTICUM REPORT

Presented to the Graduate Council of the
Graduate School of Biomedical Sciences

University of North Texas
Health Science Center at Fort Worth

in Partial Fulfillment of the Requirements for the degree of

MASTER'S IN BIOMEDICAL SCIENCES
with a focus in BIOTECHNOLOGY

By

Nancy Hudson

Fort Worth, Texas

May, 2010

ACKNOWLEDGMENTS

First of all, I would like to thank Dennis Carson and Duncan Aust of Healthpoint, Ltd. for allowing me to complete my internship at the Company's Research and Development Department. If it weren't for their gratuitous belief in a simple intern, none of this would have been possible.

I would also like to thank my mentor, Brett Kiedaisch, for his considerable help and guidance throughout the duration of this internship. He was not only there for me on a professional level, but also helped me to surpass the emotional rigors that coincide with completing a project of this magnitude. He was integral in the learning and understanding the inner workings of industrial research. In fact, this gratitude extends to all of the scientists that helped me throughout this process, especially Eric Roche and Lei Shi. Thank you to you both for always making me feel that your door was open to my often times incessant questions.

At the University of Texas Health Science Center in Fort Worth, I would like to thank all of my professors for helping me to achieve a wonderfully rich and diverse foundation with which to build on. Thank you to my Major Professor, Dr. Dimitrijevich for serving as my inspiration, and being there to put me in place when needed. I would like to also give special accolades to my wonderful advisor and confidante, Dr. Gwirtz. Her continued faith and assurance will last a lifetime. I would also like to thank Dr. McClain for him preparing me for the industrial research setting and his contributions while serving as a committee member for this research internship project.

Last but not least, I would like to thank my extraordinary family and friends—especially my grandmother, Josephine B. Davis. Without her unconditional love and support I would have never even imagined that I could have attained such a high degree of pride, dignity and excitement for what the future might hold.

Appendix I

List of frequently used acronyms:

TNF-α	Tumor Necrosis Factor-alpha
ECM	Extracellular matrix
MMP	Matrix metalloproteinases
TGF-ß	Tumor growth factor-beta
LPS	Lipopolysaccaride
ELISA	Enzyme-linked immunosorbent assay
LDH	Lactate dehydrogenase
DMSO	Dimethyl sulphoxide
VEGF	Vascular endothelial growth factor
PAF	Platelet activating factor
PDGF	Platelet derived growth factor
HPLC	High performance liquid chromatography
SEC	Size exclusion chromatography
FGF	Fibroblast growth factor
EGCG	Epigallocatechin gallate
BSA	Bovine serum albumin
FBS	Fetal bovine serum
PBS	Phosphate buffered saline

TABLE OF CONTENTS

CHAPTERS

CHAPTER I

INTRODUCTION

The goal of this practicum was to establish reliable methods for plant cell culture lines in order to obtain bioactive extracts. These extracts were screened in mammalian cell cultures to determine any medicinal advantages in wound healing.

If the *in vitro* results indicate biological potential for activity, these extracts may then be tested in a wound -healing model that has been developed by Healthpoint, Ltd. An extension of the evaluation protocol may then include animal models and eventually human subjects.

Fulfillment of these goals requires significant research in the areas of plant and animal cell biology. Specific areas include plant and animal cell culture methodologies, and evaluation methods of the effects on phases of wound healing. The establishment of dependable, reproducible and efficient bioassays is a critical task in the execution of this project, because these bioassays will be utilized by Healthpoint, Ltd. as standard protocols to discover medicinal applications of the plant species in their cryopreserved library of 60,000 samples of plant tissues.

CHAPTER II

INTERNSHIP SUBJECT

The main goal of this internship practicum was to establish working protocols for plant cell culture, as well as methods for the subsequent investigation of biomass extracts for wound healing applications. After a significant amount of time had elapsed past the internship's commencement, it was realized that the identification of specific compounds to promote wound healing would exceed by far beyond the time allotted for the internship.

The methods that follow were those that led to the most promising results after many attempts. The numerous unsuccessful trials and research that went into the development of these methods were excluded from the final practicum report.

OVERVIEW OF THE LITERATURE

PLANT BIOTECHNOLOGY

Plant biotechnology is the set of biological techniques developed originally from methods for studying cellular biology, which are applied to research and product development through the use of plants (Daurnenburg, 2008). Plants potentially contain and excrete biologically useful compounds that may be used in vaccinations, vitamin supplements, cosmetics, and consumer food products (Sharma & Sharma, 2009). The plant kingdom is vastly diverse, and as of yet, its pharmaceutical exploration is still in its infancy. It will continue to be a new and developing area of scientific research.

Before our present advances, there were formidable limitations in plant research. The utility of plant-derived biomaterials may be affected by availability due to seasonal and geographical limitations or devastation by insect populations. Likewise, harvests may be sparse because of drought, hail, flooding or some other type of inclement weather. There were and continue to be problems with inconsistent composition due to differences in plant genetics commonly seen between species located in different geographic locations (Babinard, 2001).

Some of these concerns can be compensated for by plant cell culture research. It is now possible to control the majority of the factors associated with plant growth and development. Cultures can be subjected to any desired temperature, moisture, lighting, nutrients, and so forth (Evans et al, 2003).

Advances in cell culture technology have led to the engineering of bioreactors—vessels capable of providing a superior environment for plant cell growth ranging from milliliter capacities all of the way up to tens of thousands of gallons (Eibl & Eibl, 2008). Shikonin, an expensive pigment commonly found in cosmetics, is one of the most widely biotechnologically produced plant derivatives and is manufactured in state-of-the-art 75,000 liter capacity bioreactors (Scharch et al, 2008).

PLANT CELL CULTURE

Plant cells are unique in that they are totipotent—meaning that through careful manipulation of culture conditions, a single somatic cell has the potential to regenerate an entire organism (Evans et al, 2003). Phytohormones, such as kinetin or auxin, are necessary ingredients in any plant cell culture environment. Depending on the balance of these two opposing plant hormones, the investigator may control the portion of the plant that will result. For example, increasing the concentration of auxin in the media, will generate root formation. On the other hand, shoot formation is most likely to occur if more cytokinin (a type of kinetin) is added to the culture medium. Generally speaking, an equal proportion of auxin to kinetin hormones in solution will impact only cell proliferation and result in the formation of a callus—masses of undifferentiated plant tissue (Evans et al, 2003).

After a callus is generated, it is subdivided into small sections and transferred from the surface of solid agar into a liquid medium. The subcultures are placed into a shaker (agitator) incubator that will provide adequate oxygen supply to the cells as well as control light cycles, humidity, temperature, and available carbon dioxide gas for respiration. The media for each type of plant cell culture has to be carefully defined, as establishment of a successful biomass in

liquid suspension will vary from specie to specie. The types of hormones, vitamin supplements, and electrolytes used will be derived from previously established methods or by development of a new protocol based on well-designed optimization experiments (Evans et al, 2003).

Ideally, the generated biomasses are transferred into media that will induce the synthesis of secondary metabolites—plant extracts. Plant extracts are then dried to prevent degradation of sample before being ground into finer particles (Evans et al, 2003). Compounds are extracted by solubilization in pressurized gasses or the use of polar liquid gradients. Chromatography methods such as high performance liquid chromatography (HPLC) and size exclusion chromatography (SEC) are then employed for the isolation of secondary metabolites (Romanik et al, 2007). Finally, extracts are distinguished from one another using analytical tools such as nuclear magnetic resonance, mass spectrometry, or infrared spectroscopy (Hagel & Facchuni, 2008).

It should be noted that internship time constraints did not allow for all of the described methods in plant cell line investigation to be employed. Secondary metabolites were not induced in plant cell suspensions, and the analysis of extracts was limited to simple protein quantification of biomass homogenate.

WOUND HEALING IN MAMMALS

There are four main phases that occur in wound healing that are not discrete but progress sequentially without interruption, and overlap each other: coagulation, inflammation, cell migration and proliferation, and remodeling.

The first phase of wound healing, coagulation, occurs directly after tissue injury to restore the integumentary barrier. Neighboring vasculature provides fibrin (converted from fibrinogen via thrombin) and red blood cell platelets to collect at the wound site and form a clot. This wound "plug" serves as a hemostat and transient scaffold (matrix) that allows cell migration onto and into the wound bed and holds damaged tissues together while tissue repair is underway (Schultz et al, 2005). Stem cells from bulges located half way down the shaft of hair follicles differentiate into keratinocytes, which migrate to form a bridge directly underneath the eschar. Blood platelets release singaling mediators that cause the endothelial cells in the vicinity to contract in small vessels, thereby minimalizing the blood loss to the damaged area (Hunt et al, 2000).

Upon formation of the wound plug, the inflammation phase is initiated. In this phase platelets release cytokines and growth factor including platelet derived growth factor (PDGF), platelet activating factor (PAF), and transforming growth factors (TGF) ß$_1$ and ß$_2$ (Hunt et al, 2000). These cytokines in turn recruit the immune response cells that are important for wound healing: monocytes and neutrophils. In the presence of TGF- ß cytokines, monocytes bound to the extracellular matrix (ECM) differentiate into macrophages. Mature macrophages assist neutrophils in the eradication of damaged ECM and cell debris (Singer & Clark, 1999).

Macrophages are crucial as the source of signals (cytokines) that direct the healing process and assist in transition from one phase to another. If conditions at the wound site should

require it, they secrete tumor necrosis factor-alpha (TNF-α) to perpetuate and amplify the inflammatory response (Singer & Clark, 1999). When the area has been adequately prepared for tissue remodeling, macrophages will secrete PDGF, fibroblast growth factor (FGF), and vascular endothelial growth factor (VEGF), which signal endothelial and fibroblast cells from the wound edge to migrate into the wound (Hunt et al, 2000).

Positive chemotaxis—cell movement toward a higher concentration of chemoattractant—directs the cell migration. Epidermal cells (keratinocytes) are recruited from the follicles of hair shafts or healthy marginal epidermis (Singer & Clark, 2009) to cover the wound bed. The migratory cell first binds to integrin receptors located on the extracellular matrix. The unattached portion of the cell then moves toward the wound site (where cytokines are being secreted), and attaches to collagen fibrils. Extracellular metalloproteinases (MMP's) degrade the initial bond with the extracellular matrix, which allows the keratinocyte to migrate and re-attach to the matrix more proximal to the source of chemoattractant secretion. The cell moves ahead in a "step-wise" manner, aided by the enzymatic activity by MMP's on bonds formed between the ECM and collagen fibrils that extend throughout the wound site (Schultz et al, 2005).

Once cells invade the wound site (provisional matrix/wound "plug"), they begin to proliferate, or undergo rapid mitotic division. Cells then synthesize and deposit collagen and other ECM constituents, which provide support for the influx of newly forming blood vessels (angiogenesis) (Hunt et al, 2000). Fibroblasts that are attached to the wound edges begin to contract, which pulls the edges of the wound site together, reducing the volume and surface area to be repaired. Meanwhile, granulation tissue is structured in the site while the fibrin clot is broken down by protease activity (fibrinolysis) (Singer & Clark, 2009).

The last stage of wound healing is remodeling. In this phase, the number of capillaries and ECM components such as proteoglycans and glycosaminoglycans are dramatically decreased. Cell density and metabolic activity in granular tissue also decrease in this stage. Finally, collagen is reorganized to improve the tensile strength of the dermis. At this point, the skin only has 25 percent of the original tensile strength. As the remodeling process continues, the "repair" will continue to become stronger over the next few months, but will never exceed 80 percent (Schultz, 2005).

Remodeling is the longest phase which can take weeks, months, and sometimes years depending on the size and depth of the wound, the person's age, and their overall health condition. The repair process does not recapitulate the original integrity of the tissue. Additionally, blood vessel supply is far less profuse and elasticity is significantly reduced (Schultz, 2005).

The development of pharmaceuticals to facilitate the wound healing process is presently a very popular area of research. The incidence of chronic wounds such as decubitus ulcers, or bedsores, has been on the rise as the diabetes epidemic in the United States worsens. Knowledge of the wound healing process and its integral phases is crucial to the investigation of new pharmacologics to combat such pathologies.

IN VITRO METHODS USED IN MAMMALIAN WOUND HEALING

The following four assays are the most commonly used to determine if a compound facilitates the wound healing process: cell migration, anti-inflammatory, cytotoxicity, and cell proliferation.

CELL MIGRATION ASSAY

Cell migration is a critical step in the wound healing process because it brings wound - healing phenotype of cells to the wound bed to rebuild the tissue damage. In the commercial setting, this assay is usually performed by the utilization of a manufactured kit, the "Cell BioLabs CytoSelect™ 96-well Cell Migration Assay Kit". The candidate compounds are evaluated as chemoattractants are pipetted into the wells of the lowest tray, or the feeder tray. Required numbers of target cells (generally fibroblasts and keratinocytes) are then seeded into the middle (membrane) chamber wells; and the whole apparatus is kept in an incubator overnight. The middle chamber has 8 micron sized pores that will allow cells that are being activated to migrate—to pass from the well into the bottom chamber. The following day, a detachment solution is added to the lower tray, which will cause the cells that have migrated to the underside of the middle tray wells to detach into the feeder tray. Migratory cells are lysed with a buffer, incubated with a fluorescent dye (that binds to nucleic acids), and then fluorescence is measured in a microplate reader. Fluorescence is a measure of extent of cell migration; and therefore, higher fluorescence readings indicate that candidate is a more potent chemoattractant (Entschladen et al, 2005).

ANTI-INFLAMMATORY ASSAY

A simple protocol that identifies if a compound has anti-inflammatory properties uses monocytes as target cells. Monocytes are immune cells that normally respond strongly to lipopolysaccaride (LPS—a component of gram negative bacterial cell walls) by secreting large quantities of inflammatory mediators. Prior to treatment with LPS, THP-1 monocytes are incubated for a period of time in the presence of the anti-inflammatory test compound. The cells

are then treated and incubated with LPS for 4 hours. The media is collected and analyzed by an enzyme-linked immunosorbent assay (ELISA) to detect the presence of TNF-α, a potent pro-inflammatory cytokine. The results are compared or normalized to control cells that had not been exposed to LPS. If the compound suppresses the inflammatory response, the TNF-α concentration will be significantly less when compared to the negative control group (cells that were only incubated in LPS) (Singh et al, 2005).

CYTOTOXICITY ASSAY

This protocol determines the cytotoxicity of a compound, i.e. its ability to kill cells. There are several pathways that lead to cell death (for example apoptosis, protein synthesis inhibition, DNA synthesis inhibition, etc.), but the assay kits used to measure cytoxicity do not discriminate between the specific mechanisms that have occurred. They simply quantify the number of dead cells resulting from the treatment with a test compound. One cytotoxicity method measures lactate dehydrogenase (LDH), which accumulates when the plasma membranes of the cells are compromised and cells become leaky. Basically mammalian cells are grown to the desired number, trypsinized, plated, and then incubated in the presence of a known concentration of the potentially cytotoxic agent. The supernatant is then incubated with a chromogenic dye solution that binds to formazan—the product generated when LDH reacts with the tetrazolium salts solution. Absorbance is then read using an ELISA plate reader. If the compound being tested is toxic, the amount of LDH detected will increase compared to the untreated cultures (Roche, 2008).

CELL PROLIFERATION ASSAY

The cell proliferation bioassays measures cell division. Every time that a cell divides it replicates all of its genomic material so the daughter cell will be genetically identical to the parent cell. The commercially available kits used for the proliferation assay utilizes the fact that cells in the S phase (just prior to DNA synthesis and replication) will incorporate labeled DNA precursors into their genome. The quantity of proliferating cells can then be detected utilizing an ELISA technique that is based on specific monoclonal antibodies against the labeled DNA precursor incorporated into the cellular genome (Roche, 2008).

INTERNSHIP GOAL & SPECIFIC AIMS

The practicum project can be divided into three separate phases that would normally be completed sequentially. However, to accommodate time constraints these phases were executed independently of one another but conducted simultaneously.

Goal: To establish methods in order to investigate plant cell cultures for wound healing properties.

- **Specific Aim 1: To establish plant cell culture methods**

After the initial construction and general organization of the plant cell laboratory, plant species were selected, appropriate media and growth/storage conditions procured, and cell cultures initiated from cryopreservation. Plant cell calli were then generated on agar plates, and finally transferred to an aqueous media to be grown in cell suspension.

- **Specific Aim 2: To establish extraction methods of plant cell biomass.**

Established biomasses of *Lycopersicon esculentum, Astragalus falcatus,* and *Prunella vulgaris* cell lines were shipped from Phyton Biotech, Inc. in Germany. Immediately upon arrival, the biomasses were transferred to a liquid medium for cell suspension cultures. Plant cells were grown in shaker incubators until sufficient biomasses were established and biomasses were lyophilized. Methods were then established to extract compounds from freeze-dried biomass. Finally, extracts were resuspended in a solvent that was suitable for use in *in vitro* investigation.

- **Specific Aim 3: To develop methods that will be used to determine if extracts have wound healing properties.**

Manufactured secondary plant metabolites were purchased and incubated with mammalian cells: human epidermal keratinocytes isolated from adult skin (HEK_a) and human dermal fibroblasts isolated from adult skin (HDF_a). Cell migration, anti-inflammatory, cytoxicity and cell proliferation bioassays were then conducted to examine whether the extracts have wound healing properties.

SIGNIFICANCE

Much of the research being conducted on plant-derived biologically active compounds tends to continually emerge from nations in the eastern hemisphere. It is surprising that more research in natural alternatives for wound healing and other areas of medicine are not being more aggressively pursued in the United States. For example, extracts from *Colotropis gigantean* (or milkweed) is used in India as a home remedy for indigestion, diarrhea, and fevers. New research conducted by Deshmukh et al (2009) shows that the application of extracts from this plant to cutaneous wounds significantly decreases healing time as measured by the percentage of wound closure and epithelialization rates. At high concentrations, *C. gigantean* was found to be non-toxic and actually increased the breaking strength of the healed wound by two-fold compared to the results from untreated controls (Deshmukh et al, 2009).

The application of *C. gigantean* to expedite wound closure and improve wound strength is but one potential therapeutic application of a single plant species. There are numerous examples of similar folkloric-based medicinal remedies worldwide. Collectively however, scientific research in plant-derived therapeutics is minimal and should receive significantly more attention, effort and commitment.

METHODS AND MATERIALS

LAB CONSTRUCTION PHASE

The lab space allocated for the new plant cell research laboratory was essentially a blank canvas at the beginning of the internship. While it was being constructed over the next four months, several challenges had to be met.

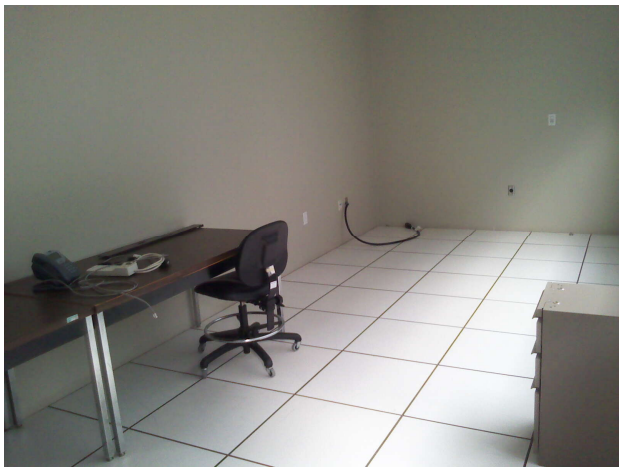


Figure 1. The plant cell laboratory at the internship's commencement.

Shelving units for storage were constructed, and separate areas in which plant media was to be prepared were cleared and cleaned. Masses of glassware (BELLCO Biotechnology, Pyrex flasks with divets) and plant cell culture tools such as scalpels, razor blades, spatulas, etc.) were moved out of long-term storage in the warehouses. Each tool and every piece of glassware

(approximately 40 of each size flask: 1 Liter, 500ml, 250 ml, and 125 ml; as well as sufficient of Buchner filter flasks) was washed and autoclaved before it was transported into the lab space. Myriad components for media such as vitamins, hormones, and minerals, and additional lab supplies such as petri dishes, ethanol spray bottles, and microporous tape were ordered and stored accordingly.

Once the electricians had finished, the lab equipment was removed from crates and transported into the room. This included two plant cell-culture hoods, two Percivals, two shelving units of glassware, and two shaker incubators. All of the equipment was thoroughly cleaned with a 10% bleach solution inside and out.

After the equipment had been inspected and certified, plant biomasses were ordered and shipped from Phyton Biotech in Germany (a company that had recently been acquired by Healthpoint, Ltd.). Upon their arrival in late December, plant cell suspension cultures were immediately initiated.

SPECIFIC AIM 1: PLANT CELL CULTURE METHODS

MAINTAINING AN ASEPTIC ENVIRONMENT

Several precautionary measures were required to prevent the contamination of the plant cell suspensions, calli, and collected plant cell biomass. Since there is no glass barrier on a plant cell culture hood, bacterial and especially fungal contamination was very likely. The following were meticulously followed to prevent the incidence of contamination:

- Prior to the commencement of any scientific investigation, all lab equipment was liberally cleaned with a 10 % bleach solution both internally and externally.
- All work surfaces were sprayed liberally with 70 % ethanol and wiped with a Kimwipe (Kimtech Science) before and after hood use.
- All items brought into the hood it were sprayed down with 70 % ethanol. This included all pipettes, flasks, media containers, tools, and especially one's own gloved hands.
- Only clean, sterile, autoclaved tools such as spatulas, scalpels, and forceps were used.
- During experiments, all tools were kept in a 95 % ethanol and were passed through an open flame before the manipulation of cell cultures of any type.
- All work in the hood was carried out as close to the most distal wall as possible—the area directly in front of the air circulation vents was the only area that was considered sterile

PREPARING MEDIA FOR PLANT CELL CULTURE

One of the most crucial tasks of plant cell culture is media preparation because several liters of media are required every week in order to sustain even a small number of plant cell cultures. The process for making the media was preparing the stocks, combining the stocks and other nutrients in the media, adjusting the media to the proper pH, aliquotting it into smaller flasks, autoclaving, and finally storing it. It is important to note that all media and its respective stocks have a shelf life of only two weeks beyond their preparation, and therefore, is best if media is made just prior (one to three days) to use.

For each specie of plant, there is a specific balance of hormones, nutrients, vitamins, etc. that is necessary for the proper growth of healthy cell cultures. If the recipe is not available for the particular species of plant that is to be cultured, a growth square can be initiated to help deduce the desirable quantities of each ingredient. A growth square is a trial and error method used to manipulate several independent variables in a single experiment to determine the most favorable conditions for culture growth (Evans et al, 2003).

	Cytokinin (µM)			
Auxin (µM)	**0**	**0.5**	**1**	**5**
0	1	2	3	4
0.5	5	6	7	8
1	9	10	11	12
5	13	14	15	16

Figure 2. Growth square of a theoretical experiment to establish optimal hormone concentrations for plant cell calli. Numbers “1” to “16” are individual petri dishes on which plant tissues will be cultured at the indicated concentrations of auxin and cytokinin hormones. The correct balance will have been achieved upon the successful generation of a plant cell callus culture.

In general, a protocol delineating composition is followed to prepare stock solutions containing hormones or vitamins and amino acids several days prior to passaging or cell suspension initiation. The stock solutions and a few additional ingredients are mixed together, and the pH is adjusted to within a particular range. Figures 3 through 5 depict examples of stock and media protocols. Note that volumes were removed for proprietary reasons.

STOCK: B5 Vitamins(100X)

Amount to make: 1 Liter(s)

Ingredient	Ingredient Lot #	Amount/L	Amount to Add	Amount Added	Init.
Myo-Inositol		g	g		
Nicotinic Acid		mg	mg		
Thiamine.HCl		g	g		
Pyridoxine.HCl		mg	mg		

Figure 3. "B5 Vitamins" stock components

STOCK: MS Macro(10X)

Amount to make: 1 Liter(s)

Ingredient	Ingredient Lot #	Amount/L	Amount to Add	Amount Added	Init.
NH4NO3		g	g		
CaCl2		g	g		
MgSO4-7H2O		g	g		
KNO3		g	g		
KH2PO4		g	g		

Figure 4. "MS Macro (10X)" stock components

NTM 37 Date: ____/____/____ Batch Number_____

Pre-Autoclave Components Volume to make:_____Liter(s)

Component Name	Lot Number	Amount/L	Amt. Needed	Amt. Addded	Init.
Sucrose		g			
MS Micro(1000X)•		ml			
B5 Vitamins(100X)•		ml			
MS Macro(10X)•		ml			
Iron Stock(50X)•		ml			
2iP(1mM)•		ml			
NAA(5mM)•		1,00 ml			

Initial pH:_____ By:_____ Adjust to pH: 5.60 ± 0.03 Final pH:_____ By:_____

Solid Media?.....Add 8 g **Agarose** And remember to add a stir bar to each flask.

Liquid Media?....If to be autoclaved remember to add an additional 8% R.O. Water. (80ml/L)

Figure 5. "NTM37" media components (• = indicates that this a stock solution, and must be prepared separately)

After the medium is made, it was advantageous to aliquot it into the different sized flasks before autoclaving. This maintained media sterility during transport, storage, and while it was being utilized in cell culture and periods of cell line expansion. Growth medium was dispensed at one fifth ($1/5^{th}$) of the total flask volume; and then 8% additional de-ionized, distilled water was added to compensate for evaporation during autoclaving. Flasks were autoclaved on a cycle that was appropriate for liquids; and then were stored in a clean, dark area at room temperature.

PREPARING AGAR PLATES FOR CALLUS INITIATION

The protocol specific to the cell line to be initiated was followed to prepare liquid media that included sugars, hormones, vitamins, and 8% extra de-ionized, distilled water to compensate for autoclave evaporation. Media was aliquotted equally into two clean, labeled 1 Liter flasks and then 4 grams of agar was added to each. Flasks were swirled to begin mixing, heat-sensing ampoules applied, and were autoclaved on a cycle that was appropriate for liquids. Autoclaved flasks were carefully transferred to a clean hood and allowed to gradually cool down in a dark environment. Flasks were agitated in a circular motion intermittently to ensure evenly distributed cooling. Once the flasks were only warm to the touch, the agar medium was poured into sterile 100 mm petri dishes until the bottom was sufficiently covered—about one-third of an inch thick. The covers were then applied to the plates, however, left slightly cracked open along the back wall of the hood to prevent condensation buildup while they congealed. Once they had completely set, the petri dishes were covered, the plates were stacked and returned to a sterile sleeve, and stored in a dark area. If plates were to be used immediately, they were kept at room temperature; or moved to 4 °C wrapped in aluminum foil for long-term storage. It is important to note the plates will expire within two weeks of pouring.

THAWING CRYOPRESERVED SAMPLES FOR CALLUS INITIATION

Adherence to a strict protocol was followed to initiate cultures from cryopreservation. Cultures were rapidly thawed to limit cell stress and death, filtered to remove cryoprotectant (sorbitol), and immediately plated on fresh agar plates. The following method has been adapted from an established method by Dr. Weiming Wang (Phyton Biotech, 2006).

Cryopreserved sample vials were removed from long-term storage and transferred directly into a portable cooler containing liquid nitrogen. It was important that the samples did not begin thawing until they have reached the hot water bath. Up to eight sample vials were placed into the hot water bath (42 °C), and intermittently stirred in the bath to ensure even heat distribution. The status of the vials was visually ascertained every half minute to monitor when the samples had thawed. When the vials had thawed but were still cool to the touch, they were quickly removed from the hot water bath. The sample vials were sterilized externally by dipping them (cap-down) into a beaker containing a 75% ethanol solution for two minutes. The vials were then moved to a sterile hood and allowed to dry before calli initiation.

CALLUS INITIATION

Flasks containing the appropriate sterile medium and a filter funnel flask setup (already assembled with a filter funnel and filter paper that has been wrapped in aluminum foil and autoclaved) were placed into the laminar air hood. Suction filtration tubing was applied to the setup and the vacuum was applied. The vial contents were poured onto the filter paper to remove the cryoprotectant solution. Additional medium was then slowly poured onto filter paper and cells to remove any remaining cryoprotectant.

A pair of sterile forcepts was used to carefully remove the filter paper (containing filtered biomass) to the surface of a petri dish containing media and agar. Petri dish lids were covered and the sides were sealed with microporous tape. The plates were then transferred to a Percival (Advanced Intellus environmental controller) set at 25°C, 43% relative humidity, and kept in dark conditions.

CALLUS MAINTENANCE

After one day had elapsed, the filters were transferred to a new plate and placed back in the Percival. Filters containing growing cell calli were then carefully transferred to fresh solid media once every seven days. After the biomass had reached confluence on their filters, cell calli were subdivided with a sterile razor blade and the smaller sections were transferred aseptically with a spatula to fresh solid media. Some callus cultures would grow vertically instead of spreading across the agar surface, which would ultimately cause the lower areas of the cell callus to dry out. In these instances, the upper, more youthful cells would be scraped off of the top of the callus and plated while the rest would be discarded. Cell calli continued to be subdivided and split until ample biomass was produced and cell suspensions could be initiated.

INITIATION OF CELL SUSPENSIONS

Once biomass from Phyton in Germany had arrived, it had to be used immediately to initiate cell suspension cultures. Being careful to observe to rules of the aseptic technique, all hood surfaces are wiped down with 70 % ethanol; and an autoclaved filtration setup, the biomass to be cultured, and autoclaved media were placed inside the hood. The filter containing the autoclaved filter paper was removed from the filtration setup and then placed on the scale. The scale was then tared, and filter was replaced back on the filtration setup. A sterile spatula was used to transfer biomass into the top of the filter setup. Fresh, autoclaved media was poured over the top of the biomass and suction was applied to remove any soluble debris. The filter containing the freshly filtered biomass was placed back on the scale to give a fresh weight reading of biomass. The lids of the flasks containing the autoclaved media were removed and the necks of the flasks were passed through an open flame several times. Small amounts of

biomass were transferred with the spatula from the filter into the new flask until the proper of biomass had been removed.

Flask size	Total Volume (after autoclaving)	Amount of Biomass to be Transferred
125 mL	25 mL	0.5 grams
250 mL	50 mL	1.0-2.0 grams
500 mL	100 mL	3.0-4.0 grams
1000 mL	200 mL	5.0-6.0 grams

Table 1. Delineates the appropriate amount of media aliquotted per flask size, as well as the amount of biomass used to initiate a plant cell culture suspension

To initiate a cell suspension for cell calli, small sections were sliced into cell calli with a sterile razor blade. The entire plate, filter paper, and subdivided callus were placed onto the scale to obtain an initial weight reading. Sterile forceps were used to transfer healthy pieces of the calli (those that were light in color and exhibited no signs of necrosis) from the filter one by one and transferred to the fresh growth medium until the scale registered that the correct amount of mass had been removed.

Note: The correct amount of callus required to initiate a cell suspension was the same quantity needed to initiate a cell suspension from biomass.

CELL SUSPENSION MAINTENANCE

After cell suspensions were initiated they were immediately moved to Kuhner Shakers (ISF-1-W, Switzwerland) and set at 25 °C, 120 rpm, and in dark conditions. Cell suspensions must never be left stationary—if they are removed from the shaker/incubator, they must be agitated on a Dual Action Shaker to limit the formation of large plant cell aggregates. Cell suspensions were checked daily for any signs of contamination, the current cell density (media viscosity), or any change in color, which usually denotes cell stress. Cell suspensions generally remained in their initial flasks eight to ten days before passaging; however, each type of specie grows at different rates. To determine the optimal period of time growth before passaging suspensions, an experiment was designed to generate that particular specie's growth curve.

GROWTH CURVE GENERATION

Once the suspension in a one-liter flask appears that is confluent, it is aseptically transferred into a sterile hood along with eight to ten 250 mL flasks containing fresh, autoclaved media. An autoclaved filtration setup was attached to a suction pump; and the filter funnel (with the filter paper) was tared on the scale and moved back to the apparatus. The cell suspension was poured onto the filtration setup under suction to remove spent media. With a sterile spatula two grams of biomass were carefully transferred into each 250 mL flask and immediately placed into a shaker incubator. Every two days, a 250 mL flask containing cell suspension was removed, filtered, and weighed; and the results were recorded and graphed.

PASSAGING OF CELL SUSPENSIONS

After the cell lines were proficiently established as described, a modified, more time efficient method was developed to passage cell suspensions. Cell suspensions were transferred from the flask containing spent media to fresh media by direct pipetting. Extreme caution for observing the aseptic technique was employed—as the likelihood of fungal contamination significantly increases when this method was used to passage cell suspensions. It was especially imperative to flame the necks of all of the flasks for several seconds, and to prevent the pipette from touching anything except the media and cells themselves. Growth curves were set up to establish the following data:

Volume of the flask	Amount to be pipetted
250 mL	7 mL
500 mL	14 mL
1000 mL	28 mL

Table 2. Delineates the amount of cell suspension to be transferred by volume of the flask when employing the pipetting method to passage cultures into fresh medium.

A sterile serological pipette (with a wide tip) was used to transfer the appropriate amount of cell suspension to fresh, autoclaved flasks with media, noting that the volume that was pipetted varied based on the density of the cell suspension being passaged.

BIOMASS COLLECTION

Cell suspensions cultures were passaged for several weeks to expand the total biomass that could be collected. The flasks of cells to be collected (several back-up flasks were always kept in the incubators) were aseptically transferred to the hood. Filtration setups, a sterile spatula, and 50 mL centrifuge tubes were also aseptically transferred into the hood. Excess media was removed from biomass by filtration as previously described. Fifty grams of the filtered biomass was then transferred into 50 mL tubes, stoppered, and and then stored in a -80 °C freezer.

Approximately one-half of the biomass collected was to be used for protein extraction, and the other half was to be lyophilized and used for the extraction of other compounds.

LYOPHILIZATION OF BIOMASSES

Fifty-milliliter centrifuge tubes containing the biomass samples to be lyophilized were transferred from -80 °C and placed on ice to thaw at a slow rate. After approximately twenty minutes, biomass was removed from the 50 mL tubes into three shallow, round dishes and broken into small, even clumps with a sterile razor blade. The biomass was spread out as evenly as possible into a layer that was roughly a half an inch in thickness. The dishes were then covered with paper towels that were secured with rubber bands, and frozen again in the -80°C freezer.

From the –80°C freezer, samples were immediately loaded into lyophilizer trays. A cycle that was programmed specifically for cell biomass (Weiming Wang, Phyton Biotech) and Catherine Van Der Kar (Healthpoint, Ltd.) was then initiated. Cycle parameters (for the VirTis advantage Plus Lyophilizer):

- Thermal treatment: -35 °C for 60 minutes
- Freeze: -30 °C (Additional time for 60 minutes), Condenser at –60 °C, Vacuum: 100 mtorr.
- Drying Cycle: -30 °C for 1200 minutes, ramp up to +20 °C for 1200 minutes followed by an additional 60 minutes at +20 °C (if temperature was not reached). The vacuum for the entire drying cycle was set at 50 m torr.
- Secondary drying: set point: +40 °C; Post Heat: 25°C degrees for 240 minutes with vacuum set at 1000 m torr.

Upon cycle completion, the freeze-dried material was removed from the lyophilization equipment, collected into fresh 50 mL centrifuge tubes, weighed, and stored at room temperature in the dark.

SPECIFIC AIM 2: ESTABLISHING EXTRACTION METHODS

ISOLATION OF BIOACTIVE COMPOUNDS FROM LYOPHILIZED BIOMASS

Extraction methods were adapted from those previously established (Long et al., 2008). Freeze-dried biomass (220 mg) were transferred into tared glass tubes, and treated with 6 mL of methanol (MeOH; HPLC Grade, Fisher Scientific) with vigorous shaking for approximately one minute, and then placed on ice for an additional three minutes. Six mL of 0.05 M Tris HCl (Sigma Aldrich) was transferred to the mixture, tubes were shaken again, and were immediately secured to a rotating apparatus for 15 minutes at 4 °C. Sixteen mL of chloroform (CHCl3; reagent ACS Spectro Grade, Fisher Scientific) was added to the mixture, the tubes were shaken again and incubated on ice for an additional 10 minutes. Samples were then centrifuged at relative centrifugal force (rcf) of 3,000 for 7 minutes at 4 °C. The resulting hyper-phase (containing the MeOH extracts) and hypo-phase (containing the $CHCl_3$ extracts) were then carefully removed by pipetting into fresh glass tubes. Care was taken to avoid contamination with the emulsified middle phase. Extracts were either stored at 4 °C or immediately moved to evaporator to isolate extracted compounds from the solvents.

EVAPORATION OF EXTRACTS

The solvents that were used (methanol and choloroform) to extract compounds from biomass would be very toxic to mammalian cells and were therefore, removed by evaporation. A GeneVac, Ltd. evaporator (HT-4X Series II System) was programmed specifically for each type of solvent, which must be run individually:

- For the hyper-phase of the collected extractions- an organic program specific for methanol and water was required: high rotor speed, ramp time of 20 minutes, heat to 15 °C (Control at 35°C), and a starting of 200 and ending at 50 mtorr.
- For the hypo-phase of the collected extractions- a program specific for chloroform ("TCM") was required: high rotor speed, ramp time of 35 minutes, cool to 32 °C (Control at 20 °C), and a pressure starting at 400 and ending at 50 mtorr.

Samples were meticulously balanced by both weight and type of solvent to maintain a constant weight throughout evaporation (methanol extracts were counterbalanced with methanol).

Noxious fumes were vented to an exhaust hood. The lids were removed from the vials and samples are loaded into centrifuge brackets. The correct program was selected, the type of container was set to "tube/vial holder" and specific volumes were entered. After evaporation was initiated, the evaporation progress was monitored intermittently.

If the samples were to be used immediately, in a bioassay for example, they were resuspended in 10 mL of filtered de-ionized distilled water. However, extracts that were prepared for long-term storage were suspended in 1 mL of dimethyl sulfoxide (DMSO) and kept at room temperature to limit the degradation of extracted compounds.

EXTRACTION OF PROTEINS FROM PLANT

A commercially available kit (P-PER® Plant Protein Extraction Kit from PIERCE Chemical Company) was purchased to extract proteins from both frozen and lyophilized plant tissue. The kit protocol and a slight modified version were followed to determine the best method for protein extractions.

Eighty mg of frozen material and 20 mg of lyophilized material were weighed into two mesh bags and two centrifuge tubes. A solution was then prepared containing three reagents (included with the kit) and a protease inhibitor (Halt Protease Inhibitor Cocktail Kit, Thermo Scientific Pierce), and was added to the respective containers. The sample in the mesh bag was ground within the bag with the back of a spatula, while the samples in the centrifuge tubes were pulverized with a plunger. Both were ground until a homogenous mixture was obtained and lysates were collected into centrifuge tubes to separate protein layer from the organic layer. The protein layer was then collected to fresh tubes and frozen in a -20 °C freezer until they were to be analyzed for protein concentration.

SPECTROMETRIC DETERMINATION OF PROTEIN CONCENTRATION

The samples obtained from the protein extraction experiments were removed from the -20°C freezer and allowed to thaw in a hot water bath. A mixture of bovine serum albumin (BSA) in water (10 µg/mL) was used to calibrate the NanoDrop (ND-1000 UV/Vis Spectrophotometer). Two microliters were transferred from the BSA mixture onto the measuring platform of the NanoDrop. The upper arm was then lowered onto the platform and the sample was analyzed to ensure that reading was equal to 10 µg/mL. After calibration, a sample of pure de-ionized, distilled water was used as a blank to serve as a starting point with which to measure protein samples from. Protein samples were then transferred 2 µL at a time (in triplicate) and the data was recorded for further analysis.

SPECIFIC AIM 3: ESTABLISHING BIOASSAY METHODS

As mentioned before, there are four main bioassays used to determine whether a compound possesses bioactivity pertinent to wound healing. These are cytotoxicity, cell proliferation, anti-inflammatory response, and cell migration bioassays.

Manufactured plant extracts were purchased from VitaDigest™. The compounds chosen to serve as positive and negative controls in future plant extract investigation were those that had been shown to have wound healing properties based on previously established research. Those were:

- "Echinacea": *Echinacea angutifolia* root/ *Echinacea purpurea* whole plant (1:1) (Nature's Answer®)
- "Astragalus": *Astragalus membranaceous* root (Nature's Answer®)
- "Chamomile": *Matricaria recutita* flower (Nature's Answer®)
- "St. John's Wort": *Hypericum perforatum* young flowering tops (Nature's Answer®)
- "Gotu Kola": *Centella asiatica* herb (Nature's Answer®)
- "Yarrow": *Achillea millefolium* flower (Nature's Answer®)

MAMMALIAN CELL CULTURE

Human dermal fibroblasts (Cascade Biologics™) isolated from neonatal foreskin and human epithelial keratinocytes (Cascade Biologics™) isolated from adult skin were seeded into 75 mm^2 cell culturing flasks in their primary passage at 150,000 cells per flask. Fibroblasts were cultured in 12 mL of 106 basal media (Cascade Biologics™) supplemented with low serum growth supplement from Cascade Biologics™ (LSGS), and keratinocytes were cultured in 12mL of 154 media (Cascade Biologics™) supplemented with Human Keratinocyte Growth

Supplement from Cascade Biologics™ (HKGS) and 200 microliters of sterile calcium chloride. Media was changed every other day until cells reached confluence. Cells were then passaged as needed into new flasks at approximately 500,000 cells until desired number of cells had been obtained. Additional cells were centrifuged and re-suspended 3 to 6 million cells per milliliter of Synth-a-Freeze™ (Cascade Biologics™) before being moved into cryopreservation.

Human monocytes taken from the peripheral blood of a patient with acute leukemia were obtained from American Type Tissue Culture (ATCC) and were initiated from their first passage in 25mm flasks. RPMI media that was supplemented with 1.7 microliters of 2-mercaptoethanol (BME) and 10% fetal bovine serum (FBS) was added to the cultures as needed to keep the cell suspensions at density between 300,000 and 500,000 cells per milliliter, and was changed every other day. These cells were cryopreserved by first suspending them in a solution containing 1ml of media and 5 percent DMSO for every 7 to 12 million cells to be frozen.

CYTOTOXICITY BIOASSAY

Living cells are essential to wound healing. Therefore an *in vitro* cytotoxicity bioassay should be used to determine if extracts would provide medicinal advantages. A Vybrant™ MTT cytotoxicty kit from Invitrogen was used to determine the toxic effects of a compound as shown by the depression of cells metabolic function. Enhancement of cells metabolic functions can also be measured with this particular assay.

Cells are seeded into the wells of a 96 well plate, using the optimum seeding density determined by conducting a trial run prior to the introduction of a test articles. Optimum seeding density experiments were initiated by seeding varying concentrations in a serial dilution into microwells for all of the following cell types: THP-1 monocytes, fibroblasts, and keratinocytes.

For example, it was found that the keratinocytes and fibroblasts have an optimal seeding density near 10,000 cells, while the THP-1 monocytes were around 75,000 cells per well.

After cells have been seeded into microwells, they were incubated for a minimum of four hours at 37 °C to allow for cell attachment. The MTT cytotoxicity is meant to be a qualitative assay because it measures chemilluminescence, which only tells the investigator how one treatment compares to each of the others. However, it was modified to quantify the number of viable cells after test candidate treatment by seeding additional wells in a series dilution (containing solely cells and media) and thus generating a standard curve with which to extrapolate more powerful data from.

Once the cells have attached and formed a monolayer, the manufactured plant extracts were prepared by filter sterilizing them and aseptically transferring different amounts (1, 5, and 10 µL) to 1 mL of serum-free, phenol red-free media in sterile tubes. All of the media and unattached cells were removed from the wells of the microplate, and 200 µL of the sample were added to each well. The plate was left undisturbed in an incubator at 37 °C for a period of 24 hours.

After the cells had incubated in the presence of the test compounds for no less than 24 hours, the MTT cytotoxicity kit protocol was followed. Viable cells produce cytosolic dehydrogenases, which reduce the kit's tetrazolium salts. The reaction causes a change in the media to varying degrees of violet hues, which is then measured in a microplate reader (Molecular devices: SPECTRAmax 384-PLUS, Program: SoftMaxPro) at 540 nanometers.

CELL PROLIFERATION BIOASSAY

A Cyquant™ cell proliferation assay kit (from Invitrogen) validates and refines the results obtained from the MTT cytotoxicity assays. Both assays can be used to determine cytotoxicity, which is the opposite response to proliferation; however, Cyquant™ is more frequently used for the evaluation of proliferation because it is designed to only measure the DNA present after treatment of the culture with the test compounds. It is more sensitive than the colorimetric-based assays, but will not discriminate between the cells that are healthy and those that have altered metabolic processes.

A standard curve was first generated to establish optimal seeding density as well as efficacy with both keratinocytes and fibroblasts. THP-1 cells were excluded from this process because an induced monocyte does not proliferate at the wound site—their role is to recruit other cells as well as to secrete the necessary cytokines to modulate inflammation.

It was found that the assay was most sensitive when each well of a 96-well microplate was seeded with 10,000 cells in 200 µL of serum-free media. The initial setup for this assay was similar to the cytotoxiciy assay since the wells were seeded with 2,500, 5,000, 10,000 and 20,000 cells to generate a standard curve. Samples are prepared following the same methods as previously described to eliminate as much variation in the results as possible. After cells have attached, the excess media and unattached cells were removed by pipetting; and 200 µL of each sample was added per well. The microplate was then incubated at 37 °C for a 24 hour period. Longer incubations can cause cell death due to the lack of nutrients. Serum-free/phenol red-free media is used in this assay to eliminate the effect of serum on cell proliferation. Phenol red affects the sensitivity of this assay due to interference with the fluorescence readout.

After incubation, all of the spent media is removed from the wells, the plate was wrapped in Parafilm and stored in a -80 °C freezer. The following day the plate was thawed at room temperature; and the reagent mixture was prepared (following the kit protocol) and added to the wells, which then lyse and label the cells and genetic contents. Finally, the fluorescence was read at excitation 480 nm and emission 520 nm spectra in a microplate reader (Tecan: Safire2; Program: XFluor4).

CELL MIGRATION BIOASSAY

A manufactured kit, the CytoSelect™ 96-well Cell Migration assay (Cell Biolabs, Inc.) was used in order to determine the effect on migration of cells that participate in the wound healing process.

The optimal seeding density for fibroblasts in this assay was found to be 10,000 cells per well. A stock suspension cell suspension of 500,000 cells/mL in serum-free media was prepared. The samples containing the potential chemoattractants were prepared exactly as described in the other assays, and 150 µL of each were pipetted into the lower level of the pre-warmed cell migration chamber. The middle chamber containing the porous membrane was then added directly on top of the lower chamber ensuring that no air bubbles were trapped. The cell suspension was continuously agitated as 100 µL were withdrawn and added to the wells of the middle chamber. The whole apparatus was covered and plates were transferred to incubator (37 °C) and incubated overnight.

The following day, the media inside the middle chamber wells was removed and the plate sections were carefully separated. The lower section containing the chemoattractants was discarded. Cell detachment solution (150 µL) was added to the wells of the harvesting tray (both

included in kit), the middle chamber was applied, and the plates were incubated at 37 °C for 30 minutes. In this step, the cells that had migrated through the 8 micron-sized pores from the underside of the middle chamber into the wells of the harvesting tray were detached.

After the incubation, the middle chamber was agitated several times to dislodge cells before being removed and discarded. A solution of 4X cell lysis buffer and Cyquant® GR dye was prepared and 50 microliters were transferred into each of the wells of the harvesting tray containing the migratory cells. The plate was then incubated at room temperature for 20 additional minutes. The mixture was then pipetted (150 µL per well) into a new 96 well plate and fluorescence was measured in a fluorescence microplate reader at 480 and 520 nanometers.

ANTI-INFLAMMATORY BIOASSAY

Inflammation is crucial in the beginning stages of the wound healing process because the cytokines that are secreted here are what recruit other cells to the wound site to begin the repair process and prevent the spread of infection. However, the use of antibiotics will negatively affect mammalian cell function, as well as chronic and excessive inflammation. An anti-inflammatory bioassay was established to determine whether extracts would hinder inflammation, but not completely eradicate it.

THP -1 monocytes were seeded (500,000 cells/mL/well) into a 12-well plate. In every experiment, three wells were reserved for the untreated controls (just cells plus media), three for the negative control (LPS), and three for positive controls. Epigallocatechin gallate (EGCG) was often used as a positive control because past research (Sing et al., 2005) has shown that it will decrease the inflammatory response by 50% (as shown by TNF-α concentration after incubation

with LPS). Other positive controls were established as well after this bioassay had determined which manufactured extracts hinder the inflammatory response.

Once the cells have been seeded, positive controls and all of the test materials were added to the wells. Neutral controls and negative controls did not receive a treatment at this point. The amount of sample that was added to each well varied based on the most conclusive results after many repetitions of this experiment. For example, it was found that EGCG should be prepared fresh for each experiment, 0.2 mg/mL in RPMI; and 200 microliters of the mixture was added to the wells. Extracts of unknown concentrations were added to the wells to test for anti-inflammatory properties each at three differing concentrations. The plates were then placed in an incubator (at 37°C) for a pre-incubation period of one hour.

LPS (5 micrograms/mL in RPMI) was then added to all of the wells, 100 microliters/well, except for the neutral controls (just cells and media). The plate was returned to the incubator for an additional four hours to allow for the monocytes to become induced and secrete cytokines. All of the samples were removed from wells and pipetted into fresh, labeled, 1.6 mL centrifuge tubes. Tubes were transferred into a microfuge and centrifuged at maximum speed (14rpm) for 15 seconds to pellet any unwanted debris or cells. The supernatant was then carefully transferred into fresh 1.6 mL tubes. The tubes were then frozen at –20 °C (-80 °C for long-term storage) until the concentration of TNF-alpha in the samples was determined by ELISA.

TNF-alpha cytokine activity concentration was evaluated using a sandwich ELISA (R&D Systems®: Human TNF-alpha DuoSet®). A 96-well plate was coated with a primary (or "capture") antibody and allowed to stand at room temperature overnight. The plate was then washed three times with a wash buffer containing 0.05% Tween 20 and PBS. Non-specific binding was then blocked with the addition of reagent diluent (1% BSA in PBS) and incubated

for at least one hour. The samples were removed from -20 °C and thawed out in a hot water bath. The samples collected from the anti-inflammatory assay contain extremely high concentrations of TNF-alpha, which fall outside of the range of the standard that is included in the kit. Therefore, they were prepared by diluting them 1:10 in RPMI.

The wells were washed again with the wash buffer and both samples and standards were loaded into the plate. The plate was incubated at room temperature for two hours to allow the TNF-alpha to bind to the primary antibody. The supernatant was removed from the plate and the wells were washed with buffer before a secondary antibody, or the detection antibody, was added to the plate. The detection antibody binds to the TNF-alpha/primary antibody complex and exposes its biotin tag. The plate then incubated at room temperature for two additional hours. The unbound antibody was washed away and a solution containing Streptavidin peroxidase and reagent diluent was then added and then left to incubate for 20 minutes at room temperature in the dark to allow it to bind to the biotin tag of the secondary antibody. A substrate solution was then added and different hues of blue result after a 20-minute incubation. Finally, a stop solution was added (sulfuric acid), and the plate was read at 450 and 540 nanometers.

RESULTS

LAB CONSTRUCTION PHASE

From the beginning of the practicum until late December, considerable efforts were required in order to install and initiate the various plant cell maintenance and investigation systems. Equipment was moved into laboratory spaces, arranged, and certified. Additional cell culture tools, supplies, and media components were purchased and organized into the allotted space as well. Racks that were constructed to store autoclaved glassware including plant cell culture flasks and filtration setups were also moved into the new research facility.

Figures 6 and 7. Two views of the Healthpoint's plant cell culture laboratory. Not pictured are the designated areas for additional shaker incubators, media preparation, lyophilization, evaporation, and cryopreservation.

SPECIFIC AIM 1: DEVELOPMENT OF PLANT CELL CULTURE METHODS

Plant Cell Calli

The purpose of these methods was to initiate plant cell calli from cryopreserved tissues. After agar plates containing the appropriate solid media were prepared, plant cell calli were successfully generated for plant cell lines: *Astragalus falcutus, Echinacea purpurea, Lycopersicon esculentum,* and *Prunella vulgaris*. Figures 8 through 11 depict images of the progress of plant cell calli from week one to week four. Note: In each figure, the lower panels are the duplicate images of the panels above; however, they have been inverted to make the progression of calli growth more visible.

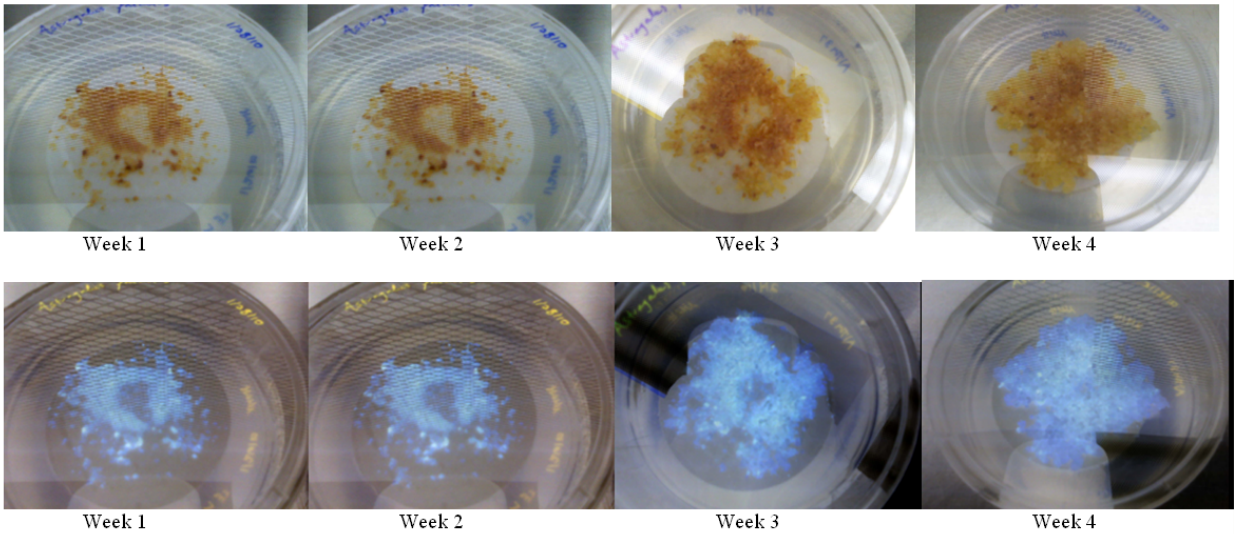


Figure 8. The progression of healthy *Astragalus falcutus* plant cell calli.

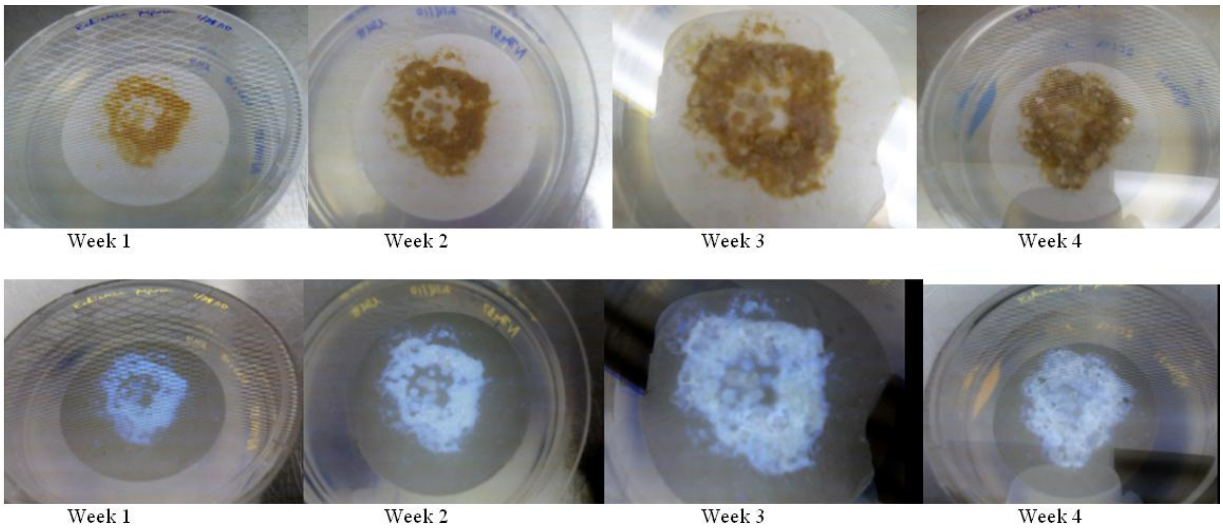


Figure 9. The progression of healthy *Echinacea purpurea* plant cell calli .

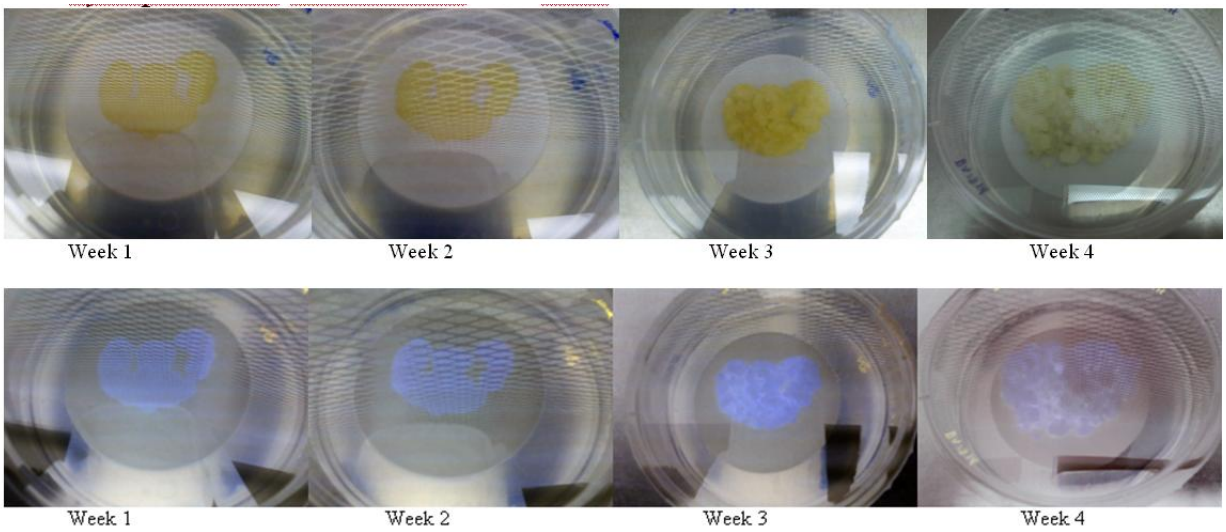


Figure 10. The progression of healthy *Lycopersicon esculentum* plant cell calli.

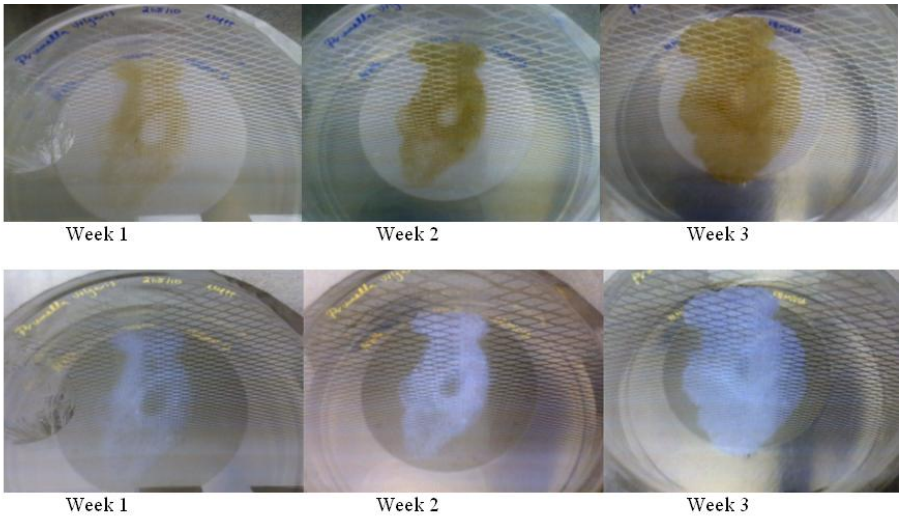


Figure 11. The progression of healthy *Prunella vulgaris* plant cell calli.

CELL SUSPENSIONS:

The methods that were developed for cell suspension cultures resulted in three established plant cell lines to be investigated for wound healing properties. Plant cell suspensions were successfully initiated from both plant cell calli and the biomass that was received from Phyton. Figures 12 and 13 are images of *Astragalus falcutus* and *Prunella vulgaris* cell suspension cultures, respectively; and Figure 14 shows an incubator that is completely occupied with *Lycopersicon esculentum* cell suspension cultures.

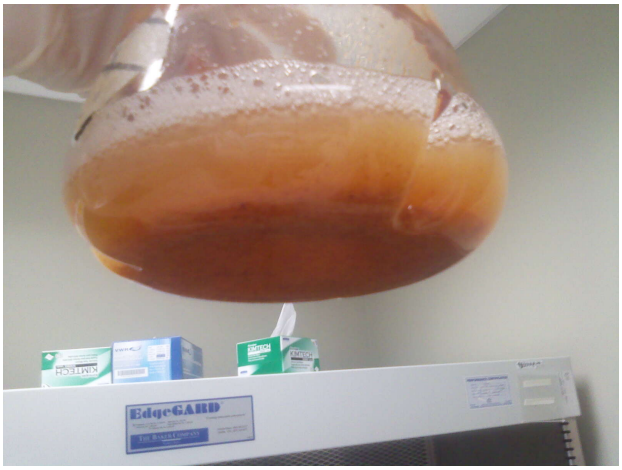
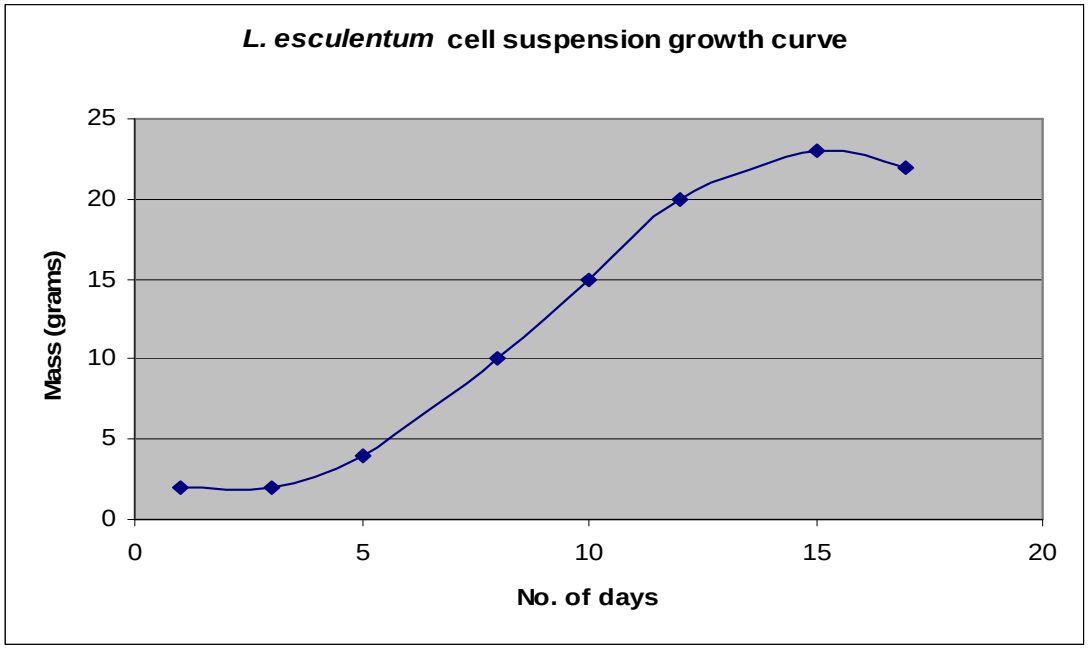


Figure 12. A healthy cell suspension of *Astraglus falcutus*.

Figrure 13. A healthy *Prunella vulgaris* cell suspension.

Figure 14. Several healthy *Lycopersicon esculentum* cell suspensions.

Cell suspension growth curve experiments were conducted to determine the optimal duration of time to elapse between passages for each type of plant specie. As shown in "Graph 1", *Lycopersicon esculentum* cell growth occurred in a standard S-shaped fashion, with no additional cell proliferation beyond day number 15 in culture.



Graph 1. *L. esculentum* cell suspension growth curve.

SPECIFIC AIM 2: PLANT CELL BIOMASS EXTRACTIONS

After plant cell biomass was collected and lyophilized, methods were developed in order to obtain extracts from plant cell biomass (See "Methods" section). Both polar (hyperphase) and nonpolar (hypophase) compounds collected from plant cell extractions were then evaporated to remove the solvents (predominately chloroform and methanol) and excess buffer. Extracts were dissolved in DMSO for long-term storage at room temperature to slow down the degradation of compounds. However, fresh extracts to be used for *in vitro* experiments were resuspended in water and immediately (the same day) used in bioassays to test for wound healing properties.

PROTEIN QUANTIFICATION

Spectrometry readings obtained by the NanoDrop-1000 (UV/Vis) detected the presence of protein, which suggests that effective methods were developed to extract proteins from plant cell biomass using the P-PER® Plant Protein Extraction Kit. It was found that the most successful protocol used (which led to the highest concentrations of protein) was when frozen tissue was ground up in the polypropelene mesh bag. The least amount of protein was extracted when lyophilized tissue was macerated in centrifuge tubes.

SPECIFIC AIM 3: ESTABLISHING WOUND HEALING BIOASSAYS

PART I: ESTABLISHING BIOASSAY METHODS WITH MANUFACTURED EXTRACTS

Bioassay experiments were conducted to establish methods using commercially available extracts (from Nature's Answer ®) of Astragalus, Echinacea, Yarrow, St. John's Wort, Gotu Kola, and Chamomile. These were executed prior to the evaluation of extracts obtained from plant cell lines established at the industrial site in order to ensure effectiveness, determine optimal seeding density of mammalian cells, and establish both positive and negative controls. The following are the results obtained from the cytotoxicity, cell proliferation, cell migration and anti-inflammatory bioassays.

MTT Cytotoxicity Bioassay

Manufactured extracts of Yarrow, St. John's Wort, Echinacea, and Astragalus were screened for cytotoxicity to mammalian cells at two different concentrations each: 5 μL and 10 μL/mL. The control groups used in this experiment were fibroblasts in serum-free/phenol red-free media only. After a 24-hour incubation, the number of viable fibroblast cells exhibiting normal metabolic functioning was determined. The results were then analyzed using paired t-test statistical analyses between the control groups and each of the groups treated with plant extracts. It was found that none of the extracts at the given concentrations were significantly cytotoxic to fibroblast cells. However, the most cytotoxic of the test articles was Astragalus at a concentration of 10 μL/mL (*p-value = 0.217*); and therefore, this extract was established to be used as a positive control in subsequent bioassays screening for cell toxicity.

Cyquant Cell Proliferation Bioassay

A CyQuant cell proliferation bioassay was conducted to determine the activity of manufactured plant extracts of Yarrow, St. John's Wort, Echinacea, and Astragalus on mammalian fibroblast cells at 5 and 10 μL/mL of media. Fibroblasts in serum-free, phenol red-free media served as the controls for this experiment. The number of viable cells remaining after a full day of treatment was then determined. These results were then analyzed using a one-tailed t-test between the control group and each of the test articles. It was found that none of the manufactured plant extracts significantly increased cell proliferation. However, the St. John's extract (5 μL/mL) did increase cell proliferation from approximately 9700 cells (triplicate average) to approximately 11,100 cells per well (triplicate average), which resulted in a *p-value* equal to *0.071*. This was the most significant value obtained, and therefore, St. John's Wort was established as a positive control for subsequent cell proliferation experiments.

Cell Migration Bioassay

Six commercially available plant extracts were screened *in vitro* for positive chemotactic activity using the CytoSelect 96-well Cell Migration assay kit. Mammalian fibroblast cells were incubated in the presence of Astragalus, Chamomile, Echinacea, Gotu kola, St. John's Wort, or Yarrow at three different concentrations: 1, 5, and 10 μL per mL of media for a period of 24 hours. The control group contained only fibroblasts and serum-free/phenol red-free media. It was not possible to set up a standard curve for this particular assay, but the resulting fluorescence for each treatment indicated cell migration. The obtained fluorescence values were then analyzed in a paired t-test to determine which extract impacted cell migration the greatest. It was found that the Echinacea extract was the most potent chemoattractant with a significance of

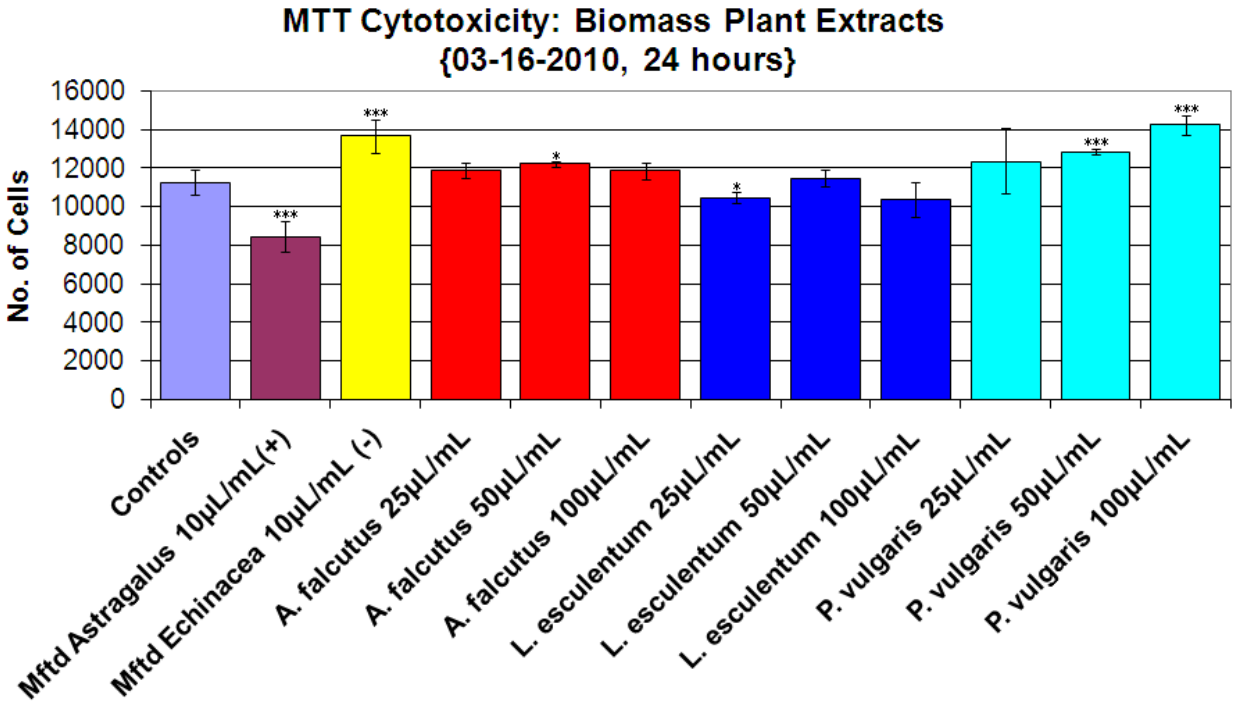
p<0.005. Therefore, this extract was established as a positive control for future bioassays to screen for cell migration.

Anti-inflammatory Bioassay

Manufactured plant extracts: Chamomile, Echinacea, Gotu kola, St. John's Wort, and Yarrow (1, 5, and 10 μL) were diluted in 1 mL of serum-free media and were screened *in vitro* for anti-inflammatory properties. After a pre-incubation in the extracts, THP-1 monocytes cells were induced with LPS for four hours. The media was then collected and the concentration of inflammatory cytokines (TNF-α) was determined using a sandwich ELISA. The control group for this experiment was the cells that were induced with LPS and received no additional treatment. The resulting TNF-α concentrations of the groups of cells that did receive a treatment were then statistically compared to the controls using paired *t*-test analyses. It was found that both St. Johns Wort and Echinacea extracts significantly reduced the secretion of inflammatory cytokines (both *p<0.005*); and therefore were established as positive controls for subsequent bioassays of this type.

PART II: BIOASSAYS FOR PLANT CELL BIOMASS EXTRACTS

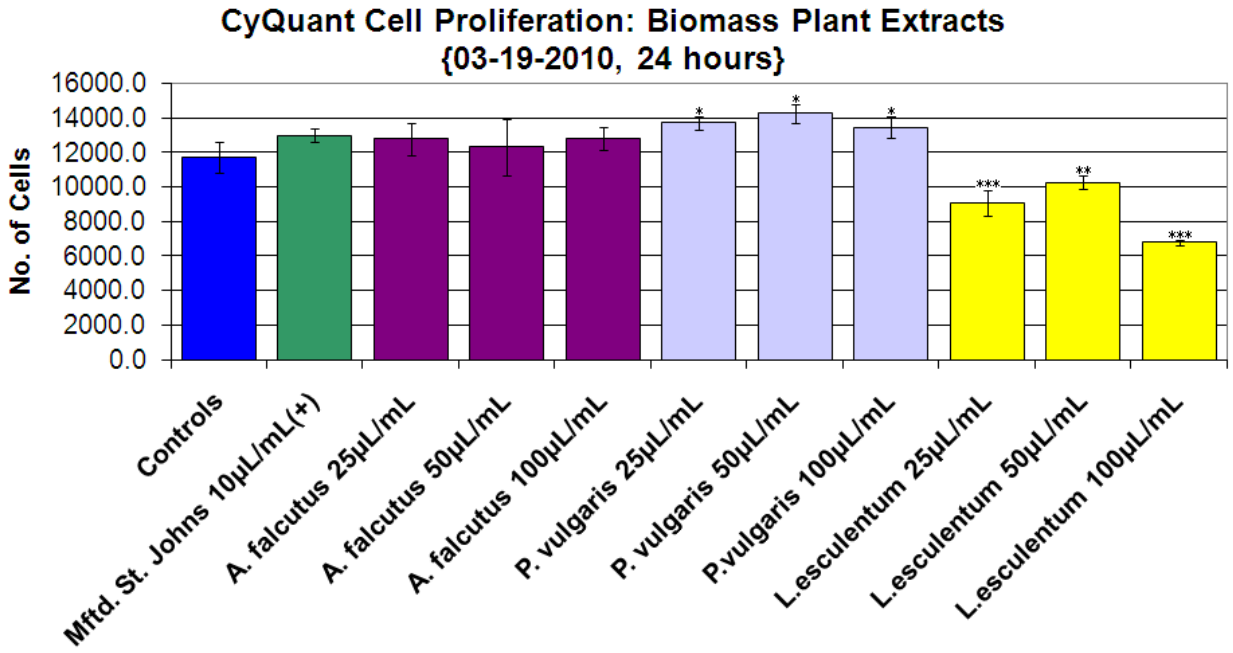
After bioassay methods were developed, the evaluation of extracts that were obtained from established plant cell lines at Healthpoint, Ltd. were executed. Previous bioassays not only established the methods that were used here, but were analyzed to establish positive and negative controls. Plant extracts obtained from plant cell biomass were investigated for wound healing applications by determining their cytotoxicity, cell proliferation, cell migration, and anti-inflammatory effects. The following four graphs are the data collected from these experiments (see "Discussion" section for interpretation of results):



Graph 2. Results of Vybrant® MTT Cytotoxicity assay of extracts obtained from plant cell biomass and commercially available plant extracts. ("Mftd" = Manufactured; * $p< 0.05$, ** $p< 0.01$, *** $p< 0.005$)

Human dermal fibroblasts were incubated in the presence of extracts obtained from *Astragalus falcutus*, *Lycopersicon esculentum* and *Prunella vulgaris* biomass. Each extract was screened for cytotoxicity at three different concentrations: 25, 50, and 100 µL/mL utilizing an MTT cytotoxicity bioassay. The controls for this experiment were fibroblasts in serum-free, phenol red-free media that were not treated with a plant extract. The data presented in "Graph 2" represents the number of viable cells that exhibited normal metabolism after 24 hours in the indicated extracts at the given concentration. These results were then compared to the negative (-) and positive controls (+) that were established during the bioassay development phase of the

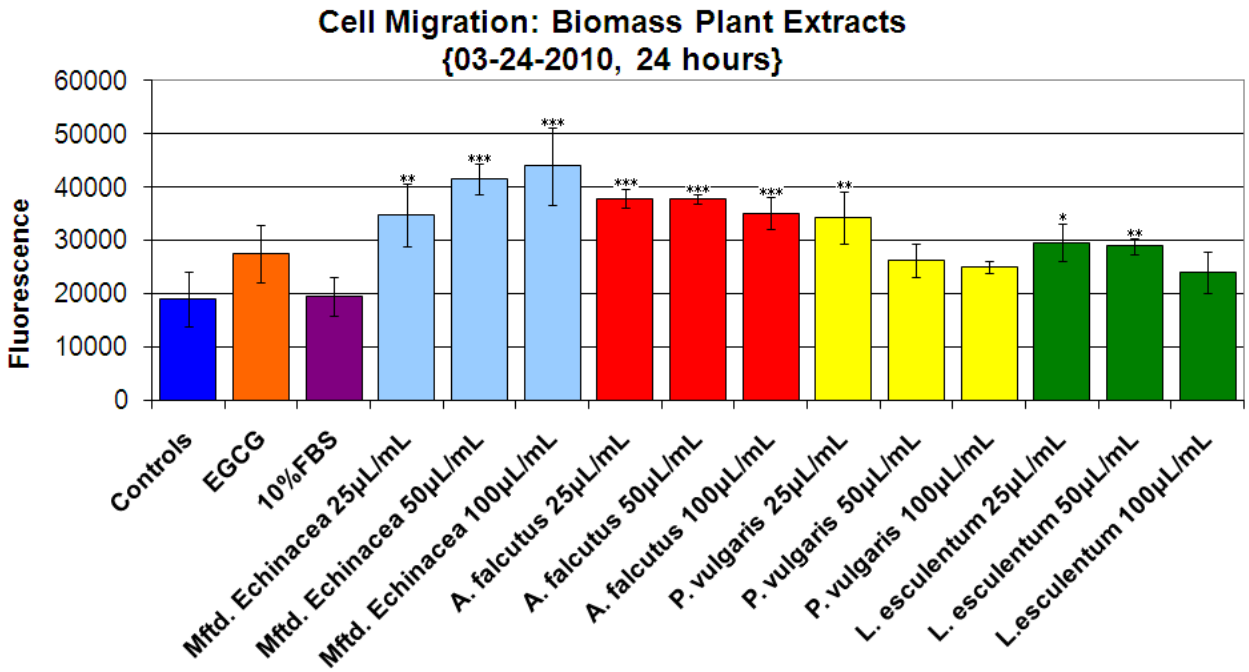
internship practicum. A paired t-test was used to determine the extracts that have significantly decreased cell viability when compared to the control group.



Graph 3. Results of CyQuant Cell Proliferation assay of extracts obtained from plant cell biomass and commercially available plant extracts ("Mftd" = Manufactured; * $p< 0.05$, ** $p< 0.01$, *** $p< 0.005$).

Extracts that were obtained from *Astragalus falcutus*, *Prunella vulgaris* and *Lycopersicon esculentum* plant cell lines were screened for their potential to enhance cell proliferation using a CyQuant® cell proliferation assay. Extracts were added to fibroblast cultures in the concentration of 25, 50, or 100 µL/mL. The control groups are those that only contain fibroblast cells in media with no addition of plant extracts. The data shown in "Graph 3" indicate the number of cells resulting from a 24-hour incubation in the presence of extracts. Since this assay does provide a reliable measure of cell death, only a positive control (+) that was established

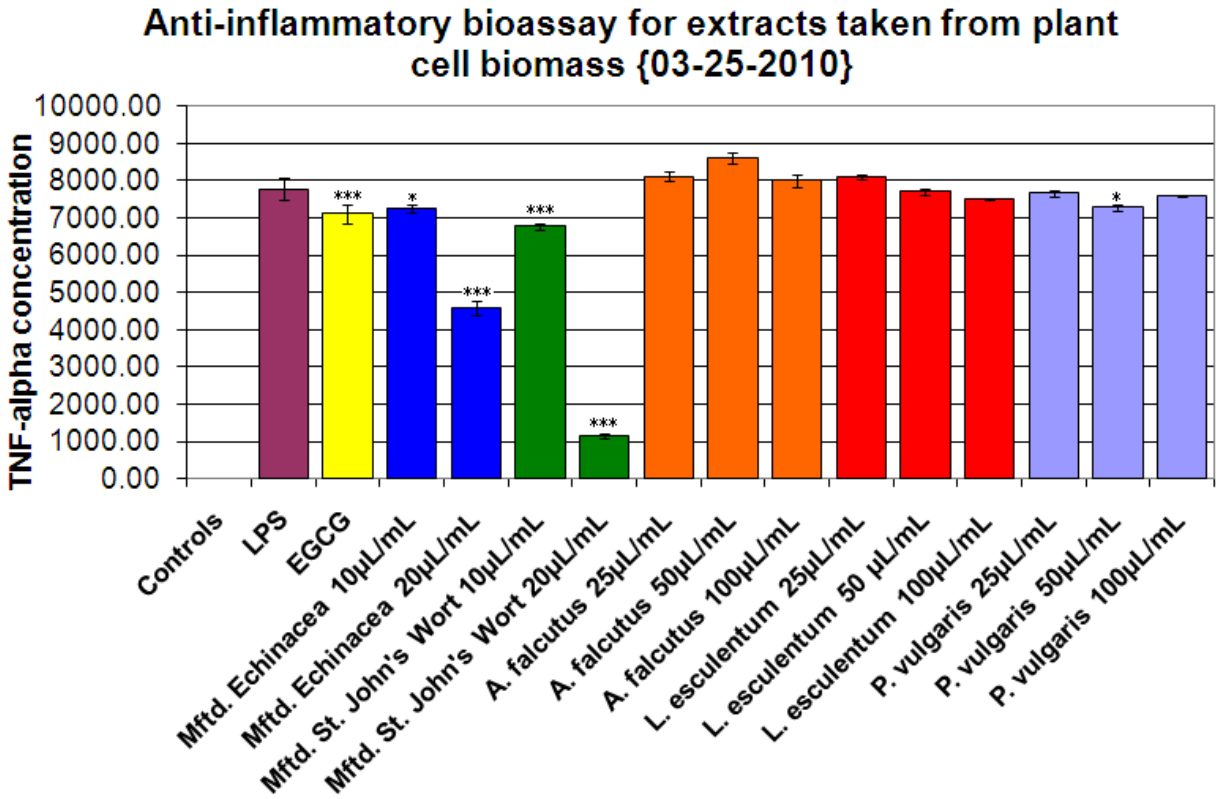
during the bioassay development phase of the practicum was used to compare against these results. A paired t-test was used to determine the extracts that have significantly increased cell proliferation when compared to the control group.



Graph 4. Results of CytoSelect Cell Migration assay of extracts obtained from plant cell biomass and commercially available plant extracts ("Mftd" = Manufactured; *$p< 0.05$, ** $p< 0.01$, *** $p< 0.005$).

Astragalus falcutus. Prunella vulgaris, and *Lycopersicon esculentum* extracts were examined for their impact on cell migration employing a CytoSelect 96-well Cell Migration assay. Fibroblasts were incubated in the presence of extracts at four different concentrations: 10, 25, 50, and 100 microliters per mL of serum-free, phenol red-free media. Established results indicated that manufactured Echinacea extract is a chemoattractant for mammalian fibroblast

cells with this particular assay; and therefore it was used a positive (+) control. EGCG and 10% FBS were also screened for their chemotactic properties in this experiment. The results in "Graph 4" indicate the relative amount of migration that took place per extract/per concentration as a function of fluorescence that was measured after incubation for 24 hours. A paired t-test was used to determine the extracts that have significantly increased cell migration when compared to the control group.



Graph 5. Results of anti-inflammatory assay for extracts obtained from plant cell biomass as compared to commercially available plant extracts ("Mftd." = Manufactured; *$p< 0.05$, **$p< 0.01$, ***$p< 0.005$).

THP-1 monocytes were incubated in the presence of extracts to measure their capacity to suppress the secretion of inflammatory cytokines (TNF-alpha) upon being induced by LPS. Extracts obtained from *Astraglaus falcutus*, *Prunella vulgaris*, and *Lycopersicon esculentum* were applied to monocyte cultures at 25, 50, and 100 μL/media concentrations. The "Controls" group reflects the concentration of TNF-alpha found in media where LPS was not added to cultures, and therefore the monocytes were not induced. The "LPS" group data shown in "Graph 9" reflects the concentration of TNF-alpha secreted after monocyte induction without a co-incubation in an extract, which served as the negative control. Published research showed that EGCG suppresses the inflammatory response and was therefore used as a positive control in this experiment (Singh et. al, 2005). Results from this bioassay during the "bioassay development" phase indicated that manufactured Echinacea extract suppresses the inflammatory response and therefore it was also used as a positive control. A paired t-test was used to determine the extracts that have significantly decreased TNF-α concentration when compared to the control ("LPS") groups.

DISCUSSION

This practicum project was designed to develop methods in order to generate established plant cell culture lines. Once the plant cell culture lines were expanded, methods were established in order to extract compounds from plant cell culture biomass. Finally, methods were developed to screen extracts for wound healing properties using *in vitro* bioassays.

PLANT CELL CALLI

The first method that was described for the first specific aim was the generation of plant cell calli. It was noted that there was a two-week lag following calli initiation where no discernible growth occurred. Beyond this "lag" period, however, growth was apparent and at times aggressive. It was found that the rate of growth varied considerably from specie to specie. For example, the calli for *Astragalus falcutus* and *Echinacea purpurea* grew at a far slower rate than the *Lycopersicon esculentum* and especially the *Prunella vulgaris* cell lines. *Prunella vulgaris*'s growth was so rapid in fact, that the cell calli had become confluent only one week after the initial "lag" phase before having to be subdivided and passaged into new solid agar plates.

This variability in growth between the different cell lines could be due to any number of reasons: naturally shorter growth and mitotic phases, the concentration and accessibility to vitamins and nutrients, the balance of auxins to cytokinin hormones, availability of carbon dioxide for gas exchange, etc.

PLANT CELL SUSPENSIONS

Plant cell suspensions were generated from plant cell biomass from cell calli, and from the tissues that were received from Phyton Biotech. Plant cell suspensions allow one to quickly expand the overall quantity of biomass for established cell lines because all of the cells in a successful suspension culture have optimal access to vitamins, hormones, and other nutrients in the media. Similar to the growth of plant cell calli, a very large degree of variation was observed in the plant cell suspensions.

Figure 15. Biomass color varieties for *Astragalus falcutus* (filtered)

For instance, it was found that the cells in a healthy *Astragalus falcutus* cell suspension (those that continued to proliferate) exhibited a color range from a light yellow/beige to a deep red. This color variation can be attributed to *Astragalus*'s formation of flavonoids that can be yellow, orange, or red (Thomas Selge, Phyton Biotech). However, on further investigation it was determined that the lighter colors of this particular cell line proved to have a higher growth index (the final fresh weight of filtered biomass divided by the initial weight of biomass) and had to be

passaged into new media every 8 to 9 days as compared to their red counterparts which were passaged every 12 to 13 days.

Unfortunately, contamination (especially by fungus) was extremely prevalent in cell suspensions. Thus, meticulous adherence to the aseptic technique was followed during cell suspension manipulation; and it was also necessary to collect several samples a week to be examined microscopically to exclude contaminated and stressed cultures from stocks.

GROWTH CURVES

Growth curve experiments were conducted for all of the cell lines to determine the optimal growth period to elapse between passages for all suspension plant cell cultures (*Astragalus falcutus, Prunella vulgaris, and Lycopersicon esculentum*). On average, it was found that upon passaging cell suspensions into new 250 mL flasks, the lag period lasted from day one through day six. Plant cells then normally entered their log phase (period of rapid cell divisions) at the beginning of the second week, and unhindered cell proliferation continued for one week. Cell culture suspensions then entered the plateau phase where cell growth arrests, and marked cell death takes place due to the depletion of nutrients in media.

With the information gained from these experiments, it was deduced that plant cell lines would benefit most from being passaged into new media every 10 to 12 days.

BIOMASS COLLECTION

After the plant cell lines had been established into healthy plant cell suspensions and their biomass expanded, it was necessary to collect the biomass for extractions. The biomass was

collected from healthy plant cell lines; and in several instances, the biomass and its respective spent media of stressed cell cultures was also collected and frozen.

Figure 16. Collected biomass and spent media from stressed (A and B) and healthy (C and D) *Prunella vulgaris* cell suspensions.

These stressed cultures were collected because of their probable composition of secondary metabolites. As in nature, plant cells excrete secondary metabolites during periods of stress (Evans et al., 2003). Any of these numerous compounds have the potential to ward off be antimicrobial, induce cell proliferation, promote cell migration or possess anti-inflammatory properties. As mentioned before, time constraints did not allow for the induction, isolation and investigation of these compounds. The further research required is a matter of future decision of Healthpoint, Ltd.

BIOASSAYS WITH EXTRACTS OBTAINED FROM PLANT CELL BIOMASS:

After methods were developed and the controls using commercially available extracts were established, the extracts from plant cell culture biomass were screened *in vitro* for wound healing properties. Recall that the expectations were low for these assays due to the fact that secondary metabolites were not generated. Normally in plant cell line investigation, cell suspensions would be passaged to induction media, which stresses the cells and leads them to secrete secondary metabolites. Time constraints did not allow for this crucial step, and instead extractions were performed on healthy plant biomass.

After the biomass was collected, freeze dried, and the extractions performed, three distinct phases were formed: the polar hyper-phase containing the methanol extracts, a middle emulsion phase, and the non-polar phase containing the chloroform extracts.

Next, the methanol and chloroform phases were evaporated. The remaining residues were each resuspended in one mL of deionized, distilled water and immediately applied to *in vitro* bioassay experiments to limit the degradation of extracted compounds. It was noted early on that chloroform and methanol evaporate at different rates—chloroform usually required three to four times the amount of time to be evaporated from extraction products. Because the extracts had to be prepared and *in vitro* bioassays conducted within the same day, only the methanol extracts were screened for wound healing actions. Some results were obtained that indicate the compounds constituting the methanol extracts from plant cell biomass may provide some benefit during the wound healing process. The following is a short discussion of the results (shown in "Graphs 2" through "5") for each of the wound healing bioassays.

MTT Cytotoxicity Bioassay

The results shown in "Graph 2" indicate that the controls established during the bioassay development phase of this practicum project were appropriate for this assay. After a 24-hour incubation, the positive control, commercially available ("Mftd") Astragalus, decreased the number of viable cells from approximately 11,000 cells (as shown in the "Controls" group) to approximately 8,500 cells with normally metabolic functioning. A paired t-test was conducted between the untreated control group and all of the treatment groups, and it was found that there was a highly significant ($p<0.005$) decrease in viable cells between the positive controls and the untreated group.

It was also found that there was an increase in the mean number of cells in the group of cells that were treated with the commercially available Echinacea (set as the negative control for this experiment). A two-tailed paired t-test was conducted and it was determined that this extract generated a significantly ($p<0.005$) higher numbers of cells than did the untreated control groups.

The results obtained from nearly all of the extracts obtained from plant cell biomass were determined to be insignificant, except for the *Lycopersicon esculentum* (25 μL/mL treated group). It is not understood why this concentration was determined to be cytotoxic (at $p<0.05$), whereas the higher concentration treatments were not.

It appeared that the *Prunella vulgaris* treated groups in "Graph 2" had actually proliferated beyond the untreated control group. A two-tailed paired t-test was conducted to analyze these results, and it was determined that the number of cells in groups treated with the 50 and 100 μL/mL extracts were significantly higher (at $p<0.005$) than those that received no

treatment. While these results show *Prunella vulgaris* to have potential to enhance wound healing, these results were next validated and duplicated utilizing a cell proliferation bioassay.

Cyquant Cell Proliferation Bioassay

In "Graph 3", a one-tailed paired t-test was conducted and it was determined that *Prunella vulgaris* was enhanced cell proliferation with a *p-value* of less than *0.05*. There was no significant difference in the results obtained between the untreated control groups and any of the *Astragalus falcutus* treated groups. Unfortunately, the positive controls for this particular assay did not result in a significant increase in cell proliferation.

There is also an apparent decrease in the number of cells resulting after a 24-hour incubation seen in the *Lycopersicon esculentum* extracts. A two-tailed t-test was conducted between these results and the untreated group, and it was found that the number of cells had significantly decreased, which such suggested that this particular extract is actually cytotoxic to cells. The results of this bioassay contradict the results obtained in the MTT assay. Therefore, this assay would need to be duplicated several times in order to determine the true nature of the *Lycopersicon esculentum* (methanol) extract.

Cell Migration Bioassay

A one-tailed paired t-test was conducted between the controls and each of the treated groups in order to analyze results for significant increases in cell migration ("Graph 4").

Manufactured Echinacea served as the positive controls (established during the bioassay development phase of this project) for this experiment and performed very well. There was an apparent dose curve generated with this extract where cell migration increases as the

concentration of Echinacea treatment increases. The statistical analyses on these groups indicated that significance (when compared to the controls) increased as the concentration of treatment increased as well.

Despite not increasing cell proliferation and possibly even being cytotoxic to cells, all concentrations of *Astragalus falcutus* significantly ($p<0.005$) increased cell migration. Likewise, *Lycopersicon esculentum* (thought to be toxic to mammalian fibroblasts) showed to have some significant impact on cell migration. It is unknown why these extracts would promote cell migration when these extracts could ultimately kill mammalian cells.

Prunella vulgaris methanol extracts, which had shown to most likely increase cell proliferation, also appeared to be positively chemotactic. At the lowest treatment concentration, 25 μL/mL, significant ($p<0.01$) cell migration resulted. At this point, this extract would be very beneficial to the wound healing process because it has been shown to be harmless to mammalian cells, increase cell proliferation, and increase cell migration.

Anti-Inflammation Bioassay

The final bioassay that was conducted to determine if extracts taken from plant cell biomass have medicinal advantage in wound healing was the anti-inflammation assay.

The group denoted “Controls” in “Graph 5” were the cells in serum-free media that received no treatment at all. As one would expect, the TNF-α concentration found in this media was essentially zero because these cells were not induced and therefore would have no need to secrete inflammatory cytokines.

The “LPS” group, however, was the group of THP-1 monocytes that were induced and did not receive a treatment of any kind. Therefore, this was the group used as the negative control group; and comparative standard when statistical analyses were conducted.

Three positive controls were established during the bioassay development phase of this project: EGCG, and manufactured St. John’s Wort and Echinacea. A one-tailed paired t-test indicated that pre-incubation with these extracts resulted in a significant decrease in TNF-α secretion. Moreover, as the concentration of the manufactured extracts increased, the resulting TNF-α concentration in media significantly decreased (a dose curve was generated).

A one-tailed paired t-test was also conducted to compare the difference in the “LPS” control group and those groups that were treated with extracts obtained from plant cell biomass. Unfortunately, no significance was found between these groups, with the exception of *Prunella vulgaris* at 50 μL/mL ($p<0.05$). This was most likely a false positive because no significance resulted for the other two concentrations that were tested. This assay would definitely need to be repeated, but these results indicate that the extracts obtained from plant cell biomass would most likely not be adequate anit-inflammatory agents.

CONCLUSIONS

The goal of this practicum project was to establish methods in order to investigate plant cell cultures for wound healing properties. After these methods were developed, they were performed to screen extracts from established plant cell lines for wound healing properties. The extracts obtained from *Prunella vulgaris* have shown the most interesting results. This particular plant cell line met three of the four expectations for an agent that might have medicinal application in wound healing. If applied to a wound site it would not be toxic to mammalian cells, would activate cell migration to the wound site, and would promote cell proliferation.

Ideally, the next course of action would be the isolation and analysis of compounds within the cell homogenate to identify individual components of the extract that were responsible for the activities identified in our studies. This is likely to be a formidable undertaking that would require proficiency in advanced chromatography methods, as well as NMR, MS or infrared spectrometry; and an investment in time and facilities/instrumentation.

Bioassays of some extracts would need to be repeated *in vitro* numerous times until results are definitive and reproducible. Compounds identified to have wound healing potential would then be applied to wounds in *in vivo* models; and if successful, would finally be screened in human clinical trials.

Even after the extracts have been successfully tested in humans, the FDA poses many challenges for pharmaceutical companies looking to introduce plant-derived therapeutics onto

the market. For example, it requires that pharmaceuticals possess uniform composition—even the slightest variation from batch to batch will thwart a company's efforts.

As experienced during this internship, plant cell cultures are easily disrupted from normal processes and will become stressed, change colors, and perhaps even expire with even the slightest change in environmental conditions. These changes can be expected to translate into the synthesis of a different profile of compounds. Controlling all of these factors is a difficult task, especially when one considers that each cell line requires different concentrations of hormones, vitamins, carbohydrates, light/temperature conditions, pressure and available gas for respiration.

Considerable preliminary research regarding plant cell culturing techniques, as well as a lot of "trial and error", was invested into the methods described in this practicum research report. Ultimately, however, it was revealed that the process for plant cell culture and its subsequent investigation is most successful if the standard protocols are used as a guideline, and experience is used to adjust the conditions according to need and response of the cultures. For instance, those cell calli that tended to grow upwards instead of laterally would dry out very quickly. However, if fresh autoclaved media was poured into the petri dishes before they were sealed, it was found that cell calli would flourish. This modification was not found in scientific literature, but was developed solely from research and experience.

In conclusion, plant cell culture can be thought of as essentially an art form. It requires substantial knowledge; and yet, success can only be achieved through creativity and tenacity.

CHAPTER III

INTERNSHIP EXPERIENCE

During this internship a broad glimpse of the inner workings of industry was experienced. Dennis Carson, director of research and development at Healthpoint, Ltd. ensured that interns participated in nearly every event that a normal employee would experience on a daily basis. This included participation in meetings with experts in the different areas of a scientific organization such as quality assurance and control, regulatory affairs, project management, as well as research and development.

Interns also participated in journal club meetings where volunteers presented new scientific articles relevant to their own research. I was selected for this task one month, where I presented literature on Lupeol (a plant cholesterol believed to be an anti-inflammatory agent when applied to mammalian wound sites) and described the basic techniques of plant cell culture research.

Several thoughtful company-wide, catered luncheons were held to commemorate employee anniversaries; and many others were held to discuss new product development and their commercialization. I was also permitted to attend additional lunches held off-site for holidays and to celebrate the addition of new employees to Healthpoint's research team.

Much of the time spent was devoted to self-study: reading journal articles, books, and other literature sources to achieve fundamental working knowledge on which to build the project. This has been a constant learning process—plant anatomy and the culturing of plant cells was an entirely new area to be mastered.

After the initial research phase, I learned and became proficient in a vast array of biotechnology techniques in Healthpoint's research and development facilities. These techniques were not only employed in the research used for my research practicum report, but also for other important projects within the Company. I am honored and thankful that I was fortunate to partake in these extracurricular ventures.

In retrospect, this has been one of the most gratifying and exciting experiences of my life. I am eager to now carry what I have learned during these past two years into the next phases of my professional journey. Now that the internship is over, I feel that I am much better prepared for an occupation in research in either the academic or industrial settings.

ADDITIONAL RESEARCH WHILE ONSITE

Establishing a plant cell culture laboratory unfortunately took a great deal of time to be constructed including approximately four months of construction. I took the opportunity that this time lag presented to partake in research that was outside the goals of my internship project.

I was very please to be able to contributed research to the collaborative whole. For instance, I conducted several experiments to determine if a certain compound is a comparable alternative to an ECM component including cell proliferation, cell migration, and anti-inflammatory bioassays. Unfortunately, most of the data from these experiments was inconclusive. For example, there were difficult problems with prevention of sample contamination that may have affected the outcome of the cell proliferation studies (Cyquant measures the DNA of lysed cells—it is unknown how pathogens could vary one's results).

Much of my extracurricular work revolved around disproving the efficacy of Medihoney. At first it appeared that this product was (as it claims) a very effective anti-inflammatory agent. However, MTT cytotoxicity bioassays indicated that the drop in TNF-alpha was not due to anti-inflammatory action, but was due to the fact that when cells of all types (keratinocytes, monocytes, fibroblasts) are incubated in the presence of this drug, it renders them incapable of normal metabolic processes. It was found that it kills cells *in vitro* at a concentration as low as 5% (in RPMI) of its normal stock preparation.

Why these trends were exhibited is not clear. They could be due to severe changes in osmotic/oncotic pressure, asphyxiation by the cells due to Medihoney's viscosity, the toxicity of certain unknown compounds to cells. Unfortunately, there is no way to truly test the toxicity of this product in a classical fashion because it is applied to wound sites as a dressing. Such conditions have to be integrated with existing methodologies so that evaluation can be performed in a laboratory setting.

A great deal of time was also spent to attempt to prove one of Healthpoint's own products (collagenase-based) constitutes an improvement when applied to wound healing. Numerous anti-inflammatory, BCA, cell proliferation, and cytotoxicity assay were conducted on this product. The results were very promising, however the details are proprietary Healthpont, Ltd. information and therefore will not be discussed.

However, a poster resulted from some of the Medihoney and collagenase research that will be presented at two events, the Wound Healing Society (WHS) and the Symposium on Advanced Wound Care (SAWC). The poster is currently being evaluated for the top, "blue" prize at the conference. An image of the poster follows (the specifics were blurred to prevent the disclosure of proprietary information):

Figure 17. “Evaluation of Anti-inflammatory Effects of *Clostridium* Collagenase and its Collagen Degradation Products” poster.

INTERNSHIP SITE DESCRIPTION

Healthpoint Ltd. is a biotechnology company located in Fort Worth, Texas. Its research and development building ("Building 4") is approximately 80,000 square feet and contains facilities for research in the areas of: Plant Cell Science, Cell Science, Biochemistry, Formulations and Analytical Chemistry, and Microbiology. This building also houses Healthpoint's Clinical, Regulatory, and Quality Control divisions. The Company also performs *in vivo* research in lab space that is leased from the University of North Texas Health Science Center.

The predominate focus of Healthpoint's R&D group is the development of products to facilitate the wound healing process, especially those wounds that are difficult to heal, or are chronic. Their products include debriding ointments, antimicrobials/sanitizers, and anti-inflammatory products. The exploration of cell-based therapies to create skin grafts and assemble extracellular matrix have recently become a main focal point. Finally, there has and continues to be a considerable effort to better understand and treat biofilms.

JOURNAL SUMMARY

08/25/2009
Orientation: Met with committee for lunch and to watch presentations:

-Dr. Patricia Gwirtz gave a presentation on behalf of UNTHSC to describe expectations of the interns, as well as of the internship site.

-Julie Garcia of Healthpoint then gave a presentation regarding the companies core values, dress code, and information regarding confidentiality of proprietary information. A preliminary tour was given of the site

08/26/2009
-Read general safety SOP's
-watched modules training for bloodborne pathogen safety
-Was given several articles to be read to build knowledge base covering wound care theories and plant cell culture. Much of the day was spent reading this material. Journal articles were also taken home to begin reading overnight.

08/27/2009
-Read more journal articles regarding the physiology of wound healing
-Completed the last of the safety training modules
-Employee appreciation banquet lasted through the majority of the day
-Met with mentor: was given a more in depth tour of building and instruments that will be used during internship. Project details were discussed in greater detail regarding expected timelines and desired outcomes for experiments.
-Completed first chapter in Plant Cell Culturing book.

08/28/2009
-Dedicated most of the day to self-study. Read through and outlined four additional chapters in the Plant Cell Culturing textbook, and tried to catch up on a few more journal articles.
-Shadowed mentor in the lab for cell culturing:

Thawed out a vial of keratinocytes from cryopreservation, spun them down to remove DMSO, resuspended in 6ml, transferred to three 75 cm^2 flasks, added 12ml of EpiLife to each flask, and then placed in incubator to be checked on Monday.

08/31/2009
-Read and outlined the last several chapters in the Plant Cell Culturing book.
-Researched background, medical application, and available extract information on the six cell lines that we will be receiving biomasses for, from Germany.

-Created a spreadsheet to organize cell line information and typed out several references that may be of use in the future.
-Read and outlined a journal article on chromatographic separation—an assay that will be employed later in the internship to analyze plant extracts.

09/01/2009
-More self study: Read articles for cosmetic applications of plant cell cultures
-Made up media to be used for 2 cells lines to be plated
-Executed separately: (one cell line completed in the morning and the other completed in the afternoon)

Removed two vials from cryo: HDFa (human dermal fibroblasts from an adult) and HEKn (human epithelial keratinocytes from neonatal foreskin), thawed out in hot water bath, spun to pellet, re-suspended and counted. The counts determined the number of flasks that were ultimately plated, media added, and flasks stored in an incubator for later use.
-Discussed bioassays and general protocol to be performed for project with mentor
-Wrote a short introduction in laboratory notebook regarding internship goals, and updated work completed in lab during the day.

09/02/09
-Self study: Read articles discussing suitable bioreactors and cylclotides production
-Checked cells: They appeared to be healthy with no signs of contamination and had very very few detached cells—plating efficiency for these particular cells is very high.
-Meeting #1: Amy Campbell with regulatory affairs. She deals with the pre-market application of industry regulations to products that are either in development, or are about to be introduced into the market.
-Meeting #2: Breda Horn with regulatory affairs. She discussed her involvement in a product's life after it has been introduced onto the market. This includes all aspects of labeling, advertising, and taking reports of adverse reactions in members of the public.
-Began working on the rough draft for research proposal.

09/03/09
-Checked on cells—all looked well. Will most likely have to passage the fibroblasts tomorrow and change keras media so they may survive the long weekend.
-Read the articles that will be highlighted at today's journal club meeting.
-Meeting: With Dr. Larry Anderson who is head of CMC (Chemistry and manufacturing controls) to discuss his role in product advancement and testing such as Microbial Limits and other USP tests.
-Journal club meeting: Listened to a presentation, and then discussed persisters in biofilms.

09/04/2009
-Self study: read articles on methods to collect secondary metabolites from plants as well as inflammatory bioassay literature.
-Lab: Changed media for both the keratinocytes and epithelial cells
-Worked on proposal rough draft
-It was a short day due to the holiday weekend, but am aiming to complete the rough draft for the proposal over the break.

09/08/2009
-Worked on rough draft for research proposal; learned to use RefWorks citation manager.
-Passaged the fibroblast cell lines. Plated 500 thousand into 6 new flasks and transferred four million into two cryo tubes to be frozen in -80 freezer overnight. Changed the keratinocyte medium.
-Prepared keratinocytes for cell migration assay tomorrow by changing one of the flasks to serum-free media, which will starve them overnight.
-Had a meeting with Michael Micheal's, who is in charge of Healthpoint's Quality Control and Quality Assurance. He discussed his involvement with the FDA as well as keeping in line with the manufacturers current GMP's.

09/09/2009
-Split the cultures of keratinocyte cells in to 6, 75 cm^2 flasks.
-Attended a luncheon with the company that included a presentation on the research and development of a new product in Switzerland, as well as future commercialization plans for this technology.
-Set up cell migration bioassay to be incubated over night
-Research proposal rough draft

09/10/2009
-Worked on cell migration assay which was largely unsuccessful.
-Also worked on the inflammatory assay using fractions collected from Healthpoint's 802 product
-Spent the entire morning in the laboratory and from lunch on working on the rough draft for the research proposal. Stayed late, and managed to finally finish.
-Emailed proposal to major professor, Dr. Dimitrijivech

09/11/2009
-Forwarded the research proposal to site mentor. No feedback from major professor as of yet
-Meeting with Forrest Goodmen from Project Management and his associate, Jennifer Holton. They spoke some about their backgrounds, and more so about what their jobs entail—which seems to be a little bit of everything.
-Lab: Shadowed mentor for a while, watching him prepare samples for product research. Changed the media on each of the twelve flasks containing keratinocyte and fibroblast cell lines.
-Extracts will arrive at the beginning of next week and the internship project will be able to begin.

09/14/2009
-Changed the media in each of the flasks—the cells were not yet confluent, made more media for the keratinocytes
-Received feedback from mentor on research proposal and implemented changes
-Meeting with Duncan Aust, who decides how and where money is to be spent for research and development. He seemed to focus four pieces of advice: Earn a PhD ASAP, self-promotion is a must, gain experience starting out in a small to mid-size biotech, and always dream big.

-Set up plates for ELISA assays to be run tomorrow for collection media from anti-inflammatory assays on “802” fractions
-On campus: Located Dr. Vishwanatha for GSBS approval on degree plan and turned in to Amanda

09/15/2009
The entire day was basically devoted to time in the cell science laboratory.
- Completed the “sandwich” ELISA that was probing for TNF-alpha, which was very time consuming due to the numerous steps involved and repetitive washes. The basic steps after the primary has been attached to the wells are: three washes, block, washes, dilute, bind TNF-alpha (in collection media) to primary, washes, incubate secondary antibody, washes, streptavidin incubation, washes, 1A:1B reagent incubation, the addition of sulfuric acid, and then read the plate.
-Passaged the keras cells: 8 vials put in -80 (approx. 20 million cells) and 3 new flasks started, each with 500K cells.

09/16/2009
-Have received extracts now. Searched many journal articles to find past research studies where extracts were used for in vitro studies—to seek out cell toxicity ranges and wound healing properties of the extracts already discovered.
-Reviewed ELISA results from yesterday with mentor and learned to complete concentration calculations via Excel software
-Made new 106 media and then changed the media for the fibroblasts that were not passaged yesterday
-Leaving early today to meet major professor on campus to discuss changes that need to be implemented in the research proposal.

09/17/2009
-Continued literature search for cell toxicity of extracts. The search so far has been largely unfruitful—will likely have to carry out series assays to determine quantities.
-Conducted the inflammation assay for plant extracts which is being done in order to find positive controls for comparison against extracts that are induced in the future. THP-1 monocytes were incubated in the presence of extracts for four hours and then in the presence of LPS for an additional hour. Supernatant was collected and frozen.
-Set up 96 well plate with primary antibody for ELISA to be run tomorrow.
-Began making corrections in the research proposal

09/18/2009
-Changed the media for all remaining flasks.
-Carried out the ELISA protocol in its entirety to determine whether extracts have anti-inflammatory capability in the presence of LPS excited THP-1 monocytes. Any measurable fluorescence indicates the presence of TNF-alpha, a potenet inflammatory cytokine. The results appeared very promising. There was no fluorescence read in the control wells and the blanks, high fluorescence in the LPS only wells, and the standards decreased in fluorescence as they should from the top of the series(A1 thru 3) to the bottom (G1 thru 3). The wells containing the

extracts (independent variable) increased in fluorescence as the concentration of the extracts decreased.
-Final calculations to be taken care of on Monday

09/21/2009
-Brett was out today due to illness
-Changed the media in all of the flasks. Cells are still not confluent yet, which is odd for fibroblast growth—they normally grow very quickly.
-Transported ELISA data into excel to create a data spreadsheet.
-Worked on research proposal corrections for the majority of the day, and finally finished
-E-mailed the corrected version of the research proposal to Dr. Gwirtz, and Dr. Dimitrijevich.

09/22/2009
-Completed calculations for research that was conducted on Friday on the TNF-alpha bioassay. Constructed a graph and tables to be used in the research practicum.
-Worked with Brett to figure out the next plan of action: Will be starting another inflammation assay tomorrow and a cell migration assay possibly as well
-Set up fibroblasts (need approximately10 million cells) for the cell migration assay to be incubated overnight in serum-free media

09/23/2009
-Set up bioassay with THP-1 monocyte cells to test extracts for anti-inflammatory properties. Used EGCG (positive control),Yarrow, Chamomile, Gotu Kola, Echinacea, and St. John's wort extracts in 10, 5, and 1 microliter increments and incubated with monocytes in two 12 well plates. Then created an inflammatory reponse by the addition of LPS to each well and left to incubate for 4 hours. Media was then collected, spun down, and frozen to be assayed with an ELISA probing for TNF-alpha concentrations.
-Cell migration assay set up in the afternoon to be used on serum starved human dermal fibroblasts. In other words, extracts were being assayed for potential chemoattractant properties.

09/24/2009
-Completed the cell migration assay individually
-Read plate on microplate reader at 480 and 520 nanometers. Results looked promising, but did notice that there are less cells migrating through the pores at higher extract concentrations suggesting cytotoxicity or negative chemotaxis occurring.
-Completed the calculations for migration assay and then created two separate graphs: one for the plant extracts and another for the samples that I ran for Brett.
-Attended the monthly company-wide luncheon where Duncan Aust, head of research and development, discussed the current news about the company
-Set up primary or "catch" antibody in microwell plates for the ELISA to be run tomorrow.

09/25/2009
-Created a universal spreadsheet to compile all of the data for all of the bioassays. Also created a single location to store all of the graphs so they are more easily located.
-Individually ran the entire ELISA to probe for TNF-alpha in media collected from cells exposed to LPS + plant extracts at 10, 5, and 1 microliter concentrations

-Changed the media on the remaining three flasks of keratinocytes and three flasks of fibroblasts
-Began performing calculations for the ELISA data in an Excel spreadsheet.

09/28/2009
-The kerainocytes were confluent and therefore they were passaged. Split one million into a single flask and transferred all of the others to cryo tubes to be frozen.
-Completed the calculations for the data that was collected on Friday from the ELISA measuring TNF-alpha from anti-inflammatory assay.
-Contacted Dr. Dan: He will finish the research proposal shortly. He believes that fibroblasts are not growing do to the lack of nutrients. Started a mini-experiment (individually): added 10% FBS to media in one of the senescent flasks—will hopefully jump start growth.
-Fibroblasts: scrapped old flasks and started anew because cell growth had arrested for some strange reason. Started new line of cells in flasks—from from an alternate origin, to be used for cell proliferation assay (hopefully this week)

09/29/2009
-Updated "bioassay" and "graph" spreadsheets in Excel with new information collected from assays ran last week.
-Checked on new flasks of keratinocytes and fibroblasts: All cells appear to be doinge well. The FBS supplemented flask of keratinocytes is not showing any visible difference in cell growth.
-Transferred keras from -80 to cryo tank, and updated log
-Passaged monocytes for "802" research: 5 million in one 25 cm flask—seeded 300k/ml of media…put the rest in -80.
-Literature searches: for extraction methods as well as induction methods for plants; specifically for the six cell lines being supplied from Germany.

09/30/2009
-Viewed the fibroblasts: cells appear well, however, there are microorganisms present—they were most likely frozen along with these cells. Washed out flasks three times vigorously with sterile PBS. Added 2mLs of pen/strep to 200 mL of 106 media before transferring to flasks.
-Moved THP-1 monocyte cells to cryopreservation and logged 16 vials. Cells passaged yesterday appear to be doing well in the 25 cm flask.
-Changed the media in the keratinocyte flask as well, which appear to be doing well.
-Continued literature search for induction methods specific to the 6 plant species cell lines that we will be receiving from Germany
-Read articles for the journal club meeting taking place tomorrow.

10/01/2009
-Out due to illness

10/02/2009
-Keratinocytes are peeling off of flask and those still attached are not growing well. Changed media and will check again on Monday
-Fibroblasts are still contaminated however, antibiotics appear to be helping because microorganism population seems to have stopped growing. Rinsed flasks several times with PBS, added new media and then transferred 100 microliters of pen/strep each.

-New kits have arrived: two different cell proliferation kits and a new type of cell migration kit. Collected product information on kits—to be reviewed over the weekend.
-Had a short day due to lingering illness.

10/05/2009
-Changed the media for the keratinocytes, and the monocytes. The flasks containing the fibroblasts were completely contaminated. Started new flasks of fibroblasts from cryopreservation, however, it is believed that the entire stock of fibroblasts are contaminated.
-Spent the majority of the day implementing changes to the research proposal that were made by Dr. Dimitrijevich over the weekend.
-E-mailed a copy of the proposal to be reviewed by Dr. Gwirtz.

10/06/2009
-Fibroblasts that were thawed out and plated yesterday appear to be doing well with no signs of contamination. Trial flask containing only 106 media was also not contaminated.
-Conducted another inflammatory assay—trying to generate a lucid dose curve. Chose the extracts with the best results from past trials: St. John's wort, Echinacea, and Yarrow. Cells were incubated in extract concentrations of 2, 4, 6, 8 and 10 microliters. The normal positive control, EGCG, was not used in this experiment.
-Set up the primary, or "catch", antibody for the sandwich ELISA that will be run tomorrow.

10/07/2009
-Ran the ELISA for TNF alpha present in the media collected yesterday and read the microwell plate at 450 and 540nm. The results initially do not appear to be very consistent with similar assays that have been conducted in the past. Will carry out the calculations tomorrow.
-Shadowed Brett in setting up the first cell proliferation assay. This assay will be conducted on fractions that were collected from product research—samples from internship project will not be analyzed for proliferative enhancing properties.

10/08/2009
-Completed calculations for the raw data generated yesterday in the ant-inflammatory assay and created a table and bar graph to organize information. The TNF-alpha present in the media collected from the cells that were only exposed to the LPS should have been the highest among all of the samples, but instead was lower. Oddly enough, the highest concentrations of TNF-alpha were found to be in Echinacea, St. John's, and Yarrow treated cell populations at low concentrations. This may indicate a problem with the LPS.
-Updated bioassay spreadsheets and graph library.
-Read the article to be presented at the Journal club meeting covering insulin-like growth factors and microencapsulation. Attended the journal club meeting this afternoon.
-Continued to shadow mentor in the lab to learn the protocol for the cell proliferation assay.

10/09/2009
-Started a new flask of THP-1 monocytes from cryo—the cells in the old flask have ruptured and there only appears to be cell debris even after aspirating the spent media and replacing it with new media.

-Made new 106 and EpiLife media. Changed the media for all of the fibroblast populations (8 flasks) as well as the keratinocytes (3 flasks).
-Continued with the CyQuant cell tracker proliferation assay, and completed the MTT cell proliferation assay. Depending on the number of viable cells, will most likely begin the Platapus cell migration assay.
-Received the SOP's for the biomasses that are being sent from Phyton in Germany and began reviewing them
-Dr. Gwirtz is reviewing the research proposal over the weekend

10/12/2009
-Volunteered to present an article at the journal club meeting taking place on November the 5th. Spent the majority of the day conducting a literature search to find an article that is pertinent to internship project.
-Completed the cell tracker proliferation assay. The results from this particular assay appear to be more promising than the proliferation assay that was executed on Friday.
Will be running additional assays later in the week.
-Started new flask of THP-1 monocyte cells. These cells are extremely finicky and have not been surviving lately. Changed plating protocol so that cells were thawed in the palm of my hand and then spun down at a much lower speed (850 rpm) before being plated.

10/13/2009
-Running five plates for assays today. For the cell migration assay (Platapus brand) will have a plate of keratinocytes, one of fibroblasts, and another that will contain both keras and fibroblast cells. Are hoping to see a synergistic effect in the plate with both cell types in it because this is what one would expect in the normal physiology of wound healing (interactions between numerous cell types). Also began two additional plates for the cell proliferation assays: one containing the keratinocytes and one with just the fibroblasts.
-Shadowed Brett while he ran numerous ELISAs for company product research.
-THP-1 cells appear to have survived so far—media is still red (instead of yellow) and cells appear healthy under the microscope.

10/14/09
-The RPMI media for THP-1 cells is still the correct color and cell morphology appears to be normal. It seems that a new protocol in handling these particular cells has been established.
-Received comments on research proposal from Dr. Gwirtz and began implementing changes into the paper.
-Aided Brett in the lab with the cell proliferation assays and cell migration assays
-Passaged new (P1) fibroblasts. Took counts for all three flasks and there were a total of 35 million cells. Initiated 10 new flasks with one million in each, and cryopreserved the remaining 25 million.

10/15/09
-A recruiter from the NIA/NIH is holding a presentation/Q & A session out at the UNT Denton campus regarding employment opportunities for graduate students within these government institutions. Took a bus to the Denton campus to attend…

10/16/2009
-Built a shelving unit and went through boxes from Phyton, Inc. with Brett in storage to locate glassware to be used in the new lab—once it is constructed.
-Changed media in all of the fibroblast (X12) flasks for the weekend
-Attended the company-wide chili cook-off luncheon. The chef from the Omni Hotel (downtown fort worth) was the judge, and the winner received an inscribed trophy
-Finally finished the research proposal and submitted it via e-mail to the entire graduate committee

10/19-10/23/2009
-Was out sick for the duration of this week due to complications of the H1N1 flu

10/26/2009
-Changed the media for the new fibroblast flasks
-Moved the 21 vials of frozen fibs from -80 to cryopreservation and logged them into the cell inventory
-Started new flasks of THP-1 monocytes; each flask was initiated via a unique process to try and determine a protocol to reanimate cells from cryopreservation. These cells have unfortunately not been surviving this transition in the past.
-Went to campus to meet with Dr. McClain to discuss research proposal and to obtain his signature on the research proposal submission form . Spoke with Dr. Dimitrijevich on campus regarding internship progress and obtained his signatures for the proposal submission as well as the "Intent to Graduate" form. Also picked up revised draft from Dr. Gwirtz's office and left forms to be signed.

10/27/2009
-Checked on the flasks of THP-1 cells that were started yesterday. All appear to be doing well as of yet, with no visible signs of cell lysis
-Took three of the flasks of the fibroblasts to begin the cell migration and cell proliferation bioassays. 802 samples are being tested as well as four of the plant extracts: Yarrow, St. John's, Echinacea, and Astragalus for both 5 and 10 microliter concentrations.
-Cells were seeded and left to attach for four hours before being incubated in the presence of candidate proliferative/migrative inducing agent overnight.
-Continued to work on presentation for next Thursday at the journal club meeting. Submitted the selected journal articles to Eric Roche via e-mail.

10/28/2009
-THP-1 monocytes still appear to be doing well still. Changed media in each of the three monocyte flasks and in each of the eleven fibroblast flasks, and made some new 106 media
-Completed the cell migration assay, read the fluorescence and carried out calculations on all of the data for both the 802 and extract sample groups. This assay will have to be repeated because unfortunately the control groups (aka "serum-free" groups) showed signs of considerable migration when they should not have had any.
-Attended the monthly Research and Development meeting where a person in R&D describes the results they have obtained regarding product research

-Continued to work on the cell proliferation assay. Cells have now been to the -80 freezer where they will remain until the final steps of the assay will be executed.

10/29/2009
-Initiated ten new flasks of P2 fibroblasts, each containing one million cells for a number of bioassays to be run next week. Froze down eleven flasks of P3 fibroblasts as well.
-Attended the "Open Mic" luncheon: a company-wide held luncheon that is held every three months or so where the "higher-ups" inform employees of recent developments at different levels within the organization.
-Updated bioassay spreadsheets and graphs, and continued to work on powerpoint for Thursday's journal club meeting
-Completed the Cyquant cell proliferation assay, and measured fluorescence multiple times with different gains.

10/30/2009
-Cyquant cell proliferation assay results were calculated and organized in graphs in several excel spread sheets. Results looked verry promising—the critical values are very low meaning there is very little variance of results within sample groups. However, the actual values are too similar to the control group and therefore this assay will have to be repeated using higher concentrations of samples to tested.
-Moved fibroblasts from -80 to cryopreservation and updated cryopreservation logs.
-Changed the media for the all of the newly initiated fibroblast flasks, as well as the THP-monocyte flasks—which are still proliferating very well.
-Obtained signatures from mentor at Healthpoint and finally Dr. Vishwanatha for the practicum research proposal and the "intent to graduate" forms. Then they were taken to the graduate school of biomedical sciences for final submission.

11/02/09
-The THP-1 cells are healthy in all three experimental trial flasks. It seems that the cells chosen from this particular passage are all viable for initiation from cropreservation.
-Changed the media in all of the flasks for the monocytes as well as the fibroblasts. Two of the fibroblast flasks, however were changed to serum-free media to starve them overnight so that the cell migration assays can be initiated tomorrow. Discussed the next mode of action with Brett for other assays to begin this week as far as testing the "honey" product in a dilution series assay to test for cytoxicity.
-Registered for classes for spring semester
-Continued to work on the presentation for Friday's journal club, and completed it in due time.

11/03/09
-Set-up cell migration assay (CytoSelect 96 well cell migration assay) for 802 samples. 1 to 1, 1 to 5, and 1 to 10 dilutions were made to hopefully result in a greater range between positive and negatice control groups than seen before.
-Also initiated the Cyquant cell proliferation assay and determined the opitimal seeding concentration by determining a standard curve.
-Platypus cell migration assay: initiated a new bioassay that determines if a compound is a chemoattractant by monitoring their physical translocation into a pre-fabricated "no growth"

zone. Various dilutions of cells were added to the wells of the plates to determine optimal seeding concentration in this assay as well.

11/04/09
-Initiated an anti-inflammatory assay to determine if "honey product" is cytotoxic to cells. Concentrations of 10, 5, 2, 1, and 0.5% were tested. Samples were collected and frozen for ELISA to be run tomorrow. ELISA primary ("catch") antibody 96 well plate was also set up.
-Completed the cell migration assay and read fluorescence at 480 and 520 nm. Will analyze results in the near future.
-Cell proliferation samples were added to the wells in several different dilutions to determine equivalence of fibrin vs. alginate compounds
-802 samples were also added to the Platypus well plate and returned to the incubator to allow cell migration to occur overnight

11/05/09
-ELISA executed for TNF-alpha for samples collected from 802 anti-inflammatory bioassay carried out yesterday
-Froze the cell proliferation plate at -80 deg—will dye/lyse tomorrow.
-Platypus cell migration assay: Cells have begun migration into the center voids. Will allow this to carry on overnight and will complete the fluorescence stain and reading tomorrow morning.
-Calculations for the cell migration assay were completed. Fibrin and the Fmc (alginate) values were very similar and therefore this assay will be considered a successful demonstration that the two are equal in there chemotactic properties and can be substituted for on another

11/06/09
-The Platypus cell migration assay was completed and the cells were fluorescently stained with Calcein AM. Cells were viewed under fluorescent microscopy to determine if the cells had indeed taken up the dye and then read by a fluorescent micropate reader. Raw data readings were then compared to microscope observations to establish what values actually correspond to positive *in vitro* results.
-Attended Paul Wilk's farewell luncheon at Pappadeaux restaurant—today is unfortunately his last day. There was also a company-wide farewell with cake and drinks afterward.
-Completed the cell proliferation assay. Will complete the calculations at a later date.
-Gave the presentation for the journal club meeting which went over decently well.
-Passaged several flasks of fibroblasts and froze several vials to be moved to cryopreservation next Monday.

11/09/09
-All of the new fibroblast flasks appear to be doing well and will be ready for new bioassays to be run later in the week. Changed their media.
-The monocyte's media was also changed and their cell numbers were quantified.
-Keratinocyte flasks were initiated from cryopreservation for the cell toxicity assays that will hopefully be run later in the week on the "Medi-honey" product.
-Healthpoint is heavily publicizing their product "Ultracept" to compete in the hand sanitizer market against Purell. Camera crews were present today to take pictures of the research and development team as well as film the site.

-Completed calculations on the cell proliferation assay ran on Friday. Results look very promising so far.

11/10/09
-A press conference was held on-site today to publicize/market Healthpoint's "Ultracept" hand sanitizer product. The R&D staff was required to occupy the labs this morning until the conference/tours were completed.
-Ran a new bioassay today to determine if "Medihoney" is cytotoxic to monocyte THP-1 cells. Cells were set up similar to the anti-inflammatory assay where they are incubated in the presence of varied concentration amounts for five hours. Cells were then counted in a hemacytometer and then live cell vs. dead cell quantities were compared.
-Set up three 96-well plates with fibroblast cells: 10,000 cells/200 microliters/ per well. Cells were then moved to the incubator to allow attachment overnight.
- The SOP's that were sent from Germany regarding plant cell culture methods were perused some as well.

11/11/09
-Took cell counts for all of the wells in the Medihoney cell toxicity assay. As expected, cells in the control groups (no medihoney treatment) proliferated while those in the treated groups died in higher quantities as concentration of the treatment increased.
-Transferred the samples from the 802 fractions to the two 96-well cell proliferation plates. A third cell proliferation plate was also loaded for the cell proliferation assay, but will be testing the medihoney product for its effects on cell growth and toxicity.
-Passaged keratinocytes to set up the Platypus cell migration plate but unfortunately did not have enough cells to initiate this assay. Cells were instead re-plated to allow for higher growth quantities.
-Froze one of the bioassay plates and allowed the other to continue to proliferate to generate a multiple time point curve.

11/12/09
-Removed contents of plate B and froze the remaining cells in the -80 degrees freezer for several hours. Thawed out both plates A and B and ran the cell proliferation assay on them. The samples were no filtered before being applied to the wells and unfortunately are believed to have contaminate the cells in the alginate wells. May have to repeat this experiment again in its entirety.
-Also completed the MTT cell proliferation assay which includes a four hour incubation in a chemi-illuminescent, the addition and mixing of contents with DMSO and then taking a reading in a microplate reader. These results are very good news because they establish that medihoney indeed is cytotoxic to cells. It is not that it is an excellent anti-inflammatory compound but rather it is preventing the cells of being able to secrete cytokines altogether.
-Began working on calculations of raw data for the proceeding experiments and analysis of results.

11/13/09

-Managed the stock of P5 firoblasts. Passaged 8 million into 4, 75 cm flasks and froze the rest in the -80 to be moved to cryopreservation on Monday. Started a new cell line of keras from "Cascade". Seeded 500,000 cells into three 75 cm flasks.
-Finished the calculations for the cell proliferation assay ran last week. The results were pretty odd because there actually more proliferation measured in plate "A" (6 hours) than what was found in plate "B" (24 hours). It is believed that there was some contamination because the samples were not filter sterilized and perhaps that lead to these strange results.
-Changed the media in all of the remaining flasks so they will survive the weekend.
-Shadowed Ryan Ermis through a brief discussion/presentation on HPLC-High performance liquid chromatography, which will be utilized a great deal later on in the internship practicum.

11/16/09
-There were too many THP-1 monocyte cell cultures running and therefore froze down 40 million cells into 8 cryopreservation vials and left approximately 20 million in culture in fresh media.
-Changed the media for all of the different passages of keratinocytes. All of the cultures appear to be doing well, but will need some time before reaching confluency. This may actually prevent several bioassays from being run this week.
-Also changed the media for all the fibroblast flasks. All of these cultures have reached confluency and are ready to be used in bioassays.
-Fibroblasts from last week were moved from -80 to cryo, and logged into cryo logs.
-Seeded 2 million fibroblast cells into two 96-well plates—equaling 10,000 cell per well, and left in incubator overnight so that cells may adhere and be ready for bioassays. Medi-honey MTT assay will be repeated as well as the cell proliferation assay this week.

11/17/09
-Loaded 802 samples into plates A and B for the time-dependent (0 hour, 6 hour, and 24 hour) cell proliferation assay to be completed in the next couple of days. Assay will be run on the alginate and fibrin samples as before to hopefully duplicate the results obtained on 11-12-09
-A third 96 well plate seeded with 10,000 fibroblast cells per well was also loaded with samples: Medihoney at varying concentrations to conduct the MTT cell proliferation assay. This assay is also being re-initiated to hopefully duplicate results that were obtained last week.
-Cells that were passaged into cryo vials and frozen at -80 were moved to cryopreservation and logged into databank.
-Plate "A" was removed from 37 degree incubator, its contents were aspirated and the plate was moved to -80 degrees Celsius freezer to prepare it for assaying tomorrow.

11/18/09
-Completed the Cyquant cell proliferation assay. The initial results are promising for many of the alginate samples showing supporting it use as an alternative for fibrin.
-Also completed the Vybrant MTT cell proliferation assay. Compared to the prior execution of this experiment, the product Medihoney is indeed cytotoxic to cells.
-Changed the media for all of the cell lines: fibroblasts, keratinocytes and THP-1 monocytes (several flasks of each). Will have to start down-sizing the back stock in time for the Thanksgiving holiday.

-Attended the R&D monthly luncheon where advancements on Ultracept in the market and "802" research we re discussed by the higher ups.
-Initiated a new 96-well plate to assay cell proliferation again for this new group of alginate samples.

11/19/09
-Finished the calculations for the MTT cell proliferation for Medihoney product. Adding the control (no cells—MTT and media only) to adjust for background noise was effective in showing the true cytotoxicity of this product. At 5 and 10% concentrations, the cells were rendered incapable of performing any normal metabolic activity.
-Added FMC/Fibrin samples to 96-well plate for the cell proliferation assay. Plate was then moved to 37 degrees for an overnight incubation. This assay is being run to validate cell proliferation assay results collected 11/18/09.
-A new cell proliferation assay was initiated using non-adherent THP-1 monocyte cells. As before, cells were incubated overninght in different concentrations of Mehihoney product diluted in RPMI.

11/20/2009
-Executed the final steps of the Cyquant cell proliferation assay. There was unfortunately very large amounts of contamination in two of the samples but it did not seem to be transferred into other test groups. Plate was frozen and then read via a fluorescent plate reader at 480 and 520 nm.
-The company had a small event to celebrate the Thanksgiving holiday where all employees met in building one to eat pie and participate in a raffle.
-The MTT cell proliferation assay utilizing the THP-1 monocytes was also completed. Because the cells were not attached to the wells before samples had to be aspirated numerous times, most of the cells were lost before chemilluminescence could be read in the microplate reader. It is also possible that this assay was not meant to be used with this cell type, so will have to run a dilution series with this assay next week to be sure.

11/23/09
-Withdrew 2 million cells from monocyte (THP-1) cell culture flasks to determine if MTT assay can even be used with this cell type. Created a dilution series starting at 100,000 cells per well down to 6,000 cells per well and incubated for four hours in presence of MTT stock solution. Will be executing both the quick protocol option (using DMSO followed by an immediate microplate reading) and the traditional method (using the SDS-HCL solution and incubation overnight before reading) with this plate.
-Changed the media in the cultures as well as froze down all of the remaining keratinocyte cell cultures. Moved the fibroblasts frozen last week from -80 to cryopreservation, and logged vials into the cryo logs.
-The plant cell culture lab has just been updated with power and proper outlet fixtures so that equipment may be moved into the space and cleaned. Will begin media preparation for plant cell cultures after the Thanksgiving holiday.

11/24/09

-Finished the MTT cell proliferation assay using the conventional protocol which has proven to be a lot more sensitive than the alternate method. It has also been proven that this assay is effective for use in monocyte cell populations.
-Moved the keratinocytes from -80 to cryopreservation and updated cryo logs

11/25/09
-Cleaned up desk area, hoods and lab areas for the Thanksgiving holidays.

11/30/09
-Had a meeting with Dr. Lei Shi regarding future experiments with competitor product: Medihoney. There are myriad papers about the application of honey-based products to facilitate the wound healing process—each with conflicting results/findings.
-Will be executing a monocyte based MTT cell proliferation first to determine Medihoney's toxicity to this cell type at timed intervals. Will also be running an anti-inflammatory assay that will be read at various time points this week
-Initiated several cultures from cryopreservation: 4 flasks of keratinocytes (each with 750K cells), 2 flasks of THP-1 monocytes (each with 11 million cells), and 2 flasks of fibroblasts (each with approximately one million cells).

12/01/09
-Worked out some of the logistics (methods) for the assays to be completed this week with Stone.
-Peformed background literature searches to seek out available research that has been conducted in "honey as a wound healing modulator". Surprisingly enough, very few experiments and peer-reviewed articles have been published regarding this subject.
-All of the cultures appear to be doing very well that were initiated yesterday. The monocytes will be ready to be assayed immediately, but the keratinocytes and fibroblasts will most likely have to wait until next week.
-Spoke to Doug this afternoon and he has promised that the equipment for the lab will be moved in by the end of this week.

12/02/09
-Moved two incubators, two Percivals, and tables from warehouse to the hall outside of the lab which has now been outfitted with power. Movers will need to come out to remove the doors and actually transfer equipment inside.
-Brought "Immunobiology" book to the site to reinforce knowledge of cytokines—particularly pro-inflammatory cytokines: IL-6 and IL-10
-Transferred and organized glassware from warehouse storage to a cart on wheels. Glassware will need to be cleaned and autoclaved before being relocated in the new lab.
-Continued literature search for articles related to honey-based product research as well as reviewed some of the SOP's that were sent from Phyton in Germany regarding plant cell culture.

12/03/09
-Out today due to sickness
12/04/09

-Changed the media for all of the cultures for the weekend: two flasks of fibroblasts, two flasks of monocytes, and four flasks of keratinocytes. Will need to passage the fibroblasts on following Monday.
-All of the equipment has been moved into the plant cell culture lab. It is a very tight fit, but will suffice. Will be needing to find other locations for storage for media, glassware, and instruments in the coming days
-Cleaned up lab areas for the weekend; and updated logs, notebook and journal.
-Planned out some of the experiments that will be needed to be conducted next week for debunking Medihoney misconceptions.

12/07/09
-Removed all of the spent media from the flasks that are currently running and replaced it with fresh media.
-Began a MTT cell proliferation (cytotoxicity) assay to test Medihoney product for its toxicity on the THP-1 monocytes. Started two 96 well plates so that both the traditional method and the quicker protocols can be conducted. Both plates will incubate for 24 hours before completion of this bioassay.
-Sprayed down all of the equipment in the new lab with a ten percent bleach solution. Autoclaved a great deal of glassware as well—especially Erlenmeyer flasks and Buchner filter flasks. Began moving other tools and equipment that will be needed for plant cell culture.
-Searched through several databases to locate any literature concerning methods to initiate plant cell cultures from cryopreservation

12/08/09
-Completed the MTT cell proliferation assay for the THP-1 monocytes for one of the 96-well plates utilizing the quicker DMSO method. Decided to assay the other plate using the overnight incubation with SDS-HCl solution. Plate will be read in the morning.
-Fibroblasts were contaminated with what appears to be a fungus. Threw these cultures out and initiated fresh ones to take their place.
-Autoclaved ample additional flasks and other glassware that will be used in the plant cell culture research laboratory
-Recalculated the components that will be used as stock solutions in the plant cell culture medium to allow more appropriate batch volumes to be made in the coming weeks.
-Also completed the calculations for the results for the "quick" MTT assay protocol and organized the data into corresponding graphs and charts.

12/09/09
-Discussed timeline with Brett regarding the plant cell culture laboratoty, the establishment of new, species-specific methods and the execution of internship goals
-finished MTT cell proliferation assays (both short and long) for the THP-1 monocytes
-Completed the calculations for the cell cytotoxicity assays—both showing a significant decrease in cell metabolic processed in as little as 5% concentration of Medihoney
-Initiated a new MTT assay following exact same experimental design as before, except added the variable of "10% concentration of Medihoney in RPMI"
-Transported and assembled the Evaporator/centrifuge that will be used to dry plant cell culture specimens before dry weights are taken, ground, and then extracts are taken.

12/10/09
-Made fresh RPMI for THP-1 monocyte cultures and aliquotted into smaller containers to be used for future bioassays
-Brett spoke to Phyton and the selected biomasses (*Ast. falcutus, Lyc. esculemtum,* and *Prun. Vulgaris*) will be arriving at the end of next week
-Completed the TNF-alpha inflammatory assay for Medihoney
-Finished the longer version of the MTT cytoxicity assay and read results in the microplate reader. Also completed calculations for this assay
-Set-up primary antibody for the ELISA that will be executed tomorrow

12/11/09
-Conducted an entire ELISA for TNf-alpha for the inflammatory experiment initiated on Thursday. Preliminary data appears promising.
-Attended the R&D holiday luncheon at a local restaurant. The head of R&D, Dennis Carson, treated the whole group as a measure of gratitude for everyone's hard work over the past year.
-Autoclaved the last of the glassware that will be needed to making media for the plant cell culture lab.
-Finished the long protocol for the MTT cytotoxicity assay. Calculated the results to reveal that there is a 75% decrease in cell metabolic functions after incubation in 10% Medihoney, and 30% decrease in 5% Medihoney. These results have successfully duplicated previous ones of the exact same experiment.

12/14/09
-Completed calculations for ELISA and discussed the results with Brett. Medihoney does appear to be causing a small inflammatory response at low concentrations. This experiment will be repeated with the added design of time dependent variables.
-Updated lab notebook with new data and began to implement its Table of Contents
-Changed the media for all of the flasks, however we have encountered a problem. All of the CO2 has been used in the incubators and there are no back-ups available. Bioassays will have to be put on hold for awhile this week.
-Began making stock solutions for the media that will be used in the plant cell culture facility including: potassium hydroxide (KOH), B5 Vitamins (for NTM37 medium), y,y-dimethylallylamino (2iP), and 1-Naphthaleneacetic acid (NAA).

12/15/09
-Began a new anti-inflammatory assay: "Collagenase vs. Medihoney". Seeded 25 million monocyte cells into 4, 12-well plates and incubated them in different concentrations of Collagenase or medihoney. Media will be collected at three different time points: 6, 24, 48 hours to be analyzed for TNF-alpha concentration via ELISA.
-Initiated the MTT cytotoxicity assay for keratinocytes by seeding 10,000 cells into 24 wells of a 96-well plate. Transferred plate to incubator and left overnight to allow cells to attach
-Also initiated the Platypus cell migration assay for keratinocytes—but only to determine the optimal seeding density for this particular cell type. Will be testing dilutions of 1.0*10^6/ml, and both 5.0 and 2.5*10^5 cells per milliliter of media.

-Made Vitamin B5 stock to be used in plant cell culture medium for when needed.

12/16/09
-Collected media (after 24 hour incubation) from anti-inflammatory assay, centrifuged samples, and froze the supernatant
-Passaged several flasks of keratinocytes into new flasks (to expand backstocks of these cells)
-Produced and applied samples for MTT cytoxicity assay using keratinocytes
-Cells have adhered well to the wells of the plate around the stopper in the 96-well cell migration plate. Created and added assay samples to each well.
-Formulated several liters of media for plant cell cultures; as well as autoclaved them and moved them into a newly cleared dark storage area
-Changed media for cell cultures including THP-1 monocytes as well as for fibroblasts

12/17/09
-Completed the keratinocytes MTT cytotoxicity assay using the quick protocol method
-The keratinocytes in the Platypus cell migration assay are behaving very strangely. Kept a careful watch on them for the duration of the day to moniter these changes and to determine what is causing them
-Completed the calculations for the MTT assay using keratinocytes incubated in Medihoney. The results are nearly identical to past MTT assays run using fibroblasts so apparently Medihoney is very toxic to these cells and a little less toxic to monocytes

12/18/09
-Changed the media for the keratinocytes and THP-1 monocytes
-Conducted an ELISA for the three time point dependent anti-inflammatory assay
-Passaged and froze down all of the fibroblast cultures
-A carbon dioxide shortage in the incubators adversely affected the cells in the Platypus cell migration assay—they had to be thrown away
-Received biomasses from Germany. Initiated cell suspensions in fresh media aseptically in the new plant cell culture lab

12/21/09
-Checked on plant cell suspensions. Cultures appeared to be doing well, and there are no visible signs of contamination. Cell aggregates are much smaller after overnight agitation.
-Had to throw away all of the remaining cultures due to the temporary CO2 deprivation.
-Transferred the fibroblasts that were passaged on Friday from -80 degrees Celsius storage to cryo, and updated cryopreservation logs.
-Performed a brief surface clean of work station and both labs for the holidays.
-Completed the calculations for the ELISA results obtained on Friday. Results are very promising: Medihoney (competitor's product) does cause some inflammation when incubated with THP-1 monocytes, whereas Collagenase (Healthpoint product) does not.

12/22/09
-The building closed early today so it was a very short day. Finished cleaning up labs and updating lab notebook.

12/23/09 – 12/25/09
-Christmas vacation

12/28/09
-Viewed plant cultures: the *Astragalus* and *Prunella* cultures appear to be growing quite well; however, the *Lycopersicum esculentum* does not appear to be proliferating at all.
-Initiated new THP-1 monocyte and keratinocyte cultures for bioassays to be conducted next week
-Autoclaved several cases of pipette tips and various pieces of glassware for both labs
-Performed background research for extraction methods for the plant cell cultures. Will be testing these to establish working S.O.P.s as early as the beginning of next week. Continued to work on research practicum thesis

12/29/09
-Spent half a day making several Liters of NTM37 and DV media, dispensing it into smaller vessels, autoclaving them, and finally storing them.
-Checked the *Prunella* cultures for cell growth and vitality by collecting four samples from the one liter flasks. Cell suspensions were then stained with Trypan blue (1:1) and viewed via microscopy. Fortunately, nearly all cells are alive and appear to be growing quickly
-Learned to passage plant cells and then passaged several flasks or *Prunella vulgaris* under the supervision of mentor, Brett Kiedaische

12/30/09
-Changed media for the THP-1 monocytes and the keratinocytes. Will be running most likely running several bioassays next week.
-Passaged all of the *Astragalus falcutus* plant cell cultures into several smaller flasks independently practicing the aseptic technique meticulously
-Formulated, aliquotted, and autoclaved two liters of DV media that will be used next week to perform time point curves to determine each cell cultures exponential and lag phases
-Washed and autoclaved a wealth of glassware for both labs

12/31/09 – 01/01/10
-Off for New Year's holidays

01/04/10
-Moved freezers and lab equipment back into place and bagged and removed all of the biological wastes for both labs
-Changed the media for the THP-1 monocyte and the keratinocyte cell cultures, and made new RPMI media
-Took samples from the *Astragalus falcutus* cell suspensions that were passaged last week because they appeared to be contaminated. Cells were left to settle and then supernatant was affixed on microscope slides. Fortunately, no contamination was detected in any of the samples taken.
-Began new fibroblast cultures from cryopreservation for Cyquant cell proliferation and MTT cytotxicity assays that will be conducted later on in the week.

01/05/10
-Prepared the plant cell culture lab for experiments that will be initiated tomorrow to determine growth curves for every specie of plant that is growing in a cell suspension presently
-Started an inflammatory bioassay to duplicate/validate past results from experiments that measured the TNF-alpha concentrations as caused by THP-1 monocytes being incubated in the presence of Medihoney versus being incubated in the presence of the Company's collagenase product.
-Began a few new THP-1 monocyte cultures—as all of the old ones were used in the inflammatory bioassay today
-Sought out additional research available on plant cell suspension extraction methods as well as additional resources for plant cell culture

01/06/10
- Passaged the plant suspensions into thirty 250ml flasks (ten for each cell line) for the time point curve experiments. These experiments will proceed over the next 20 days or so and will be used to help establish plant cell culture methods as well as allow one to plan timelines for future experimentation
-Collected the samples in the 24 hour group for the inflammatory assay, removed the debris, and froze along with previously collected samples. Will be running the TNF-alpha ELISA on Friday
-Changed the media for the keratinocytes and fibroblast cell cultures. The newly initiated THP-1 monocyte cells appear to be doing well.
-Three of the 16 flasks of *Prunella vulgaris* cell suspensions are now red in appearance for some unexplained reason, and were therefore viewed under the microscope after being stained with Trypan blue. Cell suspensions were thrown out because all cells had lysed into tiny vessicles.

01/07/10
-Cleaned and reorganized glassware for the plant cell culture laboratory,
-Set up primary antibody for the ELISA that will be run tomorrow for "Medihoney vs. Collagenase" samples
-Designed two experiments that will be run next week using fibroblasts to measure the effects of plant extracts on cell proliferation as well as toxicity if there is some.
-Reviewed expectations for practicum report and implemented several guidelines into the paper itself
-Left early due to a physician's appointment in the late afternoon

01/08/10
-Collected cells from cell suspension, removed media and took weights ("fresh") for the first time point of the growth curve experiments
-Changed the media for all of the cultures for over the weekend. The fibroblasts and monocyte cultures appear to be doing well and will be ready for bioassays next week.
-Executed a full ELISA on two, 96-well plates to measure for TNF-alpha concentrations in media collected from cells that were exposed to LPS, Medihoney, and Collagenase. Will complete calculations next week.
-Made three liters of NTM37 and passaged three flasks of the *Astragalus falcutus* of varying colors to observe changes next week]

01/11/10
-Took fresh weights for "Day # 5" for both *Prunella* and *Astragalus* cell suspensions. Prunella is growing very quickly compared to the *Astragalus* cultures
-Seeded two 96 well plates with fibroblasts for the cell proliferation and MTT cytotoxicity bioassays that will be run this week. Also prepared a series dilution of cells so data may be more quantifiable
-Passaged several flasks of *Prunella* and then set up a small experiment to help determine which color (or hue) of *Astragalus* cultures corresponds to a healthy, rapidly proliferating cell suspension. There are several different colors and consistencies apparent within cell lines—believed to be due to stress left over from their transport from Germany
-Made a huge quantity of NTM37 (6 Liters), aliquotted it into several smaller flasks and autoclaved and stored them for passaging of cells that will take place tomorrow. Another series of growth curves will be initiated tomorrow as well.

01/12/10
-Samples were prepared and transferred to the 96 well plates (previously seeded with fibroblast cells) for an overnight incubation before carrying out the MTT cytoxicity and Cyquant cell proliferation assays
-Initiated a new set of growth curves for Prunella vulgaris to help determine a more efficient method of passaging large quantities of cells using a pipetting technique—present method is very time consuming and produces a lot of dirty glassware
-Disposed of unhealthy cultures and passaged all of the remaining cell suspensions that were initiated a couple of weeks ago
-Set up primary antibody for ELISA to be run tomorrow
-Searched through the warehouse area for additional filters and filter flasks. These and dirty glassware from passaging earlier in the day were washed and then autoclaved.
01/13/10
-Executed the TNF-alpha ELISA for the samples collected from the monocytes exposed to Medihoney and collagenase at differing concentrations
-Passaged the fibroblasts into two new flasks and froze down several flasks of them. Also changed the media for all of the other cells.
-Took the "fresh weight" of the Prunella vulgaris species for the growth curve. The cells have proliferated to six times their initial mass in only one week's time
-Completed the MTT cytoxicity assay for the plant extracts using the quick protocol option (using DMSO). Results will be calculated tomorrow morning.
-Prepared the 96-well plate to be frozen overnight in the -80 freezer; will stain with Cyquant dye and read results tomorrow.
-Made new B5 vitamin stock solution for the NTM37 plant cell culture media

01/14/10
-Made new stock solutions for plant cell cultures: 2iP, NAA, and B5 Vitamins for the DV growth medium; and subsequently made six liters of the DV medium for the *Lycopersicon esculentum* cell line
-Attending a meeting with Brett and Dr. Shi concerning "Medihoney vs. Collagenase" experiments and the poster that will be created based on these experiments. This poster will be presented at a conference in March.

-Completed the Cyquant cell proliferation assay for the fibroblast cells that were incubated in the presence of various manufactured plant extracts.
-Have not had much success with the keratinocyte cultures—even at low passages of two and three. Attempted a new method of coating the flasks and allowing the matrix to congeal for a period of time before the introduction of cells. Passaged several keratinocyte cultures into these flasks.

01/15/10
-Calculations for the Cyquant cell proliferation assay were completed and it was found that Yarrow and St. John's plant extracts were greatly inhibiting cell proliferation. The results from this assay actually suggest that none of the plant extracts increase cell proliferation (according to the standard curve generated)
-Changed the media for all of the current cell cultures so they may survive the weekend
-Passaged all of the *Lycopersicon esculentum* cell suspensions using both techniques of: pipetting directly from the cell suspensions, and transferring specific quantities from a suction filtered biomasses
-Washed and autoclaved ample amounts of glassware
-Took growth curve time points and have found that pipetting 5.0 mL of is closely equivalent to the more time consuming filtering method.

01/18/10
-Keratinocytes have not attached. Therefore, treated two additional 75cm flasks with coating matrix and then initiated new keratinocyte cultures from cryopreservation in them.
-Also initiated a new flask of THP-1 monocytes for inflammatory/MTT assays later on this week. Changed the media for the fibroblast cultures.
-Took growth curve readings for the P. vulgaris cultures initiated on 01/06 (end point) and on 01/12
-Solved the plant cell culture contamination conundrum. Too much total volume is being autoclaved at a time. The temperature gets hot enough to turn the autoclave tape black, but the liquid inside the flasks is not reaching the desired temperature (or is, but not for the correct duration of time).
-Made new media: 2 liters of the NTM37 as well as 2 liters of the DV growth medium.

01/19/10
-There was a slight contamination with homophilized Clostridium and all of the surfaces in the labs had to be cleaned down with bleach.
-Numerous flasks of each of the different cell types were passaged today—leaving very little glassware for future passaging. More glassware was recovered and cleaned from storage in the warehouse areas.
-New filters arrived and were applied to their containers, wrapped in tin foil, and autoclaved for future passaging
-Finished calculations for the MTT cytotoxicity assay and found that these results very closely mirror those collected from the cell proliferation assay. Assay will be executed again to validate these current results; but this time all of the samples will be filter sterilized prior to treatment to cells.

01/20/10
-Updated journal and lab notebooks with figures and calculations from previous bioassay results
-Seeded two 96-well plates with fibroblast cells to run another cell proliferation and cytoxicity assay
-Prepared and filter sterilized samples for the assays that will be initiated tomorrow morning
-Changed the media for the keratinocyte and THP-1 monocyte cultures
-Collected the "fresh weight" readings for the *Prunella vulgaris* time curve and the 10mL cell suspension has basically stopped proliferating. There are simply too many cells and not enough media to support them.

01/21/2010
-Pre-prepared samples were thawed out and applied to 96 well plates for both MTT cytotoxicity and cell proliferation assays
-Anti-inflammatory assay was carried out for the products of collagenase that had been digested by restriction enzymes
-Additional sucrose arrived in the mail, and therefore made, autoclaved and stored several liters of NTM37 media. Will passage the remaining *Prunella vulgaris* cultures tomorrow
-Stopped by the library at UNTHSC to check out a book and check-in with Dr. Gwirtz and Dr. Dimitrijevich
01/22/2010
-ELISA for collagenase digestion products probing for TNF-alpha was executed
-MTT cell proliferation assay was carried out
-Made, autoclaved and stored 6 Liters of DV media for the *Lycopersicon esculentum* cells that will be used for passaging on Monday
-Changed the media for the mammalian cell cultures for the weekend
-96-well plate for the CyQuant cell proliferation assay was frozen after a 30 hour incubation in the presence of filter-sterilized plant extracts
-Took final readings for the *Prunella vulgaris* time curve and organized time point data into graphs

01/25/2010
-Passaged the *Lycopersicon esculentum* cell suspensions into new flasks containing 6 Liters of fresh media. It has been decided that plant cell cultures will maintained at 6 liters per cell line to better manage the project. That leaves a lot of extra biomass that must be used in some other fashion, or thrown out. Washed ample quantities of glassware.
-Collected and froze specimens from cell suspensions. They are *L. esculentum*: normal media, stressed media, normal biomass, and stressed biomass collections
-Changed the media for all of the mammalian cell lines. Initiated three new flasks of THP-1 monocytes of all different cell numbers and passages numbers.
-Completed the Cyquant cell proliferation assay for manufactured plant extracts
-Completed the calculations for the MTT cytoxicity and Cyquant cell proliferation assays and interpreted results in lab notebook

01/26/2010
-Made 6 Liters of NTM37 and DV as well as an additional liter of NTM37 containing agarose

-Spoke to Dr. Dimitrijevich regarding plans for the completion of this internship project. Two out of the three specific aims will have been met at the conclusion of this week.
-Completed the calculations for the TNF-alpha ELISA. These results were inconclusive, and it is believed that the monocytes used were not healthy enough for this bioassay
-Took inventory of all of the active plant cell suspensions and worked on setting a routine schedule for passaging.
-Worked on time point curve calculations as well
-Researched freezing protocols to find a way to store the excess cells that are being generated in cell suspensions

01/27/2010
-Collected raw data for time point curves and inputted into Excel spreadsheet
-Changed the media for current mammalian cell lines
-Made 3 Liters of NTM37 media and passaged *Astragalus falcutus* cell culture suspensions into new flasks
-Also passaged *Prunella vulgaris* and *Lycopersicon esculentum* cell suspensions into fresh media that was made yesterday
-separated a surfeit of spent media (300mL) and biomass (500g) via filtration of the *Lycopersicon esculentum* and moved samples to -80 degree freezer. Will most likely be using these to collect proteins in the future.
-split keratinocytes into coated flasks and passage fibs via normal methods

01/28/2010
-Washed, autoclaved and stored an abundance of glassware
- Attempted to initiate the first plant cell calli from cryopreserved samples of *Echinacea purpurea* and *Astragalus falcutus.* The process was carried out aseptically and no major obstacles were faced through out.
-Allocated a considerable amount on time developing a strategy for writing up the methods section of the internship thesis practicum. Contacted major professor and advisor via e-mail to update them on current status
-Made, autoclaved, and stored five liters of NTM37 media to be used to passage the *Prunella vulgaris* tomorrow and on Monday

01/29/2010
-Took pictures of cell calli, lab spaces, plant cell suspensions, etc. to be implemented into the internship practicum report
-The calli that were initiated yesterday appear to be doing well and are uncontaminated. They were transferred into fresh agar plates and sealed with microporous tape
-Collect the *Lycopersicon esculentum* growth curve time point data. The cells have doubled in mass in just two days. Updated spreadsheet in Excel
-Amassed samples to be frozen for Prunella vulgaris including “stressed” and “healthy” cell biomasses and spent media
-Made new RPMI media for the monocytes and changed the media for all of the mammalian cell cultures for the weekend

02/01/10
-Took Lycopersicon esculentum growth curve time point. The cells have most definitely begun proliferating in the log phase—there numbers have more than doubled since Friday
-Cell calli still appear to be doing well with no signs of contamination and even some possible growth. Will take pictures to monitor growth once every week.
-Changed media for healthy mammalian cell cultures. Keratinocytes had to be thrown out again because they are detaching from the flask as they grow.
-Made new vitamin stocks for both the NTM37 and DV media.
-Passaged nearly all of the *Prunella vulgaris* cell suspensions. Collected approximately 200 grams of biomass and 100mL of spent media and froze in -80 degrees Celsius. Washed and restocked ample pieces of glassware in the plant cell culture laboratory

02/02/10
-Incubated four flasks in a coating matrix and then initiated passage two keratinocyte cultures in them
-Conducted multiple research scans in order to find alternate ways of identifying plant extracts without the use of HPLC. Looking for a much shorter method such as by UV wavelength
-Took inventory of all of the plant cell cultures, as well as of available glassware and filter flasks
-Updated and made some revisions to Medihoney bioassay graphs and submitted them back to Brett and Stone
-Collected a sample of the monocyte cell culture cells, stained them with trypan blue and viewed them microscopy to determine if they are healthy or not.

02/03/10
-Collected nearly a full kilo of biomass and freeze dried *Lycopersicon esculentum* cultures for extracts
-Changed the media for the mammalian cell cultures
-Collected raw data for time point growth curve and updated cell suspension growth curve graphs
-Continued literature search for simple, quick methods to identify if plant extracts are being removed from freeze-dried cells. Focus has now shifted to predominately thin layer chromatography (TLC).

02/04/10
-Took pictures of the cell calli and added them to a small collection of pictures that will be used in the internship practicum report
-Collected several vials of normal and stressed Prunella vulgaris cultures and transferred them into the -80 degree freezer.
-Passaged *Lycopersicon esculentum* cell suspension into new media/flasks
- Took inventory of all of the samples collected so far and organized them into a more easily accessible fashion based on the type of species, amount of sample, and whether or not the culture was healthy at the time of collection.

02/05/10

-Took the mass of the *Lycopersicon esculentum* cell suspension for time point growth curve experiment. The adjusted weight was a massive 20 grams which means that in the log phase, the cells doubling time is equal to four days.
-Worked with all of my committee members to establish a date and time to give defense. Filled out paperwork and then went to campus to try and collect signatures for the "intent to defend form"
- Made 6 Liters of DV media to passage the remaining *Lycopersicon esculentum* cultures.
-Transferred filters from callus cultures to fresh NTM37 plates set with agarose

02/08/10
-Set-up lyophilizing equipment and initiated freeze drying for several samples of Lycopersicon esculentum biomasses
-Took another weight reading for the L. esculentum time point curve. It was 25 grams—up from 2 grams when it experiment was begun on day #1.
-Made 5 Liters of NTM37, aliquotted it into various smaller sized flasks, and then autoclaved it. The A. falcutus cell suspensions were all then passaged into fresh media.
-Initiated another THP-1 monocyte cell suspension culture for the inflammatory honey bioassays to be completed next week.
-changed the media for all of the mammalian cell cultures including several flasks of keratinocytes, monocytes and fibroblasts.

02/09/10
-The cells in the lyophilizer appear to be drying quite well. Researched a few extraction methods specific to *L. esculentum* to be used in the coming days
-Made 4 more liters of NTM 37 medium, however, the water boiler that is used by the autoclave and the dishwasher has a leak. All of the media was stored to be autoclaved later and used for passaging all of the *Prunella vulgaris* cell suspensions.
-All of the THP-1 monocyte cell cultures were contaminated this morning. It is believed that some of the supplements (i.e. BME) are the culprit. Removed three new vials from cryopreservation and initiated them into cell suspension.
-Spoke with Stone about bioassay graphs that will be used in an upcoming conference. Several revisions and conversions to make them more comprehensible were applied and re-submitted to the "higher ups" for evaluation

02/10/10
-Removed one glass jar from the lyophilizer and took dry weights: 20 mLs of biomass weighed a total of 690 mg.
-Collected another 500 grams *Lycopersicon esculentum* biomass and transferred it into the -86 degree freezer.
-The boiler for the autoclave and dishwasher still needs to be repaired and so a large amount of glassware had to be washed by hand.
-Took the reading for the final weight of the *L esculentum* time point growth curve and inputted data into the spreadsheet. The final results turned out well and this spreadsheet will most likely make it into the final presentation at the end of the internship
-Had a meeting with Stone and Brett to determine the next best course of action for the research that will be presented at the end of this month. There are quite a few things to do before then,

including measuring protein contents in all of the anti-inflammatory samples, execute a MTT assay that includes a collagenase variable, and re-run an anti-infammatory assay that tests collagenase digestion products.
-Changed media for all mammalian cultures and split/froze fibroblast cells.

02/11/10-02/12/2010
-Out due to snow days

02/15/2010
-Changed the media for the mammalian cell cultures. Monocytes are appearing to be very healthy (with trypan blue stain) and are proliferating quickly.
-Collected an abundance of *Prunella vulgaris* biomass and froze in -86 degree freezer
-Initiated several new calli with the *Prunella vulgaris* cell suspension. Boiler for autoclave is still out and therefore no dishware can be cleaned and media cannot be made. Plant cell culture is at a complete standstill.
-Took more pictures of calli and updated picture library
-Cleaned up lyophilizer and took dry weight readings from freeze dried samples of *Lycopersicon esculentum*.
-Continued to work on thesis. Organized all of the main themes into a concept wheel (or a skeleton of sorts)

02/16/2010
-Discussed desired variables and carefully planned out two bioassays that will be initiated tomorrow. These results will be taken and shared at the national sales conference later this month
-Developed and executed methods for extractions on lyophilized samples of Lycopersicon esculentum. The concentrations of the solvents, incubation times, and so on, needed to be modified to accommodate the large volume of these samples. It appears that extractions are working (resulting in three distinct phases), however, the products had to be stored due to a lack of time. Extracts will be evaporated tomorrow.
-New samples had to be thawed out and redistributed into separate baking tins. They were then refrozen and will be lyophilized using a new machine first thing tomorrow morning
-Organized and stored clean glassware in the plant cell lab. The autoclave is still broken and the plant cell lines are starting to suffer because they are so overdue for passaging.

02/17/2010
-Changed the media for all of the mammalian cell cultures. Checked on the plant cell cultures, and the remaining cell suspensions are becoming very dense. Cell calli still appear to be growing well, however, will need to be passaged on Friday. The autoclave is still not working and it is feared that soon cultures of all types will be lost.
-Stopped by the school to collect additional signatures for the “Intent to Defend Form”
-Began the MTT cytotoxicity assay by seeding 100K THP-1 monocytes into the wells of a 96-well plate. Samples were mixed up to desired concentrations, transferred into their respective wells, and the plate was moved to incubator overnight.

-Initiated an anti-inflammatory assay using viable THP-1 monocyte cells. This assay is being executed to test whether the company's collagenase product is more beneficial in wound healing as compared to a competitor product.
-Collaborated with another member of the R&D team to program a new lyophilizer and begin freeze-drying more plant cell biomass for extractions.

02/18/2010
-Executed an ELISA probing for TNF-alpha concentrations within samples collected from anti-inflammatory assay
-Completed the MTT cytotoxicity assay that was initiated yesterday and began working on calculations for it

-Carried all of the calculations for the ELISA that was run today, updated lab notebook, and discussed results with Stone and Brett
-The autoclave still broken, however, R & D has been given the go ahead to use it anyway. The dishwasher appeared to have been working but broke during its first trial run
-Made two liters of NTM37 media, autoclaved it, and passaged the *Prunella vulgaris*.
-Made several agar plates for the *Echinacea*, *Astragalus*, and *Prunella* plant cell calli

02/19/2010
-Passaged several million THP-1 monocytes and froze approximately 100 million more
-Changed the media for the mammalian cell cultures for the weekend
-Passaged several flasks of the Prunella vulgaris and some Astragalus falcutus cell suspensions into fresh media
-Subdivided calli into several smaller pieces with a razor blade and transferred the smaller segments into new petri dishes with agarose. Took additional pictures
-Used a BCA kit (biochonic acid protein assay kit) to quantify the proteins in the samples collected from anti-inflammatory assay. This may be a better indicator of cell activity in the presence of the compounds that were tested.
-The lyophilizer (freeze dry) cycle was completed. Collected, weighed, and stored freeze dried biomasses.

02/22/2010
-Made new vitamins and hormone supplements; and then 2 Liters of NTM37 and 1 Liter of DV media which was autoclaved. Then passaged the Prunella, Lycopersicon, and Astragalus cultures
-Prepared new agar plates with DV media components for Lycopersicon esculentum and initiated several petri dishes of plant cell calli
-Massive amounts of glassware had to be washed by hand as the dishwasher is still not functioning correctly
-Changed the media for the remaining mammalian cell cultures. Will need to initiate some newer (lower passage numbers) cultures soon to test extracts on
-Initiated lyophilization of the *Prunella vulgaris* cultures that were prepared on Friday
-Measured proteins via a BCA assay to eliminate background present in honey product as well as normal RPMI media.

02/23/2010
-Finally managed to submit the "Intent to Defend" form to the Graduate School of Biomedical Sciences. Spoke with Carla Lee about remaining requirements for graduation
-Re-ran an entire BCA to determine the protein in all of the samples obtained from the anti-inflammatory assay executed last week as the concentration of protein in Medihoney, collagenase and media alone. Dilutions were made one to ten and the conditions of experiment very closely mimicked those of anti-inflammatory assay.
-Completed calculations for the BCA results. The background values are still too high, so more brainstorming needs to be done to determine where the confounding variables lie
-Restocked several items that were missing in the plant cell lab
-Performed a series of extractions on lyophilized plant biomass samples of various dry weights. Also varied the quantities of the different solvents used in the process to determine the most efficient method of obtaining plant extracts.

02/24/2010
-Collected over 500 grams of Lycopersicon esculentum biomass in 15 mL centrifuge tube and froze them in -80 degrees Celsius for long term storage
-The boiler for the dishwasher is still not work and therefore had to handwash ample amounts of glassware
-Lyophilization on the new machine has now been completed. *Prunella vulgaris* samples were collected and dry weight masses were observed
-Initiated a MTT cytotoxicity assay in order to hopefully obtain stronger results. There was far too much variance within sample groups in the last assay of this type run
-Learned out to use a new fluorospectrometer called the "*NanoDrop*", and then utilized it to measure proteins in the anti-inflammatory samples

02/25/2010
-Made agar plates containing NTM37 media for the *Prunella, Echinacea,* and *Astragalus* calli for tomorrow
-Initiated another anti-inflammatory assay for Collagenase vs. Medihoney studies; and collected/froze the resulting spent media and induced cells
-Completed the MTT cytotoxicity assay and perform the necessary calculations to interpret the generated raw data
-evaporated the upper phase of the samples collected while doing extractions earlier this week, and resuspended compounds in acetone
-Carried out a trypan blue counting and staining for the monocytes that were incubated previously in collgagenase and Medihoney products, and set up cells in 96 well plate for possible MTT to be run tomorrow

02/26/2010
-Attempted to initiate cell suspensions from cell calli for *Prunella* and *Astragalus* plant cell lines
-The calli had grown profusely since they were originally initiated. Subdivided calli into several smaller sections and transferred these aseptically to fresh agar plates

-Initiated an anti-inflammatory assay to decide the strength that the monocytes adhere to the monolayer after they have been induced. It was determined that cells will need to be vigorously pipetted and even sloughed off with a cell scraper in future bioassays
-New fibroblast cultures were initiated today to be used in bioassays next week to test the compounds that are being extracted from plant cell cultures for wound healing properties
-Proteins were measured again via the *NanoDrop* for the samples that were collected in yesterday's anti-inflammatory assay
-Changed the media for the remaining mammalian cell cultures for the weekend

03/01/2010
-Began lyophilization of the samples of *Lycopersicon esculentum* biomass that were prepared and frozen last week
-Carried out extractions for the dried biomass of the *L. esculentum* and the *P. vulgaris*. Varying methods for these extractions were applied to determine the best as far as using the least amount of solvent and obtaining the most amount of product
-Worked on thesis for a few hours
-Evaporated the extraction products today so that they will be fresh when applied in vitro, and took dry weights to determine the ratio of product that is recovered. Resusupended extracts in DMSO and stored at four degrees Celsius.

03/02/2010
-Went on two job interviews today

03/03/2010
-Worked on thesis most of the day
-Re-started lyophilization on *Lycopersicon esculentum* because biomasses were not sufficiently freeze dried.
-Made new RPMI media for the monocytes and changed the media for all of the mammalian cell cultures that are running right now
-Washed (by hand) a great deal more glassware—the boiler pipes leading the dishwasher still have not been fixed
-Evaporated samples that contain extracts in chloroform solvent, and resuspended compounds in DMSO

03/04/2010
-Made new NAA (hormones for media), as well as one liter of NTM37 and one liter of DV media. Poured new agar plates for calli passaging tomorrow and stored at 4 degrees
-Worked on thesis—found the GSBS guidelines for formatting this paper and begun working to set up my paper to match its specifications.
-Cleaned up both lab areas and then handwashed a great deal more glassware
-Read the article for and attended the journal club meeting this afternoon discussing cross-linking polymers in tissue engineering

03/05/2010
-Passaged the L. esculentum, P. vulgaris, and Astragalus cell suspension into fresh media. Collected all of the additional biomass from these three cell lines and froze in -80.

-Transferred all four of the different cell lines of the calli cultures to fresh agar. Took pictures of the L. esculentum callus to supplement photo library for thesis
-Changed the media for the mammalian cultures for the weekend
-The lyophilization for the L. esculentum biomass—approximately 200 grams worth—has now been completed. Freeze dried material was collected in an air tight container and stored at room temperature
-Spent the latter part of the day at the campus library working on thesis and oral defense

03/08/2010
-Fibroblasts were seeded into a 96 well plates and given four hours to attach. Then samples of the extractions performed were applied at different concentrations (.01%, 1% and 10%) to the cultures and replaced back in 37 degrees Celsius for an overnight incubation. Will run an MTT assay tomorrow to determine if extracts enhance cell proliferation or if they are cytotoxic.
-Changed the media for the mammalian cell cultures
-Worked on thesis for the better part of the day

03/09/2010
-Executed a full MTT assay following the normal kit protocol (four hour incubation in phenol/serum-free media and Vybrant dye, a 10 minute incubation in DMSO, mix well and then read chemiluminescence in a microplate reader)
-Thawed out Astragalus falcutus (normal) biomass from -80 degrees Celsius and prepared them for lyophilization. After samples in shallow tins had re-frozen, they were moved to the lyophilizer to begin freeze drying
-Completed the calculations for the MTT assay and it was found that
-Continued to work on thesis

03/10/2010
-Completed the calculations for the MTT assay and it was found that the resulting numbers of cells exhibiting normal metabolic processes were all over the board. A background control to determine the concentration at which DMSO in media is toxic to cells also needed to be included in this experiment, but was unfortunately overlooked.
-Changed the media for the mammalian cell cultures
-Used the NanoDrop to reveal the protein concentrations from the samples collected yesterday when proteins were extracted from the extractions taken from plant cell biomasses
-Continued to work on thesis
-Collected and counted approximately 75 million THP-1 monocytes; and then added DMSO and frozen in -80 degrees Celsius

03/11/2010
-Made a liter of NTM37 media and then also poured additional NTM37 autoclaved with agar into fresh plates to be usesd tomorrow to passage cell calli.
-The boiler for the dishwasher is now fixed. Washed and stored a large quantity of glassware for both labs.
-The lyophilization for the *Astragalus falcutus* was completed. Freeze dried material was collected, weighed and stored for future use

-Moved and logged THP-1 monocytes that were frozen yesterday into long-term cryopreservation
-Went to the campus library to continue to work on thesis

03/12/2010
-Transferred the filters where cell calli have been growing from petri dish containing spent media/agar to fresh plates. Several of the calli had to be subdivided with a sterile razor blade and initiated on fresh plates.
-Initiated cell suspension from calli for the Astragalus falcutus and Prunella vulgaris cell lines. All of the cell suspensions containing the A. falcutus were lost when the autoclave was out of commission and therefore it is of utmost importance that this cell suspension initiation is sucessful
-conducted literature searches to find a better alternative solvent to dissolve extracts in than DMSO
-Planned out bioassays to be run next week to use fresh extracts to test for wound healing properties
-Continued to work on thesis

03/15/2010
-Concluded research on alternatives to suspend biomass extracts and have actually found that water is ideal if the extracts are to be used immediately after their resuspension.
-Changed the media for the mammalian cell cultures
-Performed extractions on three 220 mg samples of *L. esculentum, P. vulgaris,* and *A. falcutus.* The samples collected from the extractions were then immediately evaporated and resuspended in syringe filtered deionized, destilled water.
-A 96 well plate was seeded with 10,000 fibroblast cells and then moved to a 37 degree incubator to allow for cell attachment (four hours). Samples containing the extracts in serum-free/phenol red-free media at various concentrations were then added to the cell cultures and left to incubate over night. Testing extracts for cytotoxicity tomorrow
-Went to the campus library in the late afternoon to work on thesis

03/16/2010
-Executed the MTT assay to deterimine of the hyperphase of the extractions that I have been collecting is toxic to mammalian cells once they are resuspended in deionized, distilled water
-Wrapped filtration setups in almuninum foil and autoclaved them along with extra Whatmann filters and plant cell culturing tools
-Completed calculations for the MTT assay and it was found that *Lycopersicon esculentum* is most likely toxic, while *Astragalus* and *Prunella* may both actually enhance cell proliferation.
-Continued to work on thesis

03/17/2010
-Cleaned additional glassware and restored them it to the plant cell culture lab
-Performed extractions for the *L. esculentum, P. vulgaris,* and *A. falcutus* cell cultures to provide fresh extracts for bioassays
-Changed media for mammalian cell cultures

-Initiated a Cyquant cell proliferation assay to validate the results that were obtained in yesterday's MTT cytotoxicity assay. Was set up almost identically to how the MTT assay was, to hopefully limit a few confounding variables
-Went to the campus to library in the late afternoon to work on thesis

03/18/2010
-Made new vitamin and hormone stock solutions
-Removed the samples from the Cyquant bioassay microwells and froze the plate in -80 freezer. Will complete the staining and reading portion of the bioassay tomorrow
-Planned out two new bioassays to be completed next week: an anti-inflammatory and a cell migration for the extracts obtained from the plant cell biomasses
-Made, autoclaved, and stored a liter of NTM37 media as well as a liter of DV media to passage all of the remaining cell suspensions tomorrow. Made and poured a great deal of agar plates to passage all of the plant cell calli tomorrow.
-Continued to write thesis

03/19/2010
-Passaged calli to new agar plates
-Passaged *Astragulus, Prunella,* and *Lycopersicon* cell suspensions into fresh media
-Completed the Cyquant cell proliferation assay and it was found that *Prunella vulgaris* may enhance cell growth, *Astragalus falcutus* has no impact on proliferation and *Lycopersicon esculentum* hinders it
-Changed media for mammalian cell cultures for the weekend
-Worked on thesis and will try to finish most of it over the weekend

03/22/2010
-Inititated a Cytoselect cell migration assay to test the extracts obtained from plant cell biomass for chemotactic properties. Biomass extracts samples were added as well as manufactured extracts to serve as positive controls for the assay.
-Designed a new anti-inflammatory bioassay to be initiated tomorrow
-Worked on implementing bioassay graphs into internship practicum report and
-Went to the library to continue to work on thesis

03/23/2010
-Initiated an anti-inflammatory assay to determine whether extracts obtained from plant cell biomass will suppress the excretion of TNF-alpha in monocytes when they are induced by LPS
-Concluded the cell migration assay by removing the excess liquid, lysing the cell that had migrated to the underside of the middle membrane, stained the remnants, and read the fluorescence on a microplate reader
-Calculated the results for the cell migration assay and updated the biotech thesis with them. It was found that all of the extracts obtained from the plant cell biomass have chemotactic properties
-Made one liter of NTM37 media and poured several agar plates to be used for calli passaging on Friday
-Continued to work on thesis
-Set up a primary antibody for tomorrow's ELISA

03/24/2010
-Passaged the *Astragalus falcutus* cell suspensions that had been initiated from cell calli into fresh media
-Conducted and ELISA to measure the TNF-alpha concentrations in the samples that were collected in yesterday's anti-inflammatory assay
-Completed the calculations for the ELISA and it was found that none of the ethanol extracts that were prepared offer any anti-inflammatory properties show any promis of being useful in aiding the wound healing process.
-Spent the majority of the day working on the thesis

03/25/2010
-Emailed the very rough draft of the internship thesis to Dr. Dimitrijevich
-Decided which results are to be used and spent some time working on implementing them into the research internship practicum
-Made a theoretical growth square and also modified the recipes for plant cell media (to delete any proprietary information) and added both of these in the thesis.
-Went to the school library to continue to work on the thesis

03/26/2010
-Changed the media for all of the mammalian cell cultures
-Went to passage all of the cell calli and the agar had all been desiccated. Returned the plates to the Percival and will make new agar on Monday and passage calli then.
-Cleaned up lab spaces and washed additional glassware
-Collected additional biomass from the *Prunella vulgaris* cell lines into 50 mL tubes and froze them at -80 degrees Celsius.
-Drove out to Las Colinas for an interview
-Continued to work on thesis

03/29/2010
-Had an appointment with Dr. Dimitrijevich to discuss the rough draft that he received last week.
-Made one liter of NTM 37 media, aliquotted it into smaller flasks and autoclaved it. Also prepared agar plates for the calli that were not passaged on Friday.
-Took care of all of the Biohazard wastes in the labs, restocked labs, and put up cleaned glassware
-The T25 flask of THP-1 monocytes died over the weekend due to the lack of oxygen. A new culture with 12 million of these cells was initiated
-Passaged the *Astragalus* cell calli into fresh media to initiate cell suspensions. Also passaged the plant cell calli to fresh agar plates.
-Continued to work on thesis

03/30/2010 to present
-Passaged all of the plant cell calli that require NTM37 media
-Prepared the lab areas for a visit from the health inspector tomorrow
-Left early to start making revisions to the thesis at the campus library

03/31/2010
-Washed and stored a lot of glassware and cell culturing tools
-Changed the media for the remaining mammalian cell cultures
-Attended a luncheon with all of the employees of building 4
-Continued to work on thesis—actually began rewriting the whole paper to condense it and organize it in a more managed way

04/01/10 – 04/02/2010
-No work these days because of a company picnic day and an Easter Holiday. Continued to work on thesis

04/05/2010
-Made new stock solutions for NTM37 B5 Vitamins, 2iP hormones, and NAA hormones.
-The THP-1 monocytes had expired and therefore new cultures had to be initiated. The liquid nitrogen in the cryo tanks got too low and therefore several vials were used at one time.
-Made one liter of NTM37 media and a half a liter of DV media. Aliquotted autoclaved and stored. Also prepared approximately 50 agar plates to be used for passaging cell calli
-Passaged the *Lycopersicon esculentum* cell calli to fresh solid media
-Continued to work on thesis
-Emailed rough draft of internship practicum report to both Dr. Dimitrijevich and Dr. Gwirtz.

04/06/2010
-Spent the entire day at the UNTHSC library working on the internship practicum. Submitted a rougn draft to Dr. Gwirtz

04/07/2010
-Thesis work

04/12/2010
-Passaged all of the plant cell calli
-Prepared the lab spaces for a visit from the FDA
-Began working on the presentation for the oral defense and continued to revise thesis

04/13/2010
-Worked on thesis and then e-mailed it to Dr. McClain and Dr. Dimitrijevich
-Continued to work on the oral presentation

04/14/2010-04/27/2010
-Made revisions to the internship practicum report, created the presentation for the oral defense, presented the public oral defense, and essentially shut down the plant cell culture research facilities at internship site.

REFERENCES

Babinard, J. (2001). A short history of agricultural biotechnology. In Gerald C. Nelson (Ed.), *Genetically modified organisms in agriculture* (pp. 271-274). London: Academic Press.

Daurnenburg, H. (2008). Plant cell culture technology: harnessing a biological approach for competitive cyclotides production. *Biotechnology Letters, 30*(8), 1311-1321.

Deshmukh, P. T., Fernandes, J., Atul, A., & Toppo, E. (2009). Wound healing activity of calotropis gigantea root bark in rats. *Journal of Ethnopharmacology, 125*(1), 178-181.

Eibl, R., & Eibl, D. (2008). Design of bioreactors suitable for plant cell and tissue cultures. *Phytochemistry Reviews, 7*(3), 593-598.

Entschladen, F., Drell IV, T. L., Lang, K., Masur, K., Palm, D., Bastian, P., et al. (2005). Analysis methods of human cell migration. *Experimental Cell Research, 307*(2), 418-426.

Evans, D. E., Coleman, J. O. D., & Kearns, A. (2003). *Plant cell culture*. New York, New York: BIOS Scientific Publishers.

Hagel, J., & Facchini, P. (2008). Plant metabolomics: Analytical platforms and integration with functional genomics. *Phytochemistry Reviews, 7*(3), 479-497.

Hunt, T. K., Hopf, H., & Hussain, Z. (May/June 2000). Physiology of wound healing. *Advances in Skin & Wound Care, 13*(Supplement 2), 6-11.

Long, M., Millar, D.J., Kimura, Y., Donovan, G., Rees, J., Fraser, P., Bramley, P., Bolwell, G.P. (2006). Metabolite profiling of carotenoid and phenolic pathways in mutant transgenic lines of tomato: Identification of a high antioxidant fruit line. *Phytochemistry*, 67(16), 1750-1757.

Roche applied science: Apoptosis, cell death and cell proliferation**.** (2008). In Rode H. J., Eisel D. and Frost I. (Eds.), (3rd ed.). Mannheim, Germany:

Romanik, G., Gilgenast, E., Przyjazny, A., & Kaminski, M. (2007). Techniques of preparing plant material for chromatographic separation and analysis. *Journal of Biochemical and Biophysical Methods, 70*(2), 253-261.

Scharch, C., Blum, P., & Zaulli, F. (2008). Potential of plant cells in culture for cosmetic application. *Phytochemistry Reviews, 7*(3), 599-605.

Schultz, G. S., Ladwig, G. & Wysocki, A. (Tuesday, 30-Aug-2005 10:21:16 BST). *Extracellular matrix: Review of its roles in acute and chronic wounds.*http://www.worldwidewounds.com/2005/august/Schultz/Extrace-Matric-Acute-Chronic-Wounds.html

Sharma, A. K., & Sharma, M. K. (2009). Plants as bioreactors: Recent developments and emerging opportunities. *Biotechnology Advances, In Press, Corrected Proof*

Singer, A. J., & Clark, R. A. F. (1999). Cutaneous wound healing. *N Engl J Med, 341*(10), 738-746.

Singh, U., Tabibian, J., Venugopal, S. K., Devaraj, S., & Jialal, I. (2005). Development of an in vitro screening assay to test the anti-inflammatory properties of dietary supplements and pharmacologic agents. *Clin Chem, 51*(12), 2252-2256.

Soulie, M.C., G. Bompeix, G., Laval-Martin, D. (1992). Polar lipids in healthy and *Phytophthora capsici*-infected leaflets of *Lycopersicon esculentum*. *Phytochemistry*, 31(6), 1961-1967.