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The use of complementary and alternative medicine in minority populations in individuals infected with HIV is prevalent and these cultural practices require laboratory confirmation to determine effectiveness. The manipulation of HIV via nutritional supplementation could provide insight to the efficacy of cultural remedies.

Ergothioneine, a compound derived from mushrooms and a naturally occurring amino acid, was used as a positive control due to its confirmed ability to decrease HIV promoter-driven gene expression via inhibition of the NF-κB transcription factor. Four herbal remedies, which are purported to prevent or treat HIV infection by Black and Latino HIV patients and uninfected individuals, were also tested for antiviral properties. Ergothioneine, *Hypoxis hemerocallidea, Sutherlandia frutescens, Opuntia ficus-indica,* and *Uncaria tomentosa* were liquefied and used to prepare serial dilutions. The dilutions were used to treat CEM-GFP cells in cytotoxicity assays to determine ideal concentrations for treatments, that alone (i.e. in the absence of virus) had minimal effects on GFP expression. Once concentrations of each treatment were elected, CEM-GFP cells were cultured in the presence of the dilutions, followed 24 hrs. later by HIV infection and monitoring for GFP expression. In this system, increased GFP fluorescence signifies stimulation of HIV replication, and decreases in GFP fluorescence signifies inhibition of HIV replication. The results suggested that ergothioneine, *Hypoxis hemerocallidea,* and *Sutherlandia frutescens* prevented HIV proliferation post-infection while *Opuntia ficus-*

*indica* and *Uncaria tomentosa* both stabilized cells prior to infection and inhibited HIV proliferation post-infection.

# NUTRITIONAL MANIPULATION OF HIV/AIDS: THE EFFECTS OF ERGOTHIONEINE AND CULTURAL COMPLEMENTARY AND ALTERNATIVE MEDICINES ON HIV

by

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APPROVAL PAGE

This thesis written by LAUREN WEST has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

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# CHAPTER I

# INTRODUCTION

The use of complementary and alternative medicine in minority populations in individuals infected with HIV is prevalent and these cultural practices require laboratory confirmation to determine effectiveness[1-3]. The manipulation of HIV via nutritional supplementation could provide insight as to the efficacy of cultural remedies[3].

For this research, we have used the CEM-GFP cell line, which contains an engineered GFP gene driven by the HIV long terminal repeat (LTR) promoter, so that when they are infected with HIV-1, one can measure the expression of GFP fluorescence as a function of HIV activity (since HIV activates its own transcription). Ergothioneine, a compound derived from mushrooms and a naturally occurring amino acid, was used as a positive control in these studies. Based on the evidence that ergothioneine is a potent antioxidant, it is expected that ergothioneine will decrease the activity of reactive oxygen species and inhibit the NF-κB transcription factor, to inhibit the transcription and proliferation of HIV[4-8]. In ergothioneine-treated HIV-infected CEM-GFP cells, a decrease in intensity of GFP expression would signify a decline in HIV activity, confirming the expected result, if ergothioneine has antiviral activity. If successful, this experimental design and assay would serve as a positive control for the second aim.

The design of the ergothioneine assay was repeated to test 4 commonly utilized herbal compounds, *Hypoxis hemerocallidea, Sutherlandia frutescens, Opuntia ficus-indica,* and *Uncaria tomentosa*[9, 10]. As these herbs are used in Black American and Latino American populations[11], which have the highest rates of HIV infection in the United States, the ratification or denial of their purported role in inhibiting HIV activity is necessary information for the future of complementary and alternative medicine in these populations[9, 10].

# CHAPTER II

# BACKGROUND

## HIV Overview

The commonly known Human Immunodeficiency Virus (HIV) is a member of the genus *Lentivirus* included in the *Retroviridae* family[8]. While lentiviruses include a multifaceted group of viruses that afflict animals, HIV is widely known due to its involvement in the development of the Acquired Immune Deficiency Syndrome (AIDS) in humans[8, 13].

HIV-1, which differs from the HIV-2 strain mainly by the interaction of antibodies with the viral envelope glycoproteins, infects the host most commonly via binding to the CD4 (cluster of differentiation) receptors of T cells by the gp120 viral envelope protein[8, 14]. After attachment to the CD4 glycoprotein, there is subsequent fusion of the viral envelope with the T cell membrane and entry into the host as a ribonucleocapsid[8, 15]. After entry into the T cell, the HIV nucleic acids are uncoated in cytoplasm. The exposed RNA undergoes reverse transcription to form double stranded viral DNA, which accesses the T cell nucleus and is integrated into the host genome[8, 16].

Following integration of the viral DNA, replication, transcription, and translation of the T cell genome will facilitate the proliferation of HIV in the host's system[8, 16]. While antiviral therapies can be directed at any of the aforementioned steps in the HIV

infection cycle, the focus of the proposed research pertains to the inhibition of transcription.

**HIV in Society**

Aside from solely pharmaceutical interventions, a wholesome diet and healthy lifestyle is an indispensable component to HIV therapy[2]. There is a significant correlation between malnutrition and the prevalence of HIV in populations[1-3, 17]. As Colecraft (2008) states, "the interaction between HIV/AIDS and food and nutrition security increases susceptibility to HIV exposure and infection and HIV/AIDS, in turn, increases vulnerability to food insecurity.[17]" This signifies the necessity of nutritional supplementation in prevention and post-infection treatment of HIV/AIDS[1-3].

The correlation between quality of lifestyle and susceptibility to HIV/AIDS is indicative of poverty or low income being a contributing factor to likelihood of infection. Applying this ideology to populations in the United States, one can expect a higher prevalence of HIV infection and AIDS among lower income individuals. According to the National Report Card from the Stanford Center on Poverty and Inequality, Black and Hispanic Americans between the ages of 18 and 64 have high percentages of individuals living below the poverty line. By 2012, 27% of Black Americans and 25.6% of Hispanic Americans in that age category were living in poverty[18].

Juxtaposing the high poverty rates of Black and Hispanic individuals in the United States with the information regarding nutrition and HIV/AIDS, it is

understandable that domestic minorities have the highest rates of newly diagnosed infections in the country.

The CDC purports that Black Americans continually have the highest rates of HIV/AIDS compared to other races[19]. Despite only representing 12% of the U.S. population in 2010, Blacks accounted for approximately 44% of new infections that year[11, 19, 20]. Additionally, it is expected that approximately 1 in 16 black men and 1 in 32 black women living in the United States will be diagnosed with HIV in their lifetime[21]. The CDC also gives statistics exemplifying that Hispanic/Latino Americans also suffer from high rates of HIV/AIDS in the United States[19]. In 2010, Latinos represented 16% of the population and were plagued with 21% of new infections[20].The likelihood for infection was 2.9 times higher in Latino males and 4.2 times higher in Latina females than their white counterparts in 2010[11]. The death rates of Black and Latino individuals with HIV is equally jarring, with over 260,800 Blacks and 96,200 Latinos with AIDS diagnoses passing away since the epidemic began[19]. Due to these statistics, it is crucial to focus on these racial groups for antiviral nutritional intervention.

**Complementary and Alternative Medicine Use in Minorities**

Complementary and Alternative Medicine (CAM) is very common among minority populations in the United States, especially among Black Americans and Latino Americans[9, 10, 22-24]. The prevalence of herbal use and CAM is theorized to be rooted not only in cultural practice, but also as a result of discrimination in health care settings. Due

to systematic and internalized racism in American history, many domestic minorities develop a sense of mistrust of healthcare providers[25].

While estimates of CAM use in American adults ranges from 29% to 68% of the population, the prevalence among Black Americans is estimated at 67.6-71.3% of adults[9]. A 2012 study pertaining strictly to HIV positive Black Americans reflected that of 182 subjects, 94% of participants used CAM to treat their HIV and 79.7% of participants used CAM as a complement to HIV therapy, as opposed to as an alternative[23]. Similarly, the use of CAM is widely prevalent in Latino populations for antiviral purposes. A 2013 study showed 76% of 113 Latino participants with HIV used some form of CAM in their treatment[10].

Whether the commonly utilized herbs and remedies are beneficial or deleterious via drug interactions to HIV patients, it is imperative that healthcare providers document these treatments so that the proper research can be conducted[22].

**Ergothioneine**

Ergothioneine is a sulfur-containing amino acid derived from the ergot fungus at concentrations of approximately 4 mM and exists in vivo in concentrations between 1 mM and 2 mM[4, 5]. Ergothioneine is purported to have antioxidative properties, the actions of which would reduce reactive oxygen species (ROS) in the body, leading to the eventual inhibition to the function of the NF-κB pathway[5, 6, 26]. Reactive oxygen species include superoxide ($O_2^-$), nitric oxide (NO), hydroxyl radical ($OH^\cdot$), hydrogen peroxide ($H_2O_2$), hypochlorous acid (HOCl), and others[3, 5]. Shortly after infection, these ROS

increase, leading to oxidative stress which involves the generation of free radicals that contribute to the degradation of DNA and cellular damage[3, 6].

T cell transcription involves activation of the cell, which leads to a myriad of intracellular signaling events[8, 27]. Activation begins with a receptor-ligand mediated signaling event occurring on the plasma membrane. These interactions include interleukin-2 receptor (IL-2R) with the ligand IL-2, T-cell receptor (TCR) in collaboration with CD4, or transmembrane passage of molecules such as phorbol myristate acetate (PMA). TCR stimulation results in the activation of tyrosine kinases, such as Fyn ($p59^{fyn}$), ZAP-70, and Lck ($p56^{lck}$). This process is likely controlled by tyrosine phosphatases, such as CD45. Once the tyrosine kinases are activated, they are capable of phosphorylating various substrates located in the cytosol, leading to chain of signaling events often incorporating intermediate molecules[8, 27]. For example, Ras-Raf signaling pathway is initiated by the binding of Grb2 and SOS to Ras, converting it to Raf[8, 28]. The activation of Raf stimulates a cascade of serine-threonine kinases (MAP-kinase kinase, MAP-kinase, Rsk, and S6 kinase) which continue the signaling pathway and lead to the eventual activation of the DNA binding proteins and transcription factors which are responsible for the regulation of gene expression. The phosphorylation and/or the proteolysis of the inhibitor of kappa B (IκB) dissociates it from nuclear factor kappa B (NF-κB). This allows the passage of NF-κB across the nuclear membrane into the nucleus, where it binds to DNA to activate transcription and subsequently translation[8, 27]. The signal transduction pathway can also be facilitated via the activation of protein kinase C (PKC) by PMA. Once PKC is activated, it stimulates the generation of the

secondary messengers diacylglycerol (DAG) and phosponiositol triphosphate (PIP3) through the stimulation of phospholipase Cγ (PLCγ). This stimulations the mobilization of intracellular calcium ($Ca^{2+}$) and the release of phosphoinositol (PI), which also leads to the phosphorylation and/or proteolysis of IκB[8, 27].

NF-κB binds on the DNA in close proximity to the long terminal repeat region (LTR) of the HIV genome. While the LTR alone can act as its own promoter, early mRNA transcription seems to rely mainly on transcription factors, including NFAT, AP-1, SP-1, and most notably for the aforementioned specified aims, NF-κB[8, 29]. The NF-κB transcriptional factor and its inhibitor promote the expression of more than 100 target genes that are active contributors in the immune response of the host organism[26, 30]. NF-κB is also responsible for the production of cytokines, which are involved in a plethora of cellular activities and therefore essential to host survival[26]. Since NF-κB activation is an immediate early (IE) event in the immune system that is triggered quickly and leads to strong transcription of several genes, many viruses (including HIV-1) target the NF-κB pathway in order to achieve enhanced replication, facilitate survival of the host cell, and evade immune detection[30]. It is evident that NF-κB is essential, arguably vital, in the transcription of HIV-1[7, 8, 30]. For that reason, NF-κB is an ideal target for antiviral intervention.

Several studies have examined the antioxidative properties of ergothioneine. Ergothioneine was shown to be an active scavenger of hydroxyl radicals, as well as an inhibitor of metal ion complexes that allow free radical species to degrade deoxyribose[5,

[31]. Certain ROS such as hydrogen peroxide, while not a free radical itself, can lead to the formation of free radicals that readily react with DNA, causing breaks[5, 6, 32].

Ergothioneine also showed activity in binding iron and copper complexes to prevent oxidation, which is notable because under high levels of oxidative stress, cellular damage or cell death occurs. Additionally, the decrease of ROS due to oxidative stress in the cell decreases the amount of single stranded DNA breaks, failing to activate the poly(ADP-ribose) polymerase (PARP) complex[5, 6]. An activation in the PARP complex facilitates not only the apoptosis and/or necrosis of T cells, but also the activation of the NF-κB pathway[6]. An antioxidant such as ergothioneine can effectively inhibit this cascade of events, therefore preventing damage or loss of T cells, as well as deterring the proliferation of HIV throughout the host[6, 31]. In a 2006 experiment examining β-galactosidase activity in pHIVlacZ transfected cells, Xiao et al proved that the NF-κB transcription factor was inhibited by ergothioneine at μM concentrations[33].

**Cultural Herbal Remedies**

In Black populations, *Hypoxis hemerocallidea* and *Sutherlandia frutescens* have been popular in treating HIV and concomitant symptoms for roughly a decade. The use and research of the herbal compounds has been promoted by the South African Ministry of Health and herbal compounds continue to be used for HIV treatment[34].

In Latino populations, a rich variety of herbal remedies are utilized for a vast range of diseases and ailments. *Opuntia ficus*-indica (nopal, prickly pear cactus) and

*Uncaria tomentosa* (uña de gato, cat's claw) are two of the most commonly used herbal compounds purported to have antiviral properties and used to treat HIV[35].

### *Hypoxis hemerocallidea*

*Hypoxis hemerocallidea*, which has the common name "African potato," is widely used in South African and other areas of the world. It is an antioxidant and is theorized to play a role in the immune system[9, 34]. Traditional Zulu healers advise use of *Hypoxis hemerocallidea* for urinary infections, heart weakness, tumors, benign prostatic hyperplasia, cancer, hyperglycemia, and many more ailments in addition to HIV. *Hypoxis hemerocallidea* is converted to the active form, *rooperol*, by β-glucosidase during digestion[9, 37, 38]. *Rooperol* is an aglycone that is alleged to be a strong antioxidant[9, 37].

### *Sutherlandia frutescens*

*Sutherlandia frutescens* has many common names, including "cancer bush," and is used to treat numerous diseases ranging from the common flu and chronic fatigue to cancer and HIV[9]. It is widely used in South Africa, Lesotho, Namibia, Botswana, and around the world. Experimentally, *Sutherlandia frutescens* has been shown to have antiviral, anti-inflammatory, antiviral, antibacterial, and anticancer properties. The active compounds are theorized to be, L-arginine, pinitol, GABA, triterpenes, and flavonol, among others. In addition to HIV, *Sutherlandia frutescens* is used for infection prevention, stomach issues, cancer, and more[39].

### *Opuntia ficus-indica*

*Opuntia ficus-indica* is used in Mexico and the United States for HIV, hypoglycemia, stomach ulcers, diabetes, burns, asthma, indigestion, and other ailments. Ascorbic acid, polyphenolic compounds, and flavonoids are believed to be some of the most biologically active compounds in the herb. *Opuntia ficus-indica* and other closely related cacti have exhibited anti-inflammatory, neuroprotective, and antioxidative properties experimentally[40].

### *Uncaria tomentosa*

*Uncaria tomentosa* is primarily utilized in South and Central America. It is used to treat HIV, as well as inflammatory disorders, viral infections, arthritis, gastrointestinal illness, as a form of birth control, and to increase white blood cell counts. It has been experimentally proven to increase blood concentration of protease inhibitors. The active compounds are believed to be oxindole alkaloids, quinovic acid glycosides, tannins, catechins, procyanidins, plant sterols, and carboxyl alkyl ester

# CHAPTER III

# EXPERIMENTAL PREPARATION

## Cell Expansion and Experimental Materials

CEM-GFP cells were cultured in accordance with the protocol from the NIH AIDS Reagent Program based on the work of Gervaix et al in 1997[42]. The cells were removed from storage in liquid nitrogen and warmed to physiological temperature in a 37°C water bath for 20 minutes. The reagents (RPMI 40 medium, fetal bovine serum, 200 mM L-glutamine, and penicillin-streptomycin) were also be warmed in a 37°C water bath for 20 minutes. The cell suspension was prepared in a T-25 tissue culture flask by adding 88% RPMI 1640 medium, 10% fetal bovine serum, 1% 200 mM L-glutamine, and 1% penicillin-streptomycin. The CEM-GFP cells were added to achieve a 1:10 mixture. Cell media was replaced every 2-4 days for maintenance and growth as recommended in the aforementioned protocols. The expansions and passaging were performed in a sterile hood and under sterilized conditions.

The cells were passaged and counted every three to four days to maintain healthy and viable cells for experimental use. In passaging, cells were centrifuged and supernatant was removed.

Powder ergothioneine, CEM-GFP cells, and HIV-$1_{LAI}$/PBMC were in the possession of the Taylor Research Group at the Joint School of Nanoscience and

Nanoengineering. The CEM-GFP cells and HIV-$1_{LAI}$ were acquired from the NIH AIDS Reagent Program, along with the applicable protocols. The reagents required for growth of the CEM-GFP cells in suspension include RPMI 40 medium, fetal bovine serum, 200 mM L-glutamine, and penicillin-streptomycin. These reagents were also in possession of the Taylor Research Group. *Hypoxis* (African Potato) and *Sutherlandia* (Cancer Bush) were purchased from Lyndsay Wilson of MamaJackal, an online store based in South African that sells common African herbs used in teas. Fresh prickly pear cactus pads and Baida Cat's Claw tea were purchased from SuperGMart, an international food store located in Greensboro. β-glucosidase was purchased from MP Biomedicals.

**Cell Viability**

The viability of cells was measured using the Nexelom Cellometer Vision. During the passaging protocol, after cells were pelleted in the centrifuge, cells were resuspended in 1 mL of growth media. 20 μL of cells suspended in media were combined with 20 μL of Trypan blue, a stain which colors dead cells, can only entered cells with compromised membranes. The stained cells were loaded into a reading plate, which was inserted into the Cellometer Vision. A readout was produced giving the total number of cells, the number of live cells, the number of dead cells, the concentration per milliliter of total cells, live cells, and dead cells, and a percentage labeled "viability" representing the fraction of live cells to total cells. This result is based on the number of Trypan blue stained cells in the brightfield image.

**Preparation of Dilutions**

Serial dilutions of ergothioneine, *Hypoxis hemerocallidea, Sutherlandia frutescens,* β-Glucosidase*, Opuntia ficus-indica,* and *Uncaria tomentosa* were prepared prior to beginning experiments. The dilutions were prepared to reach an ideal concentration when combined with 200 μL of media already in each well of a 96-well plate.

**Ergothioneine Dilutions**

Concentrations for ergothioneine dilutions of 6 mM, 5 mM, 4 mM, 3 mM, 2.5 mM, 2.0 mM, 1.5 mM, 1.0 mM, 0.5 mM, 0.1 mM and 0.05 mM were derived from the experimental concentrations of ergothioneine used by Xiao et al[33]. A $6.0x10^{-1}$ M stock solution of ergothioneine was prepared by dissolving 137.58 mg of powder ergothioneine in 800 μL of autoclaved water and adding 200 μL of autoclaved water to bring the solution to 1 mL. The 1 mL solution was filtered with a single use needle, a syringe, and a 0.22 μm syringe-filter. When 2 μL of this solution was combined with 200 μL of media in each well of a 96-well plate, this yielded a 6.0 mM concentration treatment of ergothioneine. The stock solution was used for a serial dilution to produce the other desired concentrations. 840 μL of $6.0x10^{-1}$ M stock solution was combined with 160 μL of autoclaved water to produce a $5.0x10^{-1}$ M solution of ergothioneine which produced 1 mL of a 5.0 mM solutions of ergothioneine when 2 μL of the solution was added to a well containing 200 μL media. 800 μL of the $5.0x10^{-1}$ M ergothioneine solution was combined with 200 μL of autoclaved water to produce 1 mL of a $4.0x10^{-1}$ M

ergothioneine solution that yielded a 4.0 mM treatment when 2 μL of the solution was added to well containing 200 μL of media. 750 μL of the $4.0x10^{-1}$ M ergothioneine solution was combined with 250 μL of autoclaved water to produce 1 mL of a $3.0x10^{-1}$ M ergothioneine solution that yielded a 3.0 mM treatment when 2 μL of the solution was added to well containing 200 μL of media. 840 μL of the $3.0x10^{-1}$ M ergothioneine solution was combined with 160 μL of autoclaved water to produce 1 mL of a $2.5x10^{-1}$ M ergothioneine solution that yielded a 2.5 mM treatment when 2 μL of the solution was added to well containing 200 μL of media. 800 μL of the $3.0x10^{-1}$ M ergothioneine solution was combined with 200 μL of autoclaved water to produce 1 mL of a $2.0x10^{-1}$ M ergothioneine solution that yielded a 2.0 mM treatment when 2 μL of the solution was added to well containing 200 μL of media. 750 μL of the $2.0x10^{-1}$ M ergothioneine solution was combined with 250 μL of autoclaved water to produce 1 mL of a $1.5x10^{-1}$ M ergothioneine solution that yielded a 1.5 mM treatment when 2 μL of the solution was added to well containing 200 μL of media. 667 μL of the $1.5x10^{-1}$ M ergothioneine solution was combined with 333 μL of autoclaved water to produce 1 mL of a $1.0x10^{-1}$ M ergothioneine solution that yielded a 1.0 mM treatment when 2 μL of the solution was added to well containing 200 μL of media. 500 μL of the $1.0x10^{-1}$ M ergothioneine solution was combined with 500 μL of autoclaved water to produce 1 mL of a $0.5x10^{-1}$ M ergothioneine solution that yielded a 0.5 mM treatment when 2 μL of the solution was added to well containing 200 μL of media. 200 μL of the $0.5x10^{-1}$ M ergothioneine solution was combined with 800 μL of autoclaved water to produce 1 mL of a $0.1x10^{-1}$ M

ergothioneine solution that yielded a 0.1 mM treatment when 2 µL of the solution was added to well containing 200 µL of media. 500 µL of the $0.1x10^{-1}$ M ergothioneine solution was combined with 500 µL of autoclaved water to produce 1 mL of a $0.05x10^{-1}$ M ergothioneine solution that yielded a 0.05 mM treatment when 2 µL of the solution was added to well containing 200 µL of media.

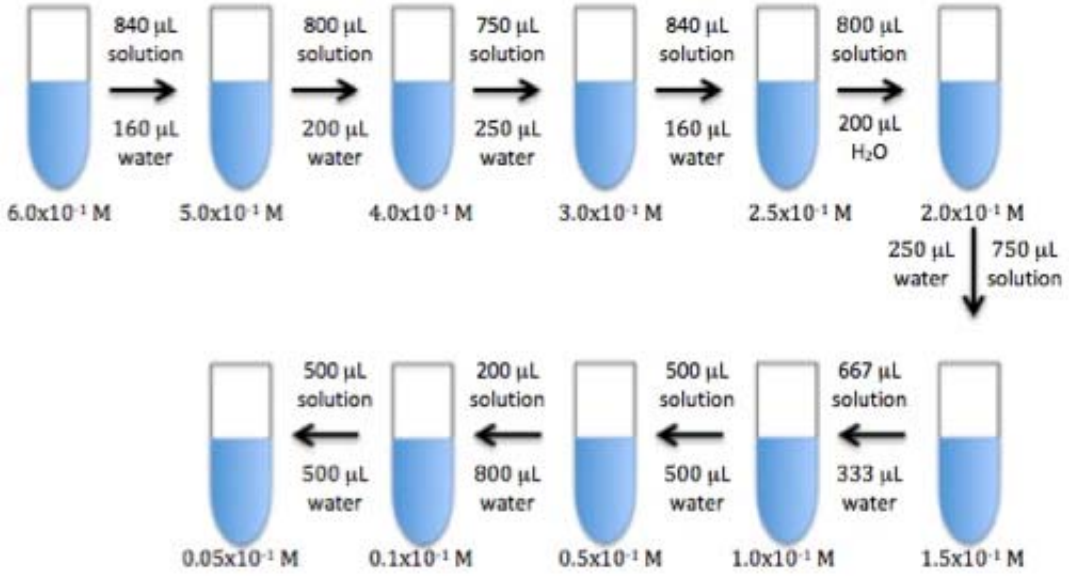



**Figure 1. Preparation of Serial Dilutions of Ergothioneine**

### *Hypoxis hemerocallidea, Sutherlandia frutescens,* and β-Glucosidase Dilutions

Dilution concentrations for *Hypoxis hemerocallidea* and *Sutherlandia frutescens* were based around the values for experimental concentrations of teas utilized by Si *et al*[43]. A stock solution of the same concentration of *Hypoxis hemerocallidea* and *Sutherlandia frutescens* was prepared by measuring 6.2510 g of dried *Hypoxis hemerocallidea* and 6.2521 g of dried *Sutherlandia frutescens* (approximately 6250 mg)

and boiling each in 50 mL of autoclaved water in a 150 mL beaker for 20 minutes. After cooling for 10 minutes, a hand sieve was used to strain the boiled herb and collect the tea. More autoclaved water was added to bring the final volume of the tea to 100 mL. This gave a stock solution with a concentration of 62.5 mg/mL. 1 mL of the stock solution was collected and filtered. 800 μL was taken to give a concentration of 50 mg/800 μL, which became 50 mg/mL when 2 μL of the solution was added to a well containing 200 μL of media. Another 1 mL of the stock solution was filtered and 800 μL was taken. The 800 μL with concentration 50 mg/mL was combined with 200 μL of autoclaved water to produce 1 mL of a 40 mg/mL solution that yielded a 40g/mL treatment when 2 μL of the solution was added to a well containing 200 μL of media. 750 μL of the 40 mg/800 μL solution was combined with 250 μL of autoclaved water to produce 1 mL of a 30 mg/800 μL solution that yielded a 30 mg/mL treatment when 2 μL of the solution was added to a well containing 200 μL of media. 667 μL of the 30 mg/800 μL solution was combined with 333 μL of autoclaved water to produce 1 mL of a 20 mg/800 μL solution that yielded a 20 mg/mL treatment when 2 μL of the solution was added to a well containing 200 μL of media. 500 μL of the 20 mg/800 μL solution was combined with 500 μL of autoclaved water to produce 1 mL of a 10 mg/800 μL solution that yielded a 10 mg/mL treatment when 2 μL of the solution was added to a well containing 200 μL of media. 500 μL of the 10 mg/800 μL solution was combined with 500 μL of autoclaved water to produce 1 mL of a 5 mg/800 μL solution that yielded a 5 mg/mL treatment when 2 μL of the solution was added to a well containing 200 μL of media. 200 μL of the 5

mg/800 μL solution was combined with 800 μL of autoclaved water to produce 1 mL of a 1 mg/800 μL solution that yielded a 1 mg/mL treatment when 2 μL of the solution was added to a well containing 200 μL of media. In order to convert *Hypoxis hemerocallidea* to the active rooperol form, equal dilutions of β-Glucosidase were prepared in the aforementioned manner and added along with the corresponding concentration of *Hypoxis hemerocallidea*. This method of action was influenced by the precedent of adding equal concentrations of *Hypoxis hemerocallidea* and β-Glucosidase utilized by Boukes in his 2010 experiments[44].

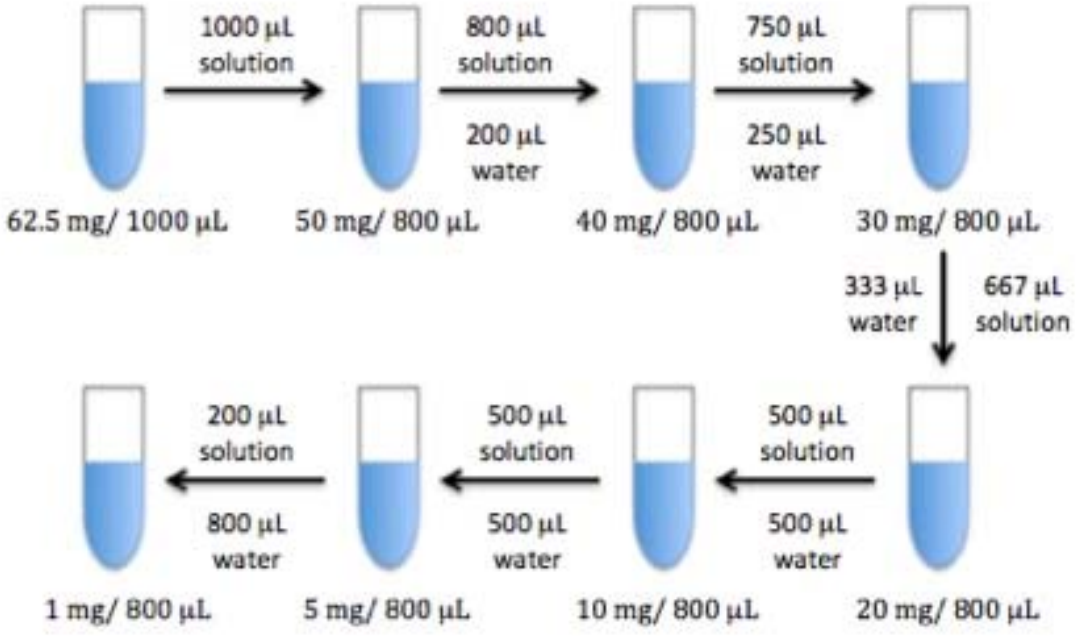



**Figure 2. Preparation of Serial Dilutions of *Hypoxis hemerocallidea, Sutherlandia frutescens* and β-Glucosidase**

### *Opuntia ficus-indica* Dilutions

Three pads of *Opuntia ficus-indica* were purchased and weighed to determine the general mass of a prickly pear cactus pad. The three pads weighed 73.5824 g, 89.0023 g, and 107.0625 g. From these weights, the desired dilution concentrations of 100 g/250 mL, 75 g/ 250 mL, 50 g/250 mL, 25 g/250 mL, 10 g/250 mL, 5 g/250 mL and 1 g/250 mL were developed. 250 mL was used as the measurement of 1 metric cup, as there is variation between a "customary cup" and a "legal cup" in the United States[45]. A stock solution of prickly pear cactus was prepared by placing 40.0194 g of a pad stripped of needles in a blender with 100 mL of autoclaved water for approximately 5 minutes on varied settings until all visible particles were entirely obliterated. A hand sieve was used to strain large particles out of the juice, meant to mimic a "licuado,[46]" and 100 mL of juice was used. Due to the relatively large particles of prickly pear cactus in the sieved solution, this solution was not filtered to be sterile to prevent extricating biologically active compounds. 100 mL of juice containing approximately 40 g of prickly pear cactus is equivalent to a 100 g/249.8 mL solution. When 2 μL of this solution was added to a well containing 200 μL of media, a treatment of 100 g/250 mL, or 100 g of prickly pear cactus per cup of water, was produced. 750 μL of the 40 g/100 mL stock solution was combined with 250 μL of autoclaved water to p•roduce 1 mL of a 30 g/ 100 mL solution. This was the equivalent of a concentration of 75 g/249.8 mL solution that yielded a 75 g/ 250 mL treatment when 2 μL of the solution was added to a well containing 200 μL of media. 667 μL of the 30 g/100 mL solution was combined with 333 μL of autoclaved water to produce 1 mL of a 20 g/ 100 mL solution. This was the equivalent of a

concentration of 50 g/249.8 mL solution that yielded a 50 g/ 250 mL treatment when 2 μL of the solution was added to a well containing 200 μL of media. 500 μL of the 20 g/100 mL solution was combined with 500 μL of autoclaved water to produce 1 mL of a 10 g/ 100 mL solution. This was the equivalent of a concentration of 25 g/249.8 mL solution that yielded a 25 g/ 250 mL treatment when 2 μL of the solution was added to a well containing 200 μL of media. 400 μL of the 10 g/100 mL solution was combined with 600 μL of autoclaved water to produce 1 mL of a 4 g/ 100 mL solution. This was the equivalent of a concentration of 10 g/249.8 mL solution that yielded a 10 g/ 250 mL treatment when 2 μL of the solution was added to a well containing 200 μL of media. 500 μL of the 4 g/100 mL solution was combined with 500 μL of autoclaved water to produce 1 mL of a 2 g/ 100 mL solution. This was the equivalent of a concentration of 5 g/249.8 mL solution that yielded a 5 g/ 250 mL treatment when 2 μL of the solution was added to a well containing 200 μL of media. 200 μL of the 2 g/100 mL solution was combined with 800 μL of autoclaved water to produce 1 mL of a 0.4 g/ 100 mL solution. This was the equivalent of a concentration of 1 g/249.8 mL solution that yielded a 1 g/ 250 mL treatment when 2 μL of the solution was added to a well containing 200 μL of media.

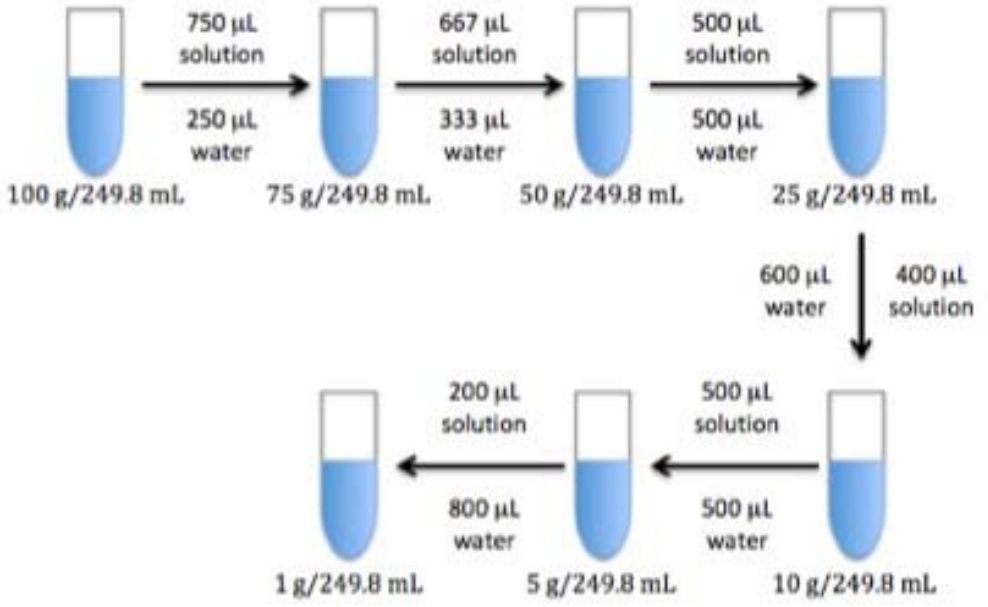



**Figure 3. Preparation of Serial Dilutions of *Opuntia ficus-indica.***

### *Uncaria tomentosa* Dilutions

*Uncaria tomentosa* was sold as a Baida tea product labeled "Cat's Claw" and packaged in individual tea bags. This packaging quantity was used to develop the desired concentrations of 4 bags/250 mL, 3 bags/250 mL, 2 bags/250 mL, 1 bag/250 mL, ½ bag/ 250 mL, ⅓ bag/250 mL, and ¼ bag/250 mL. To prepare a stock solution of 4 bags/249.8 mL, a 150 mL beaker was filled with 50 mL of autoclaved water. One bag of cat's claw was placed in the water and boiled for 20 minutes. After cooling for 10 minutes, the bag was strained of liquid with a spatula and the final volume of the tea was brought up to 62.3 mL. This yielded a concentration of 1 bag/ 62.5 mL, or 1 bag/250 mL, when 2 µL of the solution was added to a well containing 200 µL of media. 1 mL of

the stock solution was filtered and used for serial dilutions. 749 µL of the 1 bag/62.3 mL stock solution was combined with 251 µL of autoclaved water to produce 1 mL of a 1 bag/83.13 mL solution. This was the equivalent of a concentration of 3 bags/249.8 mL solution that yielded a 3 bags/250 mL solution when 2 µL was added to a well containing 200 µL of media. 666 µL of the 1 bag/83.13 mL stock solution was combined with 334 µL of autoclaved water to produce 1 mL of a 1 bag/124.8 mL solution. This was the equivalent of a concentration of 2 bags/249.8 mL solution that yielded a 2 bags/250 mL solution when 2 µL was added to a well containing 200 µL of media. 500 µL of the 1 bag/124.8 mL stock solution was combined with 500 µL of autoclaved water to produce 1 mL of a 1 bag/249.8 mL solution. This was the equivalent of a concentration of 1 bag/249.8 mL solution that yielded a 1 bag/250 mL solution when 2 µL was added to a well containing 200 µL of media. 500 µL of the 1 bag/249.8 mL stock solution was combined with 500 µL of autoclaved water to produce 1 mL of a 1 bag/499.8 mL solution. This was the equivalent of a concentration of ½ bags/249.8 mL solution that yielded a ½ bags/250 mL solution when 2 µL was added to a well containing 200 µL of media. 667 µL of the 1 bag/249.8 mL stock solution was combined with 333 µL of autoclaved water to produce 1 mL of a 1 bag/749.8 mL solution. This was the equivalent of a concentration of $^{1}/_{3}$ bags/249.8 mL solution that yielded a $^{1}/_{3}$ bags/250 mL solution when 2 µL was added to a well containing 200 µL of media. 750 µL of the 1 bag/749.8 mL stock solution was combined with 250 µL of autoclaved water to produce 1 mL of a 1

bag/999.8 mL solution. This was the equivalent of a concentration of ¼ bags/249.8 mL solution that yielded a ¼ bags/250 mL solution when 2 µL was added to a well containing 200 µL of media.

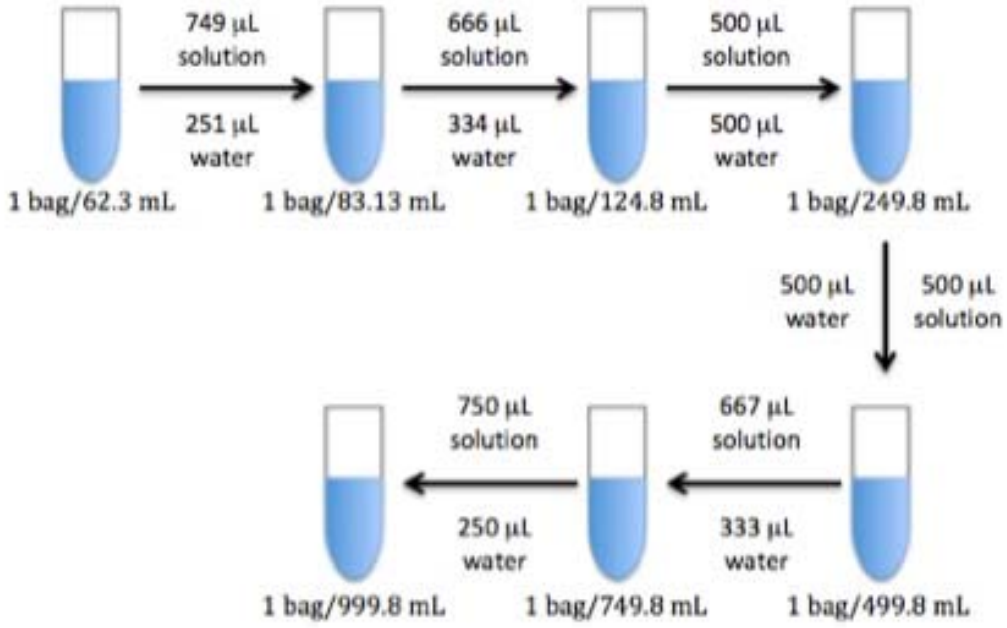



**Figure 4. Preparation of Serial Dilutions of *Uncaria tomentosa***

After all dilutions were prepared, they were stored in the lab in a refrigerator at approximately 4°C.

## CHAPTER IV

## ASSAYS TO DETERMINE CYTOTOXICITY OF TREATMENTS AND TO ELECT APPROPRIATE CONCENTRATIONS FOR EXPERIMENTAL USE

In order to ensure the herbal treatments didn't have significant effects on the viability of the CEM-GFP cells, cytotoxicity assays were performed with various concentrations of each treatment. The objective of these assays was to examine various concentrations of each treatment and select the concentration that had little effect on the GFP expression in comparison to the control trials.

### Materials

96-well plates were seeded with CEM-GFP cells at approximately 30,000 cells per well in 200 μL of media. After approximately 24 hours, the cells were treated with varying concentrations of chemical or herbal dilutions. 2 μL of the dilution was placed in each well. 24 hours after adding treatments, the plates were read by the BioTek Synergy Mx for three to four days. For the preparation of each plate, the following calculations were used.

***30,000 cells x 96 wells = 2,880,000 cells total***

***200 μL media x 96 wells = 19,200 μL media total***

***19,200 μL → 19.2 mL***

From these calculations, the ideal concentration of $2.88x10^6$ cells in 22 mL of media was derived for seeding plates.

**Methods**

To evaluate appropriate concentrations of each treatment, cytotoxicity assays were performed with 96-well plates, of which the BioTek readouts were analyzed.

**Ergothioneine Cytotoxicity Assay**

For the ergothioneine cytotoxicity assay, a culture of CEM-GFP cells with a viability of 99.4% was used. The Cellometer determined that the concentration of live cells was $1.75x10^7$ cells/mL. In order to seed the 96-well plate with 30,000 cells in 200 μL of media per well, the following calculations were performed.

$$c_1v_1 = c_2v_2$$

$$(1.75x10^7 \text{ cells/mL})(v_1) = (2.88x10^6 \text{ cells}/22 \text{ mL})(22 \text{ mL})$$

$$v_1 = 0.16457143 \text{ mL} \rightarrow 165 \ \mu\text{L of cells}$$

165 μL of cells was taken from the suspension and added to 22 mL of media and mixed thoroughly via inversion. A 96-well plate was seeded with 200 μL of media in each well. Each of the 12 columns of the well plate contained a different concentration of ergothioneine. The first column served as a control with no treatment added while the remainder of the columns were treated with an increasing concentration of the ergothioneine dilution.

**Table 1. 96-Well Plate Experimental Setup for the Ergothioneine Cytotoxicity Assay**

| | Control | 0.05 mM | 0.1 mM | 0.5 mM | 1.0 mM | 1.5 mM | 2.0 mM | 2.5 mM | 3.0 mM | 4.0 mM | 5.0 mM | 6.0 mM |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Trial 1 | | | | | | | | | | | | |
| Trial 2 | | | | | | | | | | | | |
| Trial 3 | | | | | | | | | | | | |
| Trial 4 | | | | | | | | | | | | |
| Trial 5 | | | | | | | | | | | | |
| Trial 6 | | | | | | | | | | | | |
| Trial 7 | | | | | | | | | | | | |
| Trial 8 | | | | | | | | | | | | |

***Hypoxis hemerocallidea* (with β-Glucosidase), *Opuntia ficus-indica, and Uncaria tomentosa* Cytotoxicity Assays**

For the *Hypoxis hemerocallidea* with β-Glucosidase, *Opuntia ficus-indica,* and *Uncaria tomentosa* cytotoxicity assays, a culture of CEM-GFP cells with a viability of 98.2% was used for all three assays. The Cellometer determined that the concentration of live cells was $3.57 \times 10^7$ cells/mL. In order to seed the three 96-well plates with 30,000 cells in 200 μL of media per well, the following calculations were performed.

***30,000 cells x 96 wells x 3 plates = 8,640,000 cells total***

***200 μL media x 96 wells x 3 plates = 57,600 μL media total***

***57,600 μL → 57.6 mL***

From these calculations, the ideal concentration of $8.64 \times 10^6$ cells in 60 mL of media was derived for seeding plates.

$$c_1v_1 = c_2v_2$$

$$(3.57x10^7 \text{ cells/mL})(v_1) = (8.64x10^6 \text{ cells/60 mL})(60 \text{ mL})$$

$$v_1 = 0.2420168067 \text{ mL} \rightarrow 242\ \mu\text{L of cells}$$

242 μL of cells was taken from the suspension and added to 60 mL of media and mixed thoroughly via inversion. Three 96-well plates were seeded with 200 μL of media in each well. Each of the 8 rows of each well plate contained a different concentration of *Hypoxis hemerocallidea (*with β-Glucosidase), *Opuntia ficus-indica, and Uncaria tomentosa*. The first row of each plate served as a control with no treatment added while the remainder of the rows were treated with an increasing concentration of the each dilution.

**Table 2. 96-Well Plate Experimental Setup for the *Hypoxis hemerocallidea* with β-Glucosidase Cytotoxicity Assay**

| | Trial 1 | Trial 2 | Trial 3 | Trial 4 | Trial 5 | Trial 6 | Trial 7 | Trial 8 | Trial 9 | Trial 10 | Trial 11 | Trial 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | | | | | | | | | | | | |
| 1 mg/mL | | | | | | | | | | | | |
| 5 mg/mL | | | | | | | | | | | | |
| 10 mg/mL | | | | | | | | | | | | |
| 20 mg/mL | | | | | | | | | | | | |
| 30 mg/mL | | | | | | | | | | | | |
| 40 mg/mL | | | | | | | | | | | | |
| 50 mg/mL | | | | | | | | | | | | |

**Table 3. 96-Well Plate Experimental Setup for the *Opuntia ficus-indica* Cytotoxicity Assay**

| | Trial 1 | Trial 2 | Trial 3 | Trial 4 | Trial 5 | Trial 6 | Trial 7 | Trial 8 | Trial 9 | Trial 10 | Trial 11 | Trial 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | | | | | | | | | | | | |
| 1 g/ 250 mL | | | | | | | | | | | | |
| 5 g/ 250 mL | | | | | | | | | | | | |
| 10 g/ 250 mL | | | | | | | | | | | | |
| 25 g/ 250 mL | | | | | | | | | | | | |
| 50 g/ 250 mL | | | | | | | | | | | | |
| 75 g/ 250 mL | | | | | | | | | | | | |
| 100 g/ 250 mL | | | | | | | | | | | | |

**Table 4. 96-Well Plate Experimental Setup for the *Uncaria tomentosa* Cytotoxicity Assay**

| | Trial 1 | Trial 2 | Trial 3 | Trial 4 | Trial 5 | Trial 6 | Trial 7 | Trial 8 | Trial 9 | Trial 10 | Trial 11 | Trial 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | | | | | | | | | | | | |
| ¼ bag/ 250 mL | | | | | | | | | | | | |
| ⅓ bag/ 250 mL | | | | | | | | | | | | |
| ½ bag/ 250 mL | | | | | | | | | | | | |
| 1 bag/ 250 mL | | | | | | | | | | | | |
| 2 bags/ 250 mL | | | | | | | | | | | | |
| 3 bags/ 250 mL | | | | | | | | | | | | |
| 4 bags/ 250 mL | | | | | | | | | | | | |

### *Sutherlandia frutescens* Cytotoxicity Assay

For the *Sutherlandia frutescens* cytotoxicity assay, a culture of CEM-GFP cells with a viability of 80.4% was used. The Cellometer determined that the concentration of live cells was $2.38 \times 10^6$ cells/mL. In order to seed the 96-well plate with 30,000 cells in 200 μL of media per well, the following calculations were performed.

$$c_1v_1 = c_2v_2$$

$$(2.38 \times 10^6 \text{ cells/mL})(v_1) = (2.88 \times 10^6 \text{ cells}/22 \text{ mL})(22 \text{ mL})$$

$$v_1 = 1.210084034 \text{ mL} \rightarrow 1210 \ \mu\text{L of cells}$$

Since 1 mL of cells at the aforementioned concentration was available, it was not possible to seed the 96-well plate with 30,000 cells per well. Alternatively, calculations were performed to seed the 96-well plate with 25,000 cells in 200 μL of media.

$$25{,}000 \text{ cells} \times 96 \text{ wells} = 2{,}375{,}000 \text{ cells total}$$

$$200 \ \mu\text{L media} \times 96 \text{ wells} = 19{,}200 \ \mu\text{L media total}$$

$$19{,}200 \ \mu\text{L} \rightarrow 19.2 \text{ mL}$$

From these calculations, the ideal concentration of $2.375 \times 10^6$ cells in 60 mL of media was derived for seeding plates.

$$c_1v_1 = c_2v_2$$

$$(2.38 \times 10^6 \text{ cells/mL})(v_1) = (2.375 \times 10^6 \text{ cells}/22 \text{ mL})(22 \text{ mL})$$

$$v_1 = 0.9978991597 \text{ mL} \rightarrow 998 \ \mu\text{L of cells}$$

998 μL of cells was taken from the suspension and added to 22 mL of media and mixed thoroughly via inversion. A 96-well plate was seeded with 200 μL of media in each well. Each of the 8 rows of the well plate contained a different concentration of *Sutherlandia frutescens*. The first row served as a control with no treatment added while the remainder of the rows were treated with an increasing concentration of the *Sutherlandia frutescens* dilution.

**Table 5. 96-Well Plate Experimental Setup for the *Sutherlandia frutescens* Cytotoxicity Assay**

| | Trial 1 | Trial 2 | Trial 3 | Trial 4 | Trial 5 | Trial 6 | Trial 7 | Trial 8 | Trial 9 | Trial 10 | Trial 11 | Trial 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | | | | | | | | | | | | |
| 1 mg/mL | | | | | | | | | | | | |
| 5 mg/mL | | | | | | | | | | | | |
| 10 mg/mL | | | | | | | | | | | | |
| 20 mg/mL | | | | | | | | | | | | |
| 30 mg/mL | | | | | | | | | | | | |
| 40 mg/mL | | | | | | | | | | | | |
| 50 mg/mL | | | | | | | | | | | | |

## Results

Based on the readouts generated by the BioTek Synergy Mx, the GFP expression values for each dilution were averaged for each day. The averages of each dilution were used to assess the trends in viability over the reading window. BioTek readouts for the results of the ergothioneine (EGT) cytotoxicity assay for days 1-4 are provided in Appendix A in tables 12-14. The readouts for the *Hypoxis hemerocallidea* (AP),

*Sutherlandia frutescens* (CB), *Opuntia ficus-indica* (PPC), and *Uncaria tomentosa* (CC) cytotoxicity assays are also provided in Appendix A, with tables 15-17 representing days 1-3 for *Hypoxis hemerocallidea*, tables 18-21 representing days 1-4 for *Sutherlandia frutescens*, tables 22-24 representing days 1-3 for *Opuntia ficus-indica*, and tables 25-27 representing days 1-3 for *Uncaria tomentosa*. Each readout depicts numbers in the wells of the 96-well plate that signify the GFP expression in relative fluorescence units (RFUs). A drastic decrease in GFP expression in comparison to the values for the control trials was regarded as cell death, meaning the concentration decreased the viability of the cells in that well. The graphs for the analysis of the BioTek readouts are provided in Appendix B with Figures 10, 12, and 14 representing the daily readings for ergothioneine and Figures 11, 13, and 15 representing the averages of daily trials for ergothioneine. Figure 16 represents the expression trends for the ergothioneine cytoxicity assay. Additionally, Figures 17, 19, and 21 represent the daily readings for *Hypoxis hemerocallidea* with Figures 18, 20, and 22 representing the averages of daily trials. Figure 23 represents the expression trends for the *Hypoxis hemerocallidea* cytotoxicity assay. Figures 24, 26, 28, and 30 represent the daily readings for *Sutherlandia frutescens* with Figures 25, 27, 29, and 31 representing the averages of daily trials. Figure 32 represents the expression trends for the *Sutherlandia frutescens* cytotoxicity assay. Figures 33, 35, and 37 represent the daily readings for *Opuntia ficus-indica* with Figures 34, 36, and 38 representing the averages of daily trials. Figure 39 represents the expression trends for the *Opuntia ficus-indica* cytotoxicity assay. Figures 40, 42, and 44 represent the daily readings for *Uncaria tomentosa* with Figures 41, 43, and 45

representing the averages of daily trials. Figure 46 represents the expression trends for the *Uncaria tomentosa* cytotoxicity assay.

After examination of the trends in viability and the difference between the average GFP expression of treated cells and the control GFP expression, dilutions of each treatment were selected for further experimentation. Selection criteria involved dilutions that yielded an average GFP expression readout close to the control trials over the reading period, as well as the highest concentration of treatment that could be given without significantly lowering GFP expression, which was interpreted as cell death. Based on these criteria, the concentrations of each dilution were selected to be 0.1 mmol for ergothioneine, 5 mg/mL for *Hypoxis hemerocallidea*, 1 mg/mL for *Sutherlandia frutescens*, 10 g/250 mL for *Opuntia ficus-indica*, and ½ bag/250 mL for *Uncaria tomentosa*.

# CHAPTER V

# MTT ASSAY TO VERIFY CELL VIABILITY WITH SELECTED HERBAL CONCENTRATIONS

While the cytotoxicity assays assisted in electing appropriate concentrations of treatments, the assays were only designed to give GFP expression. A change in GFP expression could be attributed to various factors; however the use of an MTT assay could verify that changes in GFP expression were not affected by cell viability. The purpose of an MTT assay is to ascertain cell viability through a colorimetric experiment, which functions by exposing the cells to tetrazolium dye and monitoring for color changes linked to the formation of formazan crystals[47]. The reaction is facilitated by mitochondrial dehydrogenase enzymes and monitored by the change of color in the wells, which is more significant when there are a greater number of viable cells[47].

## Materials

The cytotoxicity assay was performed with a Vybrant MTT Cell Proliferation Assay Kit from Life Technologies . The kit contained 10 vials of Component A, each of which contained 5 mg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, MW = 414) and 10 vials of Component B, each of which contained 1 g of sodium dodecyl sulfate (SDS, MW = 288). The materials were stored at 4ºC in a dry, dark area.

Required materials not included in the kit included sterile phosphate-buffered saline (PBS), sterile 0.01 M hydrogen chloride (HCl), and RPMI 1640 without phenol red, all of which were in possession of the Taylor Research Group[48].

**Methods**

The experimental protocol for the MTT assay found on the Life Technologies website was closely adhered to.

**Reagent and Cell Preparation**

To prepare the reagents for the experiment, 1 mL of PBS was added to 1 vial of Component A, yielding a 12 mM stock solution of MTT. The solution was mixed by vortexing. Additionally, 10 mL of 0.01 M HCl was added to 1 vial of Component B, yielding an SDS solution, which was mixed by gentle inversion.

In order to perform the assay, a 96-well plate seeded with CEM-GFP cells cultured in RPMI complete media without phenol red was needed. Based on the protocol provided by Life Technologies, the desired experimental design called for 10,000 cells in 200 μL media per well.

***10,000 cells/well x 72 wells = 720,000 cells total***

***200 μL media/well x 72 wells = 14,400 μL media total***

***14,400 μL → 14.4 mL***

CEM-GFP cells used for the MTT assay were obtained from a cell culture that was determined to contain $6.55x10^6$ live cells with a viability of 51.2%. The following calculations were used to seed a 96-well plate for the assay.

$$c_1v_1 = c_2v_2$$

$$(6.55x10^6 \text{ cells/mL})(v_1) = (7.2x10^5 \text{ cells/15 mL})(15 \text{ mL})$$

$$v_1 = 0.1099236641 \text{ mL} \rightarrow 110\ \mu L$$

110 µL of cells was taken from suspension and combined with 15 mL of phenol red free media. This suspension of cells was used to seed 72 wells of a 96-well plate with 10,000 cells per well in 200 µL media. Approximately 24 hours later, the previously determined dilutions of each treatment were added to five separate rows, with the first row serving as a control. 48 hours after the initial seeding of the plate, the MTT assay was performed.

**Table 6. 96-Well Plate Setup for MTT Assay. EGT – Ergothioneine, AP – *Hypoxis hemerocallidea* (African potato), CB – *Sutherlandia frutescens* (Cancer Bush), PPC – *Opuntia ficus-indica* (Prickly Pear Cactus), CC – *Uncaria tomentosa* (Cat's Claw)**

| MTT Assay | Trial 1 | Trial 2 | Trial 3 | Trial 4 | Trial 5 | Trial 6 | Trial 7 | Trial 8 | Trial 9 | Trial 10 | Trial 11 | Trial 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control (CEM-GFP) | | | | | | | | | | | | |
| EGT (0.1 mmol) | | | | | | | | | | | | |
| AP (5 mg/mL) | | | | | | | | | | | | |
| CB (1 mg/mL) | | | | | | | | | | | | |
| PPC (10 g/cup) | | | | | | | | | | | | |
| CC (1/2 bag/cup) | | | | | | | | | | | | |
| MTT Negative Control (Media only) | | | | | | | | | | | | |

**Labeling Cells**

Since CEM-GFP cells are suspension cells, the microplate was centrifuged at 1000 RPM for 5 minutes. The media was removed from each well and replaced with 100 μL of fresh RPMI media. An additional row of 100 μL of media alone was added to the plate to serve as a negative control. 10 μL of the 12 mM MTT stock solution was added to each well and the plate was incubated at 37ºC for 4 hours. After the incubation, 100 μL of the SDS-HCl solution was added to each well and mixed in thoroughly with the pipette. The plate was incubated for another 4 hours at 37ºC and after removal, each well was again mixed thoroughly with a pipette. The plate was read in the BioTek Synergy Mx at an absorbance of 570 nm.

**Results**

The readout from the BioTek Synergy Mx was acquired and analyzed in order to determine whether the selected concentrations were appropriate for further experimentation.

**Table 7. BioTek Absorbance Readout for MTT Assay in Absorbance Units (AUs). Highlighted Wells Represent Trials that were Considered Outliers and Not Used for Calculation of Mean, Standard Deviation, and Standard Error.**

| MTT Assay | Trial 1 | Trial 2 | Trial 3 | Trial 4 | Trial 5 | Trial 6 | Trial 7 | Trial 8 | Trial 9 | Trial 10 | Trial 11 | Trial 12 | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control (CEM-GFP) | 0.13 | 0.113 | 0.361 | 0.129 | 0.152 | 0.267 | 0.133 | 0.187 | 0.124 | 0.233 | 0.245 | 0.09 | 0.188545455 |
| EGT (0.1 mmol) | 0.07 | 0.714 | 1.057 | 0.131 | 0.302 | 0.59 | 0.457 | 0.599 | 0.112 | 0.085 | 0.099 | 0.323 | 0.406272727 |
| AP (5 mg/mL) | 0.138 | 1.048 | 0.866 | 1.586 | 0.544 | 0.973 | 1.077 | 0.379 | 1.112 | 0.689 | 0.228 | 0.195 | 0.659 |
| CB (1 mg/mL) | 0.102 | 0.665 | 0.103 | 0.764 | 0.184 | 1.074 | 1.073 | 0.095 | 0.987 | 0.407 | 0.43 | 0.226 | 0.509166667 |
| PPC (10 g/cup) | 0.41 | 0.224 | 0.1 | 0.117 | 0.476 | 1.019 | 1.059 | 0.958 | 0.582 | 0.082 | 0.53 | 0.082 | 0.469916667 |
| CC (1/2 bag/cup) | 0.177 | 0.14 | 0.318 | 0.58 | 1.347 | 0.449 | 0.474 | 0.298 | 1.088 | 0.105 | 0.501 | 0.337 | 0.3379 |
| MTT Negative Control (media only) | 0.446 | 0.101 | 0.791 | 0.062 | 0.783 | 0.056 | 0.058 | 0.058 | 0.091 | 0.087 | 0.141 | 0.108 | 0.1208 |

After outliers were eliminated from calculations, the absorbance of each treatment for every trial was assessed. The averages for each treatment were also assessed.

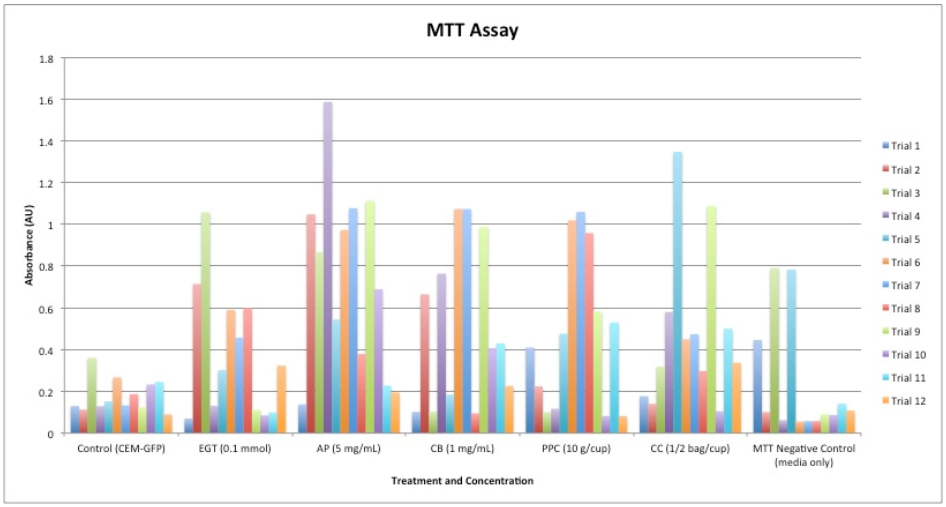



**Figure 5. Graph of BioTek Readouts for MTT Assay for Each Day of Reading**

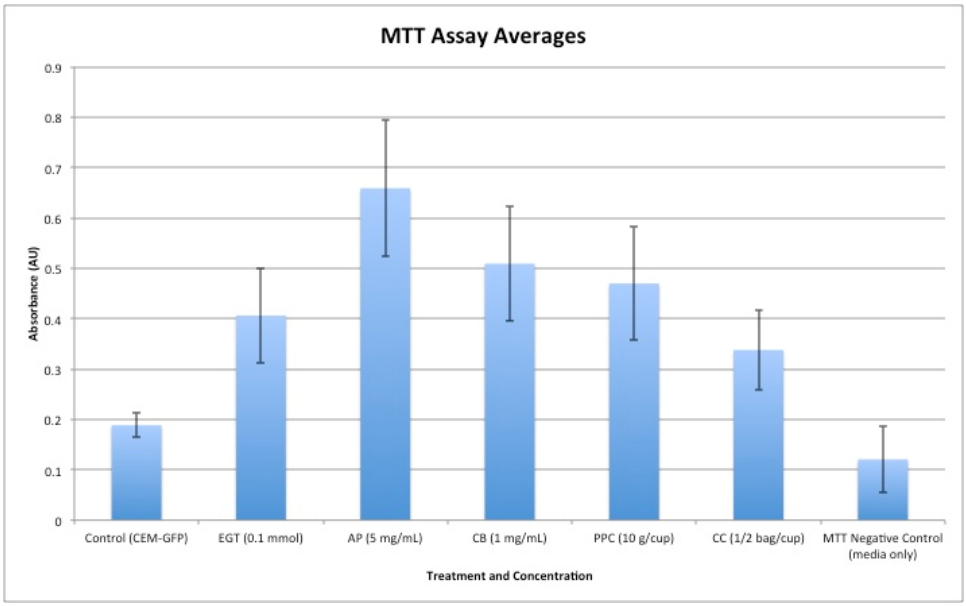



**Figure 6. Graph of BioTek Readouts for MTT Assay Averages for Each Day of Reading**

From the analysis of the BioTek readout, it was determined that the selected dilutions of treatments did not negatively affect the viability of the cells, due to the average absorbance of treated cells being higher than that of the control. In an MTT assay, the higher the value in absorbance units correlates to the ability of viable cells to metabolize the tetrazolium dye[47]. The reading, performed at 570 nm, measures absorbance for the presence of the salt product from the reaction with the dye. Since a higher absorbance correlates to cell viability and the control trials of CEM-GFP cells alone produced an average readout of approximately 0.189 absorbance units, the concentrations of dilutions used to treat the cells were deemed appropriate due to higher average readouts in absorbance. Additionally, there was an implication that some of the herbal treatments stabilized cells in a way that promoted a higher viability than untreated cells, resulting in a higher concentration of CEM-GFP cells in the experimental wells.

It is important to keep in mind that the increased viability seen in the herbal treatments of ergothioneine, *Hypoxis hemerocallidea*, *Sutherlandia frutescens*, *Opuntia ficus-indica*, and *Uncaria tomentosa*, these results are vital in the assessment of the effect of herbal treatments on HIV infected cells. The increased number of CEM-GFP cells that is achieved through unknown mechanisms of the herbal treatments could possibly affect the GFP expression readouts generated by the BioTek.

These results will be reiterated in the conclusion as a function of explaining limitations and possible discrepancies in the GFP expression readouts for the experiment investigation the action of the herbal remedies when used to treat CEM-GFP cells prior to HIV infection.

# CHAPTER VI

# EXPERIMENTAL INVESTIGATION OF EFFECT OF HERBAL TREATMENTS ON CELLS PRIOR TO HIV INFECTION

After it was confirmed that the herbal treatments were not decreasing the viability of the cells or significantly effecting the function of the GFP protein, the selected concentrations of herbal treatment were tested for effectiveness with live HIV. As the cells were treated before viral infection, the ability of the herbal treatments to prevent entry of HIV into the cell or inhibition of HIV proliferation was monitored through analysis of GFP expression.

## Materials

Powdered polybrene and HIV-$1_{LAI}$ was previously acquired from the NIH AIDS Reagent Program by the Taylor Research Group. Polybrene (hexadimethrine bromide) is a polymer that is commonly used to increase susceptibility of cells to a retroviral infection in cell culture[49].

## Methods

In order to perform the assay, 72 wells of a 96-well plate seeded with CEM-GFP cells cultured in RPMI complete media and treated with polybrene was needed.

**Plating and Treatment**

To plate a 96-well plate with 30,000 cells in 200 μL per well in 72 wells, the following calculations were performed.

$$30{,}000 \text{ cells/well} \times 72 \text{ wells} = 2{,}160{,}000 \text{ cells total}$$

$$200\ \mu\text{L media/well} \times 72 \text{ wells} = 14{,}400\ \mu\text{L media total}$$

$$14{,}400\ \mu\text{L} \rightarrow 14.4 \text{ mL}$$

Based on these calculations, it was decided that the cells would be prepared in 15 mL of media. CEM-GFP cells used for the HIV assay were obtained from a cell culture that was determined to contain $6.55 \times 10^6$ live cells with a viability of 51.2%. The following calculations were used to seed 72 wells of a 96-well plate for the assay.

$$c_1 v_1 = c_2 v_2$$

$$(6.55 \times 10^6 \text{ cells/mL})(v_1) = (2.16 \times 10^6 \text{ cells}/15 \text{ mL})(15 \text{ mL})$$

$$v_1 = 0.329770924 \text{ mL} \rightarrow 330\ \mu\text{L}$$

330 μL of cells was taken from suspension and combined with 15 mL of media. The cells were then treated with a previously prepared stock solution of polybrene with a concentration of 500 μg/mL of media. The stock solution was prepared by dissolving 100 mg of powder polybrene into 200 mL of RPMI 1640, without FBS, L-Glutamine, or penicillin-streptomycin. After preparation, the mixture was sterilized with a 0.2 μL filtration unit and aliquoted into 15 mL tubes. The stock solution was stored at 2-4°C pending use. After the 330 μL of CEM-GFP cells were taken from suspension and

combined with 15 mL of media, polybrene was added according to the predetermined ratio of 40 μL polybrene per $10x10^6$ cells currently under use by the Taylor Research Group.

$$40\ \mu L \text{ of polybrene}/\ 10x10^6 \text{ cells} = x\ \mu L \text{ of polybrene}/2.16x10^6 \text{ cells}$$

$$x = 8.64\ \mu L \text{ of polybrene}$$

The cell suspension was treated with 8.64 μL of polybrene and mixed by gentle inversion. This suspension of cells was used to seed 72 wells of a 96-well plate with 30,000 cells per well in 200 μL media. Approximately 48 hours later, the previously determined dilutions of each treatment were added to five separate rows, with the first row serving as a control. 72 hours after the initial seeding of the plate, the cells were infected with HIV.

**HIV Infection**

As treatment with polybrene represents Day 0, on Day 3 (72 hours) after polybrene treatment, the plated cells were infected with HIV. Based on previous research conducted by the Taylor Research Group, a standard of 1 μL of live virus per 10,000 cells was used to determine the amount of live virus needed. With the guidance of mentor Lakmini Premdasa, the plated cells were estimated to be at approximately 100,000 CEM-GFP cells per well, requiring 10 μL of cells for each well. The following calculations were performed in order to prepare the HIV particles for infection.

***1 μL of virus per 10,000 cells***
***100,000 cells per well → 10 μL of virus per well***
***72 wells x 10 μL of virus = 720 μL virus total***
***100 μL of media x 72 wells = 7200 μL of media → 7.2 mL of media***

Based on calculations, 800 μL of cells was mixed with 8.0 mL of media and 100 μL of media containing virus was pipetted into each well. The cells were incubated and read for 7 days following infection.

**Table 8. 96-Well Plate Experimental Setup for HIV Experiment**

| HIV Assay | Trial 1 | Trial 2 | Trial 3 | Trial 4 | Trial 5 | Trial 6 | Trial 7 | Trial 8 | Trial 9 | Trial 10 | Trial 11 | Trial 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control (CEM-GFP with HIV) | | | | | | | | | | | | |
| EGT (0.1 mmol) | | | | | | | | | | | | |
| AP (5 mg/mL) | | | | | | | | | | | | |
| CB (1 mg/mL) | | | | | | | | | | | | |
| PPC (10 g/cup) | | | | | | | | | | | | |
| CC (1/2 bag/cup) | | | | | | | | | | | | |

## Results

Readings for 7 days following the infection were taken with the BioTek Synergy Mx. BioTek readouts are provided in Appendix A with Tables 28-34 representing the GFP expression readouts for reading days 1-7. Graphs for each day and daily averages are provided in Appendix B, with Figures 47, 49, 51, 53, 55, 57, and 59 representing the daily readings for days 1-7 and Figures 48, 50, 52, 54, 56, 58, and 60 representing the averages derived from each trail on days 1-7 . Averages for each day were also assessed for trends.

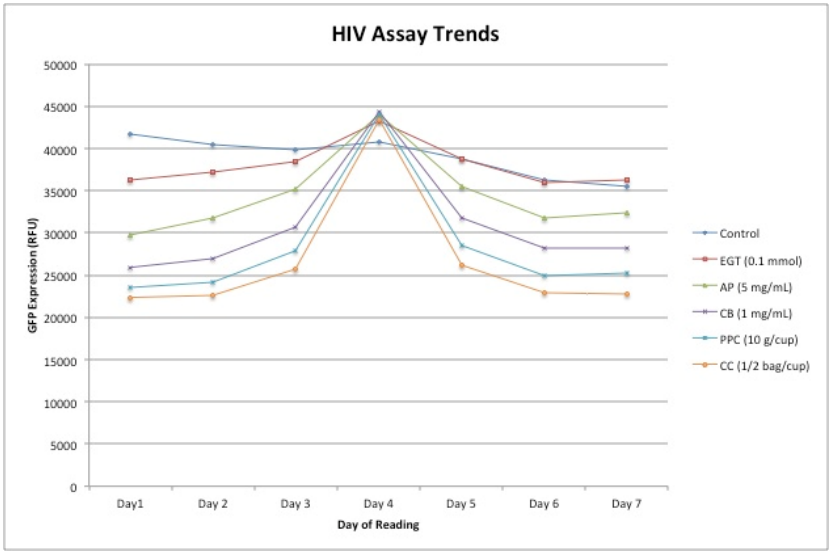



**Figure 7. Graph of HIV Experiment Trends for Each Day of Reading**

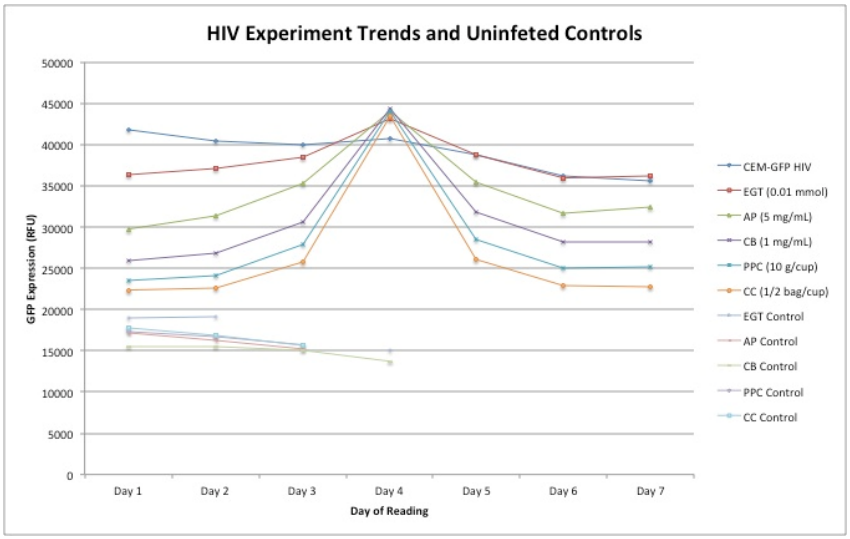



**Figure 8. Graph of HIV Experiment Trends for Each Day of Reading, Including Control CEM-GFP Cells from Cytotoxicity Experiments**

The trend graphs for the HIV experiment signify some protective ability of the experimental herbal treatments in preventing infection or stabilizing cellular stress in the first few days of active infection. The peak at day 4 could represent more viable cells in

the experimental wells in comparison to the control due to the higher GFP expression on that day by the cells treated with herbal remedies. After day 4, the control trials decrease gradually, signifying cell death. However the rapid decrease in GFP expression exemplified by *Hypoxis hemerocallida*, *Sutherlandia frutescens*, *Opuntia ficus-indica*, and *Uncaria tomentosa* implies some antiviral activity or inhibition in viral propagation within those wells. However this could also be a function of the increased viability facilitated by these herbal treatments that was demonstrated in the MTT assay.

# CHAPTER VII

# CONCLUSION

## Summary

Several conclusions can be drawn from the BioTek readout trends. All of GFP expressions exceeded the maximum for previous uninfected, untreated CEM cells, confirming a successful HIV infection. The peak exhibited at Day 4 by nearly every treatment corresponds with that day being the height of GFP expression and likely the point at which all cells are infected with virus[42]. The control, untreated CEM-GFP cells infected with HIV, showed a general decline in GFP expression throughout the 7 days, signifying cell death over time.

## Ergothioneine

Cells treated with ergothioneine showed a gradual increase of GFP expression leading up to Day 4 and a decrease in expression following that day. While this could signify cell death after peak infectivity, given what is known about the inhibition of the NF-κB transcription factor, the decrease in expression could also be interpreted as a decrease in viral activity due to hindered transcription. This conclusion is difficult to make due to the trend line for ergothioneine corresponding so closely to the trend line for the negative control. However, the lower levels of GFP expression in the ergothioneine

treated cells prior to Day 4 could speak to ergothioneine's properties as an antioxidant and imply a role in stabilizing cells exposed to HIV and the closeness to the negative control trendline could signify that several other conclusions. These conclusions include, but are not limited to, the possibility that ergothioneine is not as effective in inhibition HIV as previously hypothesized or that while the NF-κB transcription factor is inhibited by ergothioneine, there are other transcription factors or cellular mechanisms compensate and carry out viral transcription through other means.

**Cultural Herbal Remedies**

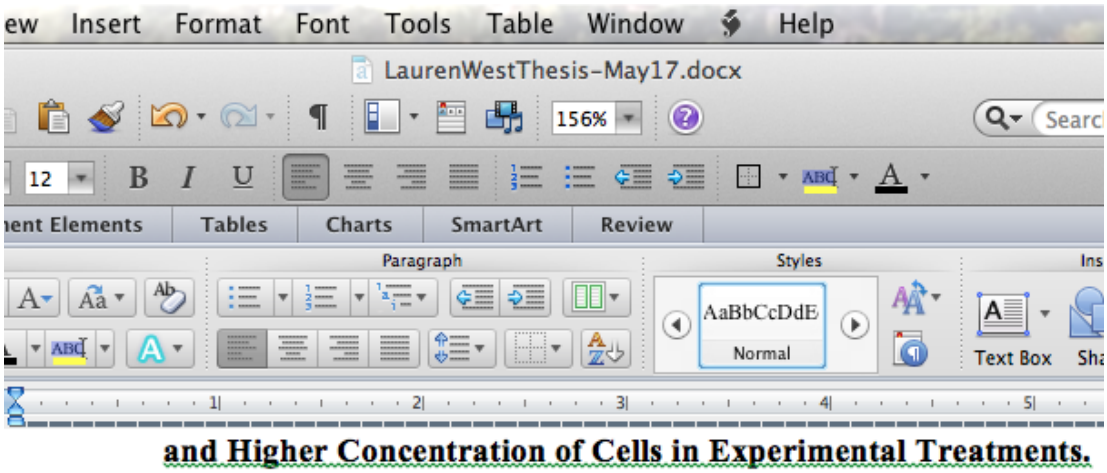
The results for *Hypoxis hemerocallidea, Sutherlandia frutescens, Opuntia ficus-indica, and Uncaria tomentosa* exhibit similar trends and imply that all herbal remedies provide some degree of stability to the cell in the early stages of HIV infection. The GFP expression on Day 4 of reading was higher than the experimental control, however for reading Days 5-7, all herbal remedies returned to GFP expression levels that were similar to reading Days 1-3. While this could be interpreted as immediate cell death after complete infection, the results of the MTT assay suggest that there is a potential for the herbal remedies to be hindering the proliferation of HIV in some way. Figure 8 includes the trends for the HIV experiment along with the trends for the untreated, uninfected CEM-GFP controls from all cytotoxicity assays. As all of the GFP expression levels from the HIV assay remain significantly higher than uninfected, untreated CEM-GFP cells, the rapid death of cells is unlikely. However it is possible that for the HIV-infected cells, there are fewer viable cells than the uninfected, untreated cells, but that they are

fluorescing more brightly thus producing a higher level of GFP expression. This is not very likely, as the MTT assay demonstrates an increased viability in the treated cells compared to the negative controls.

**Adjustments to HIV Experiment Results based on MTT Results**

Based on the results from the MTT assay, it is important to consider the results of the HIV experiment could be influenced by concentration of cells per well. As the herbal treatments in every case appeared to yield a higher number of viable cells in terms of absorbance units, this has a possible influence on the GFP expression displayed in the HIV experiment. Although the GFP expressions were not as low in the cytotoxicity assays for herbal treatments, the drastic decrease in the HIV experiment creates a need for normalization in the number of cells per well. In order to assess relative changes in GFP expression based on the MTT results, the results from the BioTek readout were normalized to acknowledge the absorbance unit value for the negative control CEM-GFP cells as 1.

**Table 9. Normalization of the MTT Assay Results to Account for Increased Viability and Higher Concentration of Cells in Experimental Treatments.**




Considering that a normalization of the MTT assay results gives a relationship regarding the ratio of cells treated with herbal remedies to negative control cells, these relationships were applied to the GFP expression results given by the HIV experiment.

**Table 10. Averages from Each Reading Day of HIV Experiment.**

| Treatment (Conc.) | Day 1 Mean | Day 2 Mean | Day 3 Mean | Day 4 Mean | Day 5 Mean | Day 6 Mean | Day 7 Mean |
|---|---|---|---|---|---|---|---|
| Control (no treatment) | 41746.17 | 40463.42 | 39920.83 | 40752.75 | 38780.5 | 36200.5 | 35520.08 |
| EGT (0.1 mmol) | 36295.75 | 37158.92 | 38471.17 | 43202.17 | 38714 | 35953.75 | 36232.67 |
| AP (5 mg/mL) | 29760.17 | 31758.83 | 35222.75 | 44074.67 | 35467.25 | 31713 | 32430.42 |
| CB (1 mg/mL) | 25921.92 | 26905.17 | 30677.75 | 44286.92 | 31763.92 | 28182.58 | 28206 |
| PPC (10 g/ cup) | 23521.5 | 24153.08 | 27825.08 | 44093.5 | 28512.33 | 25018.75 | 25181.42 |
| CC (1/2 bag/cup) | 22364 | 22572.33 | 25770.33 | 43447.92 | 26120.42 | 22976.75 | 22759.17 |

**Table 11. Averages Normalized with Values Determined from the MTT Assay.**

| Treatment (Conc.) | Day 1 Adjusted | Day 2 Adjusted | Day 3 Adjusted | Day 4 Adjusted | Day 5 Adjusted | Day 6 Adjusted | Day 7 Adjusted |
|---|---|---|---|---|---|---|---|
| Control (no treatment) | 41746.17 | 40463.42 | 39920.83 | 40752.75 | 38780.5 | 36200.5 | 35520.08 |
| EGT (0.1 mmol) | 16844.34973 | 17244.93485 | 17853.93171 | 20049.52262 | 17966.62572 | 16685.63231 | 16815.07518 |
| AP (5 mg/mL) | 8514.636451 | 9086.469988 | 10077.52681 | 12610.13602 | 10147.48033 | 9073.357637 | 9278.61757 |
| CB (1 mg/mL) | 9598.94064 | 9963.04015 | 11360.03433 | 16399.53816 | 11762.24533 | 10436.06772 | 10444.74019 |
| PPC (10 g/ cup) | 9437.569564 | 9690.97943 | 11164.30194 | 17691.70646 | 11440.04837 | 10038.31361 | 10103.58196 |
| CC (1/2 bag/cup) | 12478.92883 | 12595.17527 | 14379.63308 | 24243.58351 | 14574.98043 | 12820.83832 | 12699.43046 |

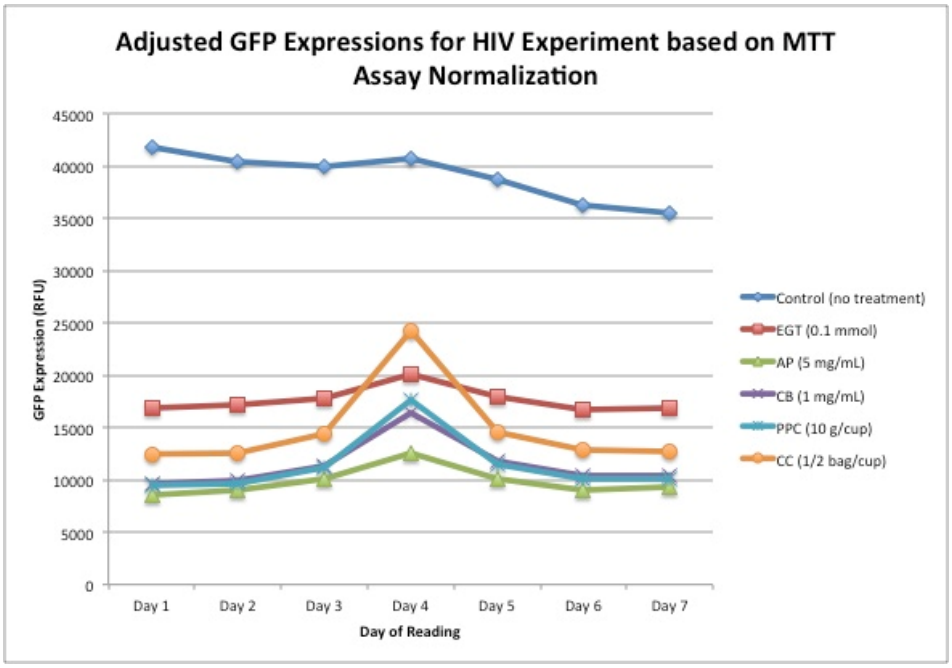



**Figure 9. By Dividing the GFP Expression Values by the Normalization Values Determined from the MTT Assay, the Trend Graph above was Constructed to Account for Higher Concentrations of Cells in Experimental Trials.**

By taking the increased quantity of cells in the experimental wells according the MTT assay results, the effectiveness of the herbal treatments on GFP expression is more visible. An examination of the GFP expression distributed evenly by the theoretical number of cells shows that herbal treatments are very effective in decreasing cellular stress, namely in the presence of the virus. However, this is all mathematical speculation and would require laboratory confirmation in future trials.

## Limitations

While the preparation of dilutions was meant to mimic the way in which each herb was consumed, the use of autoclaved water for solutions possibly hindered the

assessment of hydrophobic biologically active compounds in each treatment. It would take a great deal of planning and preparation to mimic *in vivo* conditions for digestions of each herb *in vitro*.

Additionally, the conversion of *Hypoxis hemerocallidea* to rooperol by use of β-Glucosidase was unmonitored, meaning that there is no way of knowing what portion of *Hypoxis hemerocallidea*, if any, was successfully converted to rooperol. A varying rate of conversion would likely produce a different GFP expression, signifying how much rooperol is present.

Lastly, despite quantifiable differences in GFP expression, this assay does not provide mechanisms with which to determine the cause of variation in GFP expression. There is no definite way of knowing for certain that a decrease in expression signifies inhibition of HIV or a decrease in cell viability. However, the latter seems unlikely, because the treatments appear to significantly *increase* the number of viable cells in the MTT assays (Chapter V).

**Future Research Directions**

Future research should involve preparing dilutions to account for hydrophobic compounds in each herb. Also, rooperol should be obtained directly or successfully derived prior to experimentation. Other assays should be used to determine whether or not HIV is being actively inhibited.

Additionally, herbs should be subjected to separation to determine biologically active compounds. Herbs should also be tested against existing antiretroviral therapies for significant interactions to ensure safety of use as complementary herbal remedies.

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# APPENDIX A

## BIOTEK READOUTS FOR ALL CYTOTOXICITY ASSAY READINGS AND ALL HIV EXPERIMENT READINGS

**Table 12. Day 1 BioTek Readout for Ergothioneine Cytotoxicity Assay**

| EGT Day 1 | 0.0 mM | 0.05 mM | 0.1 mM | 0.5 mM | 1.0 mM | 1.5 mM | 2.0 mM | 2.5 mM | 3.0 mM | 4.0 mM | 5.0 mM | 6.0 mM |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Trial 1 | 16899 | 18729 | 16897 | 18185 | 17517 | 17492 | 17615 | 17194 | 17709 | 17083 | 16547 | 15822 |
| Trial 2 | 18836 | 19969 | 19779 | 19014 | 18977 | 19400 | 19329 | 19140 | 19515 | 17921 | 17576 | 17035 |
| Trial 3 | 19452 | 19893 | 19682 | 19394 | 18946 | 20118 | 19196 | 18754 | 18908 | 19279 | 18634 | 16737 |
| Trial 4 | 19751 | 21537 | 19446 | 19872 | 19590 | 19010 | 20126 | 18772 | 20006 | 18432 | 19109 | 16853 |
| Trial 5 | 19188 | 19586 | 19898 | 19269 | 20148 | 20107 | 20067 | 20413 | 20368 | 18599 | 18855 | 17210 |
| Trial 6 | 19312 | 20027 | 19563 | 22091 | 19896 | 20091 | 20559 | 20091 | 18505 | 17631 | 19042 | 17005 |
| Trial 7 | 19703 | 19971 | 19581 | 19908 | 19207 | 20503 | 20223 | 19830 | 18351 | 18274 | 18507 | 17097 |
| Trial 8 | 18211 | 18653 | 18411 | 16897 | 18104 | 18491 | 18597 | 18206 | 17810 | 18002 | 17497 | 16414 |
| Mean | 18919 | 19795.625 | 19157.125 | 19328.75 | 19048.125 | 19401.5 | 19464 | 19050 | 18896.5 | 18152.625 | 18220.875 | 16771.625 |

**Table 13. Day 2 BioTek Readout for Ergothioneine Cytotoxicity Assay**

| EGT Day 2 | 0.0 mM | 0.05 mM | 0.1 mM | 0.5 mM | 1.0 mM | 1.5 mM | 2.0 mM | 2.5 mM | 3.0 mM | 4.0 mM | 5.0 mM | 6.0 mM |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Trial 1 | 17022 | 18639 | 17274 | 18398 | 18092 | 17669 | 17612 | 17502 | 17242 | 16799 | 16889 | 15789 |
| Trial 2 | 19150 | 20122 | 19934 | 19693 | 19621 | 19759 | 19495 | 19523 | 19680 | 18220 | 18160 | 17149 |
| Trial 3 | 19960 | 20552 | 20550 | 20306 | 20031 | 20709 | 21017 | 19551 | 19471 | 19682 | 18830 | 17030 |
| Trial 4 | 19945 | 21877 | 20106 | 20371 | 20364 | 20229 | 20662 | 19844 | 20006 | 18468 | 19451 | 17148 |
| Trial 5 | 19507 | 20051 | 20270 | 20303 | 20542 | 20358 | 20414 | 20465 | 20583 | 19382 | 19914 | 17168 |
| Trial 6 | 19698 | 20340 | 20138 | 22223 | 20413 | 20856 | 20845 | 20392 | 19236 | 18130 | 19301 | 17442 |
| Trial 7 | 19373 | 20408 | 19837 | 19280 | 19935 | 20394 | 20519 | 19989 | 19132 | 19001 | 18532 | 17081 |
| Trial 8 | 17839 | 18472 | 18128 | 16896 | 18042 | 18357 | 18791 | 17874 | 18000 | 17874 | 17462 | 16367 |
| Mean | 19061.75 | 20057.625 | 19529.625 | 19683.75 | 19630 | 19791.375 | 19919.375 | 19392.5 | 19168.75 | 18444.5 | 18567.375 | 16896.75 |

**Table 14. Day 4 BioTek Readout for Ergothioneine Cytotoxicity Assay**

| EGT Day 4 | 0.0 mM | 0.05 mM | 0.1 mM | 0.5 mM | 1.0 mM | 1.5 mM | 2.0 mM | 2.5 mM | 3.0 mM | 4.0 mM | 5.0 mM | 6.0 mM |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Trial 1 | 13711 | 15493 | 14663 | 14911 | 15195 | 14965 | 14690 | 14941 | 14174 | 14318 | 14325 | 12577 |
| Trial 2 | 15774 | 17308 | 17512 | 17374 | 16985 | 17135 | 16883 | 16966 | 17019 | 16605 | 16004 | 14484 |
| Trial 3 | 17678 | 17930 | 18238 | 17824 | 17931 | 18229 | 19231 | 17644 | 16613 | 16243 | 15099 | 13074 |
| Trial 4 | 14594 | 16284 | 15440 | 16400 | 17834 | 18199 | 18413 | 18065 | 17774 | 16501 | 17010 | 14252 |
| Trial 5 | 14967 | 17388 | 18011 | 18251 | 18429 | 18342 | 18181 | 17807 | 16971 | 15950 | 15371 | 13161 |
| Trial 6 | 14345 | 15766 | 15601 | 16154 | 17179 | 18457 | 18605 | 18080 | 17454 | 15927 | 16731 | 13799 |
| Trial 7 | 14947 | 17646 | 17339 | 17350 | 17951 | 18274 | 17908 | 17504 | 16513 | 16205 | 15086 | 12845 |
| Trial 8 | 14973 | 15511 | 15437 | 14557 | 14868 | 15468 | 16141 | 15163 | 15628 | 15095 | 14113 | 13327 |
| Mean | 15123.625 | 16665.75 | 16530.125 | 16602.625 | 17046.5 | 17383.625 | 17506.5 | 17021.25 | 16518.25 | 15855.5 | 15467.375 | 13439.875 |

**Table 15. Day 1 BioTek Readout for *Hypoxis hemerocallidea* Cytotoxicity Assay**

| AP Day 1 | Trial 1 | Trial 2 | Trial 3 | Trial 4 | Trial 5 | Trial 6 | Trial 7 | Trial 8 | Trial 9 | Trial 10 | Trial 11 | Trial 12 | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 mg/mL | 14829 | 17186 | 17327 | 17741 | 17719 | 17137 | 17517 | 17509 | 17424 | 17394 | 17162 | 16375 | 17110 |
| 1 mg/mL | 17259 | 17873 | 18507 | 18525 | 18645 | 18901 | 18903 | 19043 | 18922 | 18700 | 18725 | 18130 | 18511.08333 |
| 5 mg/mL | 18946 | 21061 | 20683 | 20446 | 19089 | 18659 | 18874 | 19408 | 19443 | 18850 | 17549 | 17492 | 19208.33333 |
| 10 mg/mL | 18442 | 15872 | 18905 | 19571 | 20935 | 22575 | 27508 | 22869 | 20389 | 25204 | 22722 | 22299 | 21440.91667 |
| 20 mg/mL | 19209 | 21245 | 23073 | 23473 | 23127 | 27078 | 25917 | 21648 | 20444 | 19800 | 20396 | 19521 | 22077.58333 |
| 30 mg/mL | 19852 | 24170 | 23318 | 22566 | 22717 | 25241 | 24801 | 23961 | 25147 | 29019 | 26627 | 25402 | 24401.75 |
| 40 mg/mL | 20856 | 22168 | 25233 | 23449 | 25717 | 23845 | 23059 | 21128 | 18097 | 22069 | 22512 | 22350 | 22540.25 |
| 50 mg/mL | 23499 | 25015 | 24381 | 24983 | 25099 | 25500 | 25777 | 24935 | 25706 | 25501 | 23584 | 22568 | 24712.33333 |

**Table 16. Day 2 BioTek Readout for *Hypoxis hemerocallidea* Cytotoxicity Assay**

| AP Day 2 | Trial 1 | Trial 2 | Trial 3 | Trial 4 | Trial 5 | Trial 6 | Trial 7 | Trial 8 | Trial 9 | Trial 10 | Trial 11 | Trial 12 | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 mg/mL | 14085 | 16377 | 16427 | 17158 | 16855 | 16501 | 16713 | 16506 | 16613 | 16862 | 16402 | 15138 | 16303.08333 |
| 1 mg/mL | 15678 | 16400 | 17509 | 17453 | 17411 | 18514 | 18719 | 18588 | 18935 | 18459 | 18475 | 16940 | 17756.75 |
| 5 mg/mL | 17798 | 20983 | 20976 | 20908 | 19784 | 18796 | 18433 | 18498 | 18171 | 17612 | 16767 | 16043 | 18730.75 |
| 10 mg/mL | 17662 | 15585 | 18303 | 18880 | 19877 | 21234 | 27173 | 22826 | 20396 | 24673 | 21125 | 20658 | 20699.33333 |
| 20 mg/mL | 18287 | 18952 | 20277 | 21024 | 20635 | 23737 | 23242 | 20053 | 19461 | 19095 | 18636 | 17749 | 20095.66667 |
| 30 mg/mL | 18153 | 22209 | 22023 | 20713 | 20702 | 22251 | 21431 | 20708 | 21774 | 24386 | 22390 | 23100 | 21653.33333 |
| 40 mg/mL | 18793 | 19514 | 20872 | 19477 | 21044 | 19742 | 19519 | 18470 | 15174 | 19135 | 19080 | 18649 | 19122.41667 |
| 50 mg/mL | 20866 | 22008 | 21464 | 21429 | 20993 | 21169 | 20965 | 20642 | 20683 | 20673 | 19251 | 18489 | 20719.33333 |

**Table 17. Day 3 BioTek Readout for *Hypoxis hemerocallidea* Cytotoxicity Assay**

| AP Day 3 | Trial 1 | Trial 2 | Trial 3 | Trial 4 | Trial 5 | Trial 6 | Trial 7 | Trial 8 | Trial 9 | Trial 10 | Trial 11 | Trial 12 | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 mg/mL | 13075 | 15052 | 15535 | 16069 | 15692 | 15378 | 15658 | 15512 | 15770 | 15689 | 15148 | 14163 | 15228.41667 |
| 1 mg/mL | 14893 | 15839 | 16598 | 16793 | 17040 | 18002 | 18144 | 18003 | 18212 | 17864 | 17703 | 16076 | 17097.25 |
| 5 mg/mL | 17503 | 20503 | 20742 | 20861 | 19588 | 18618 | 18982 | 18824 | 18217 | 17530 | 16612 | 15772 | 18646 |
| 10 mg/mL | 17413 | 15558 | 16654 | 18834 | 19811 | 21900 | 28212 | 23487 | 20853 | 23040 | 21689 | 19949 | 20616.66667 |
| 20 mg/mL | 18528 | 19767 | 21565 | 22880 | 22579 | 25452 | 24526 | 21205 | 20260 | 19910 | 19562 | 18448 | 21223.5 |
| 30 mg/mL | 18604 | 23294 | 23257 | 22064 | 22091 | 23777 | 23816 | 23559 | 24071 | 26478 | 23582 | 23746 | 23194.91667 |
| 40 mg/mL | 19411 | 20820 | 22408 | 21181 | 22775 | 21589 | 21172 | 20157 | 16076 | 20932 | 20952 | 20260 | 20644.41667 |
| 50 mg/mL | 22559 | 24482 | 23843 | 24164 | 23627 | 23857 | 23460 | 23228 | 22909 | 22645 | 20885 | 19628 | 22940.58333 |

**Table 18. Day 1 BioTek Readout for *Sutherlandia frutescens* Cytotoxicity Assay**

| CB Day 1 | Trial 1 | Trial 2 | Trial 3 | Trial 4 | Trial 5 | Trial 6 | Trial 7 | Trial 8 | Trial 9 | Trial 10 | Trial 11 | Trial 12 | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 mg/mL | 13839 | 14871 | 15289 | 15376 | 15383 | 15458 | 15607 | 14940 | 15252 | 16700 | 17442 | 16229 | 15532.16667 |
| 1 mg/mL | 15890 | 15738 | 16689 | 16544 | 16391 | 16420 | 16417 | 16696 | 17735 | 18019 | 17769 | 15810 | 16676.5 |
| 5 mg/mL | 16890 | 15304 | 13888 | 14061 | 14310 | 14826 | 14594 | 14408 | 16927 | 17210 | 17126 | 16561 | 15508.75 |
| 10 mg/mL | 16573 | 17416 | 16245 | 14933 | 14915 | 13319 | 11917 | 17846 | 17291 | 16816 | 16415 | 16890 | 15881.33333 |
| 20 mg/mL | 16445 | 18408 | 15606 | 17259 | 17346 | 17118 | 16168 | 16905 | 17080 | 20782 | 18903 | 19857 | 17656.41667 |
| 30 mg/mL | 16052 | 16533 | 16540 | 16050 | 16529 | 19469 | 19840 | 21082 | 16704 | 18633 | 17453 | 19097 | 17831.83333 |
| 40 mg/mL | 15990 | 16335 | 16748 | 16966 | 16052 | 16428 | 15921 | 17847 | 16820 | 17278 | 16866 | 20847 | 17008.16667 |
| 50 mg/mL | 15576 | 16023 | 16068 | 15828 | 15733 | 16071 | 15723 | 16512 | 13925 | 17982 | 18746 | 17797 | 16332 |

**Table 19. Day 2 BioTek Readout for *Sutherlandia frutescens* Cytotoxicity Assay**

| CB Day 2 | Trial 1 | Trial 2 | Trial 3 | Trial 4 | Trial 5 | Trial 6 | Trial 7 | Trial 8 | Trial 9 | Trial 10 | Trial 11 | Trial 12 | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 mg/mL | 13849 | 14798 | 15376 | 15729 | 15045 | 15212 | 15291 | 15115 | 15074 | 16823 | 17576 | 16124 | 15501 |
| 1 mg/mL | 14116 | 14465 | 15306 | 15436 | 15792 | 16406 | 16808 | 17331 | 18370 | 18064 | 18378 | 16256 | 16394 |
| 5 mg/mL | 15823 | 15628 | 14333 | 14433 | 14869 | 15377 | 14576 | 13575 | 14869 | 14633 | 14690 | 14056 | 14738.5 |
| 10 mg/mL | 13530 | 14390 | 13438 | 12956 | 13086 | 12339 | 11535 | 17432 | 16802 | 15892 | 14790 | 14205 | 14199.58333 |
| 20 mg/mL | 13683 | 15825 | 13619 | 14977 | 14674 | 14479 | 13589 | 13969 | 14033 | 16927 | 15205 | 15774 | 14729.5 |
| 30 mg/mL | 12463 | 12855 | 12961 | 12774 | 13350 | 16123 | 16445 | 17702 | 14032 | 15599 | 14035 | 14910 | 14437.41667 |
| 40 mg/mL | 12238 | 12887 | 13226 | 13409 | 12537 | 12957 | 12458 | 13657 | 12813 | 12855 | 12554 | 15221 | 13067.66667 |
| 50 mg/mL | 12397 | 12419 | 12118 | 12246 | 12044 | 11972 | 12006 | 12173 | 10636 | 13578 | 13705 | 12873 | 12347.25 |

**Table 20. Day 3 BioTek Readout for *Sutherlandia frutescens* Cytotoxicity Assay**

| CB Day 3 | Trial 1 | Trial 2 | Trial 3 | Trial 4 | Trial 5 | Trial 6 | Trial 7 | Trial 8 | Trial 9 | Trial 10 | Trial 11 | Trial 12 | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 mg/mL | 13422 | 14776 | 15070 | 15307 | 14743 | 15065 | 14739 | 14683 | 15092 | 16508 | 16134 | 14842 | 15031.75 |
| 1 mg/mL | 12650 | 13154 | 13783 | 13683 | 13815 | 14484 | 15309 | 16508 | 18137 | 18080 | 18286 | 15476 | 15280.41667 |
| 5 mg/mL | 13385 | 12913 | 12138 | 11939 | 12332 | 12617 | 12130 | 11738 | 13067 | 13090 | 12892 | 11592 | 12486.08333 |
| 10 mg/mL | 11270 | 12277 | 11446 | 10760 | 10808 | 9878 | 8948 | 13324 | 12993 | 12481 | 12494 | 12588 | 11605.58333 |
| 20 mg/mL | 11157 | 13130 | 11127 | 12320 | 11798 | 11671 | 10925 | 11169 | 11103 | 13326 | 12226 | 12692 | 11887 |
| 30 mg/mL | 9591 | 10124 | 10208 | 10174 | 10521 | 12655 | 12466 | 13868 | 10984 | 11851 | 10982 | 11841 | 11272.08333 |
| 40 mg/mL | 9566 | 10053 | 10234 | 10366 | 9788 | 10099 | 9686 | 10187 | 9977 | 9954 | 9690 | 11770 | 10114.16667 |
| 50 mg/mL | 8663 | 8864 | 8759 | 8683 | 8906 | 8695 | 8910 | 8923 | 8029 | 10045 | 10257 | 9701 | 9036.25 |

**Table 21. Day 4 BioTek Readout for *Sutherlandia frutescens* Cytotoxicity Assay**

| CB Day 4 | Trial 1 | Trial 2 | Trial 3 | Trial 4 | Trial 5 | Trial 6 | Trial 7 | Trial 8 | Trial 9 | Trial 10 | Trial 11 | Trial 12 | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 mg/mL | 11857 | 13477 | 13868 | 13971 | 13408 | 13742 | 13466 | 13811 | 13757 | 15137 | 14670 | 13602 | 13730.5 |
| 1 mg/mL | 12658 | 13379 | 14099 | 14391 | 14599 | 15533 | 16032 | 16508 | 17228 | 17121 | 17358 | 14413 | 15276.58333 |
| 5 mg/mL | 14384 | 14793 | 14040 | 13922 | 14217 | 14642 | 14057 | 12954 | 13843 | 13650 | 13447 | 12187 | 13844.66667 |
| 10 mg/mL | 12322 | 13878 | 12796 | 12152 | 12522 | 11421 | 10914 | 16709 | 15944 | 14875 | 14425 | 13263 | 13435.08333 |
| 20 mg/mL | 12151 | 15285 | 13116 | 14623 | 13703 | 13737 | 13008 | 12965 | 13180 | 15610 | 14076 | 14549 | 13833.58333 |
| 30 mg/mL | 11071 | 11951 | 12098 | 12254 | 12664 | 15502 | 15247 | 17122 | 13291 | 14635 | 13101 | 13480 | 13534.66667 |
| 40 mg/mL | 11042 | 12213 | 12551 | 12530 | 11833 | 12347 | 11766 | 12679 | 12013 | 11996 | 11786 | 13954 | 12225.83333 |
| 50 mg/mL | 11095 | 11039 | 11122 | 11097 | 10961 | 10625 | 10975 | 11020 | 9969 | 12146 | 12370 | 11503 | 11160.16667 |

**Table 22. Day 1 BioTek Readout for *Opuntia ficus-indica* Cytotoxicity Assay**

| PPC Day 1 | Trial 1 | Trial 2 | Trial 3 | Trial 4 | Trial 5 | Trial 6 | Trial 7 | Trial 8 | Trial 9 | Trial 10 | Trial 11 | Trial 12 | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 g/cup | 15704 | 17422 | 17429 | 17800 | 17657 | 17483 | 17231 | 17922 | 17511 | 17617 | 17774 | 16298 | 17320.66667 |
| 1 g/cup | 17636 | 17852 | 17612 | 18445 | 18930 | 18385 | 18774 | 19786 | 18736 | 18204 | 18904 | 13998 | 18105.16667 |
| 5 g/cup | 18687 | 18975 | 18234 | 18376 | 19136 | 18940 | 20584 | 18539 | 17451 | 16744 | 16038 | 15755 | 18121.58333 |
| 10 g/cup | 15970 | 15857 | 16977 | 17428 | 17692 | 18775 | 18836 | 19758 | 18126 | 18473 | 19385 | 16780 | 17838.08333 |
| 25 g/cup | 16421 | 18236 | 18175 | 9622 | 14273 | 19105 | 18094 | 17189 | 15526 | 16985 | 16399 | 15877 | 16325.16667 |
| 50 g/cup | 16269 | 16430 | 16293 | 16464 | 17797 | 18555 | 20042 | 20266 | 20070 | 18910 | 18523 | 16409 | 18002.33333 |
| 75 g/cup | 16481 | 17658 | 18788 | 19932 | 20231 | 17484 | 18402 | 17129 | 18331 | 16885 | 16953 | 16470 | 17895.33333 |
| 100 g/cup | 18883 | 20434 | 20003 | 19737 | 19634 | 19198 | 19974 | 20493 | 19800 | 19605 | 19009 | 16551 | 19443.41667 |

**Table 23. Day 2 BioTek Readout for *Opuntia ficus-indica* Cytotoxicity Assay**

| PPC Day 2 | Trial 1 | Trial 2 | Trial 3 | Trial 4 | Trial 5 | Trial 6 | Trial 7 | Trial 8 | Trial 9 | Trial 10 | Trial 11 | Trial 12 | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 g/cup | 15096 | 17087 | 17174 | 17268 | 17048 | 16907 | 16690 | 16880 | 17002 | 16888 | 17078 | 15514 | 16719.33333 |
| 1 g/cup | 15783 | 16033 | 16018 | 17022 | 17580 | 18103 | 18480 | 18906 | 18722 | 17821 | 18467 | 13733 | 17222.33333 |
| 5 g/cup | 16852 | 18477 | 18023 | 18266 | 18490 | 18968 | 19614 | 17305 | 16139 | 15548 | 14854 | 14444 | 17248.33333 |
| 10 g/cup | 14797 | 14737 | 15513 | 15483 | 16096 | 16753 | 17726 | 19153 | 17522 | 17543 | 17239 | 15013 | 16464.58333 |
| 25 g/cup | 15126 | 15914 | 15680 | 8302 | 12161 | 16223 | 15345 | 14836 | 13955 | 15111 | 14949 | 14255 | 14321.41667 |
| 50 g/cup | 14424 | 14790 | 14577 | 14534 | 15235 | 15937 | 16544 | 16493 | 16689 | 15692 | 15436 | 14455 | 15400.5 |
| 75 g/cup | 14618 | 15381 | 15473 | 15872 | 16280 | 14443 | 15696 | 14597 | 15416 | 14673 | 14777 | 14051 | 15106.41667 |
| 100 g/cup | 16267 | 17094 | 16924 | 15948 | 15947 | 15216 | 16015 | 15397 | 15444 | 14865 | 15319 | 13932 | 15697.33333 |

**Table 24. Day 3 BioTek Readout for *Opuntia ficus-indica* Cytotoxicity Assay**

| PPC Day 3 | Trial 1 | Trial 2 | Trial 3 | Trial 4 | Trial 5 | Trial 6 | Trial 7 | Trial 8 | Trial 9 | Trial 10 | Trial 11 | Trial 12 | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 g/cup | 14320 | 16387 | 16147 | 16086 | 15915 | 16086 | 15778 | 15798 | 16083 | 15967 | 15701 | 14425 | 15724.41667 |
| 1 g/cup | 16995 | 17639 | 17415 | 17991 | 18056 | 17990 | 17970 | 18095 | 18274 | 17485 | 17891 | 13095 | 17408 |
| 5 g/cup | 17829 | 18187 | 17541 | 17503 | 18121 | 18424 | 18925 | 18122 | 17267 | 16393 | 15364 | 14633 | 17359.08333 |
| 10 g/cup | 14710 | 14812 | 15217 | 15734 | 16881 | 17525 | 17888 | 18400 | 17314 | 17650 | 18659 | 15924 | 16726.16667 |
| 25 g/cup | 15285 | 17424 | 17283 | 9421 | 13546 | 18062 | 17606 | 16530 | 14630 | 15628 | 15226 | 14571 | 15434.33333 |
| 50 g/cup | 14873 | 15341 | 15122 | 15402 | 16675 | 17677 | 18631 | 18377 | 18979 | 18118 | 18019 | 14146 | 16780 |
| 75 g/cup | 15108 | 16797 | 18361 | 18880 | 19901 | 17463 | 18210 | 16908 | 16887 | 16397 | 16309 | 15384 | 17217.08333 |
| 100 g/cup | 16971 | 18420 | 18562 | 18035 | 18179 | 17820 | 18592 | 18384 | 18238 | 18111 | 17718 | 15113 | 17845.25 |

**Table 25. Day 1 BioTek Readout for *Uncaria tomentosa* Cytotoxicity Assay**

| CC Day 1 | Trial 1 | Trial 2 | Trial 3 | Trial 4 | Trial 5 | Trial 6 | Trial 7 | Trial 8 | Trial 9 | Trial 10 | Trial 11 | Trial 12 | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 bags/cup | 16011 | 17744 | 17788 | 18572 | 17939 | 17876 | 17136 | 17229 | 18073 | 18378 | 17779 | 18063 | 17715.66667 |
| ¼ bag/cup | 18370 | 19136 | 19601 | 19036 | 19267 | 20214 | 19425 | 20252 | 19283 | 20369 | 19081 | 18383 | 19368.08333 |
| 1/3 bag/cup | 20414 | 20528 | 21684 | 20903 | 19435 | 20719 | 21267 | 20701 | 18993 | 19176 | 17803 | 17345 | 19914 |
| ½ bag/cup | 16669 | 17474 | 17354 | 20649 | 20884 | 21613 | 20959 | 21533 | 20146 | 19847 | 19899 | 19014 | 19670.08333 |
| 1 bag/cup | 18140 | 21709 | 22439 | 22914 | 22561 | 22444 | 21110 | 21078 | 20321 | 18610 | 19106 | 17603 | 20669.58333 |
| 2 bags/cup | 20605 | 21406 | 20857 | 20454 | 23072 | 23876 | 24263 | 24451 | 23948 | 23733 | 30098 | 21036 | 23149.91667 |
| 3 bags/cup | 21508 | 25018 | 25842 | 25358 | 25786 | 25486 | 26246 | 25135 | 24512 | 23987 | 23892 | 22876 | 24637.16667 |
| 4 bags/cup | 26014 | 25239 | 26604 | 26584 | 26043 | 27165 | 25443 | 27127 | 26452 | 26301 | 25180 | 24897 | 26087.41667 |

**Table 26. Day 2 BioTek Readout for *Uncaria tomentosa* Cytotoxicity Assay**

| CC Day 2 | Trial 1 | Trial 2 | Trial 3 | Trial 4 | Trial 5 | Trial 6 | Trial 7 | Trial 8 | Trial 9 | Trial 10 | Trial 11 | Trial 12 | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 bags/cup | 14983 | 17420 | 17216 | 17551 | 17440 | 17332 | 16530 | 16772 | 17101 | 16998 | 17010 | 16560 | 16909.41667 |
| ¼ bag/cup | 16736 | 17855 | 18591 | 18159 | 19011 | 19503 | 19059 | 19574 | 18948 | 19474 | 18728 | 18054 | 18641 |
| 1/3 bag/cup | 18965 | 19648 | 21212 | 20399 | 19473 | 20274 | 20592 | 19154 | 17780 | 17395 | 16450 | 15768 | 18925.83333 |
| ½ bag/cup | 15497 | 16681 | 16472 | 19119 | 19126 | 20000 | 20312 | 20900 | 19856 | 19676 | 19077 | 16217 | 18577.75 |
| 1 bag/cup | 16698 | 19153 | 20706 | 21768 | 21470 | 21025 | 19263 | 18600 | 18202 | 17390 | 17230 | 16169 | 18972.83333 |
| 2 bags/cup | 18955 | 19818 | 19275 | 18963 | 20399 | 21533 | 22683 | 23077 | 23002 | 21790 | 25028 | 17891 | 21034.5 |
| 3 bags/cup | 19122 | 21280 | 21366 | 21938 | 21989 | 21536 | 21749 | 20345 | 20340 | 19924 | 19799 | 18898 | 20690.5 |
| 4 bags/cup | 23978 | 23541 | 23698 | 23962 | 23205 | 23889 | 23119 | 24381 | 23806 | 22543 | 20823 | 19576 | 23043.41667 |

**Table 27. Day 3 BioTek Readout for *Uncaria tomentosa* Cytotoxicity Assay**

| CC Day 3 | Trial 1 | Trial 2 | Trial 3 | Trial 4 | Trial 5 | Trial 6 | Trial 7 | Trial 8 | Trial 9 | Trial 10 | Trial 11 | Trial 12 | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 bags/cup | 13562 | 15790 | 15862 | 15684 | 16165 | 16039 | 15633 | 15686 | 16319 | 15808 | 16099 | 15195 | 15653.5 |
| ¼ bag/cup | 15367 | 17035 | 17856 | 17856 | 18224 | 18123 | 18361 | 18144 | 18252 | 18450 | 18155 | 17002 | 17735.41667 |
| 1/3 bag/cup | 20228 | 14496 | 18952 | 18330 | 18477 | 19215 | 19201 | 18565 | 17822 | 16801 | 16284 | 15126 | 17791.41667 |
| ½ bag/cup | 16395 | 12576 | 15320 | 18194 | 18468 | 19879 | 19267 | 19706 | 19414 | 19257 | 18959 | 16287 | 17810.16667 |
| 1 bag/cup | 17527 | 15288 | 19426 | 20292 | 19205 | 20252 | 19459 | 18367 | 17562 | 17166 | 16666 | 15406 | 18051.33333 |
| 2 bags/cup | 19631 | 15599 | 19107 | 19181 | 19887 | 22054 | 22465 | 21413 | 22321 | 21174 | 25391 | 17330 | 20462.75 |
| 3 bags/cup | 18239 | 17092 | 21984 | 22788 | 22216 | 23134 | 21969 | 20965 | 20619 | 19874 | 19510 | 18957 | 20612.25 |
| 4 bags/cup | 25560 | 19874 | 18045 | 23755 | 22172 | 23656 | 21962 | 22831 | 23361 | 22457 | 21927 | 19493 | 22091.08333 |

**Table 28. Day 1 BioTek Readout for HIV Experiment**

| HIV Assay Day 1 | Trial 1 | Trial 2 | Trial 3 | Trial 4 | Trial 5 | Trial 6 | Trial 7 | Trial 8 | Trial 9 | Trial 10 | Trial 11 | Trial 12 | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control (CEM-GFP with HIV) | 39171 | 42629 | 42275 | 41659 | 41945 | 41652 | 42858 | 41876 | 41924 | 41858 | 41411 | 41696 | 41746.16667 |
| EGT (0.1 mmol) | 32873 | 32797 | 33588 | 34422 | 34936 | 35575 | 35492 | 33423 | 36835 | 42816 | 41471 | 41321 | 36295.75 |
| AP (5 mg/mL) | 33135 | 23837 | 33461 | 33323 | 32259 | 32178 | 31214 | 30371 | 29663 | 29382 | 20973 | 27326 | 29760.16667 |
| CB (1 mg/mL) | 24672 | 24073 | 25070 | 27048 | 26409 | 25821 | 26705 | 25983 | 27507 | 28268 | 28178 | 21329 | 25921.91667 |
| PPC (10 g/cup) | 24327 | 24639 | 25006 | 21076 | 24524 | 24355 | 23784 | 23207 | 22971 | 22789 | 22808 | 22772 | 23521.5 |
| CC (1/2 bag/cup) | 21035 | 21908 | 21838 | 21918 | 22511 | 23216 | 22770 | 22771 | 23004 | 23163 | 22334 | 21900 | 22364 |

**Table 29. Day 2 BioTek Readout for HIV Experiment**

| HIV Assay Day 2 | Trial 1 | Trial 2 | Trial 3 | Trial 4 | Trial 5 | Trial 6 | Trial 7 | Trial 8 | Trial 9 | Trial 10 | Trial 11 | Trial 12 | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control (CEM-GFP with HIV) | 37669 | 41449 | 41068 | 40802 | 41082 | 40480 | 41293 | 41075 | 40463 | 40779 | 40559 | 38842 | 40463.41667 |
| EGT (0.1 mmol) | 32113 | 32419 | 33640 | 34027 | 34129 | 34804 | 36781 | 40203 | 42600 | 42873 | 41518 | 40800 | 37158.91667 |
| AP (5 mg/mL) | 33496 | 33386 | 34135 | 34136 | 33748 | 33112 | 31682 | 30982 | 30376 | 29900 | 28622 | 27531 | 31758.83333 |
| CB (1 mg/mL) | 24573 | 25048 | 25213 | 27985 | 26620 | 26354 | 27325 | 27389 | 27904 | 28744 | 28085 | 27622 | 26905.16667 |
| PPC (10 g/cup) | 24717 | 25127 | 25753 | 24786 | 24794 | 24500 | 24446 | 23738 | 23015 | 23412 | 22759 | 22790 | 24153.08333 |
| CC (1/2 bag/cup) | 21362 | 22114 | 22274 | 22253 | 22616 | 23658 | 23122 | 23182 | 23256 | 23135 | 22563 | 21333 | 22572.33333 |

**Table 30. Day 3 BioTek Readout for HIV Experiment**

| HIV Assay Day 3 | Trial 1 | Trial 2 | Trial 3 | Trial 4 | Trial 5 | Trial 6 | Trial 7 | Trial 8 | Trial 9 | Trial 10 | Trial 11 | Trial 12 | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control (CEM-GFP with HIV) | 36921 | 40660 | 40901 | 40590 | 40621 | 40284 | 40766 | 40260 | 40560 | 40472 | 38979 | 38036 | 39920.83333 |
| EGT (0.1 mmol) | 33806 | 34369 | 35517 | 35753 | 35843 | 37019 | 39976 | 42365 | 42960 | 43710 | 41130 | 39206 | 38471.16667 |
| AP (5 mg/mL) | 35565 | 36476 | 37833 | 38365 | 38584 | 36991 | 35269 | 33962 | 33820 | 33385 | 31431 | 30992 | 35222.75 |
| CB (1 mg/mL) | 28171 | 28672 | 29273 | 32425 | 30396 | 30642 | 31280 | 31921 | 31611 | 32576 | 31080 | 30086 | 30677.75 |
| PPC (10 g/cup) | 27737 | 28818 | 29435 | 28786 | 29107 | 28796 | 28279 | 27511 | 26821 | 27340 | 25402 | 25869 | 27825.08333 |
| CC (1/2 bag/cup) | 24370 | 25691 | 25712 | 25760 | 26368 | 26966 | 26807 | 26816 | 26695 | 25991 | 25094 | 22974 | 25770.33333 |

**Table 31. Day 4 BioTek Readout for HIV Experiment**

| HIV Assay Day 4 | Trial 1 | Trial 2 | Trial 3 | Trial 4 | Trial 5 | Trial 6 | Trial 7 | Trial 8 | Trial 9 | Trial 10 | Trial 11 | Trial 12 | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control (CEM-GFP with HIV) | 38050 | 42020 | 42061 | 41416 | 41464 | 40237 | 40688 | 41348 | 41554 | 41530 | 39716 | 38949 | 40752.75 |
| EGT (0.1 mmol) | 42818 | 43808 | 44032 | 43960 | 43192 | 42546 | 42713 | 43968 | 44374 | 44191 | 42310 | 40514 | 43202.16667 |
| AP (5 mg/mL) | 44276 | 44668 | 44580 | 44582 | 44702 | 43247 | 44164 | 44031 | 43766 | 44786 | 42602 | 43492 | 44074.66667 |
| CB (1 mg/mL) | 43906 | 44400 | 44506 | 47563 | 44211 | 43283 | 43561 | 44111 | 44041 | 44909 | 44035 | 42917 | 44286.91667 |
| PPC (10 g/cup) | 43397 | 44956 | 45297 | 43726 | 44413 | 44247 | 44435 | 43704 | 43524 | 44775 | 43013 | 43635 | 44093.5 |
| CC (1/2 bag/cup) | 42433 | 44634 | 43926 | 44104 | 44475 | 45436 | 44909 | 44398 | 44664 | 43115 | 41985 | 37296 | 43447.91667 |

**Table 32. Day 5 BioTek Readout for HIV Experiment**

| HIV Assay Day 5 | Trial 1 | Trial 2 | Trial 3 | Trial 4 | Trial 5 | Trial 6 | Trial 7 | Trial 8 | Trial 9 | Trial 10 | Trial 11 | Trial 12 | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control (CEM-GFP with HIV) | 34958 | 39199 | 40424 | 39940 | 39902 | 37781 | 38264 | 39585 | 39997 | 39771 | 38115 | 37430 | 38780.5 |
| EGT (0.1 mmol) | 33644 | 35824 | 36072 | 36840 | 36531 | 35361 | 39917 | 42830 | 43314 | 43480 | 41605 | 39150 | 38714 |
| AP (5 mg/mL) | 35834 | 37491 | 38464 | 39226 | 38128 | 36227 | 34914 | 34312 | 33990 | 33804 | 31901 | 31424 | 35476.25 |
| CB (1 mg/mL) | 29262 | 29660 | 30542 | 33286 | 31409 | 30752 | 32296 | 32713 | 33069 | 33859 | 33079 | 31240 | 31763.91667 |
| PPC (10 g/cup) | 28124 | 29771 | 30797 | 29743 | 27522 | 29643 | 29425 | 28437 | 27713 | 28233 | 26467 | 26273 | 28512.33333 |
| CC (1/2 bag/cup) | 24796 | 25862 | 26234 | 26200 | 26303 | 27609 | 27294 | 27285 | 27549 | 26542 | 25583 | 22188 | 26120.41667 |

**Table 33. Day 6 BioTek Readout for HIV Experiment**

| HIV Assay Day 6 | Trial 1 | Trial 2 | Trial 3 | Trial 4 | Trial 5 | Trial 6 | Trial 7 | Trial 8 | Trial 9 | Trial 10 | Trial 11 | Trial 12 | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control (CEM-GFP with HIV) | 33739 | 36775 | 37692 | 37268 | 39121 | 37242 | 36777 | 37774 | 37746 | 37569 | 30852 | 31851 | 36200.5 |
| EGT (0.1 mmol) | 29620 | 33087 | 33561 | 34500 | 33916 | 32390 | 36067 | 38910 | 42827 | 40965 | 40236 | 35366 | 35953.75 |
| AP (5 mg/mL) | 31558 | 34042 | 34205 | 35283 | 34989 | 32838 | 32270 | 31045 | 30325 | 29811 | 28470 | 25720 | 31713 |
| CB (1 mg/mL) | 25289 | 26153 | 26940 | 29179 | 27729 | 28106 | 28854 | 29354 | 29735 | 29854 | 29288 | 27710 | 28182.58333 |
| PPC (10 g/cup) | 24183 | 26325 | 26683 | 26399 | 25023 | 26542 | 26163 | 25013 | 24761 | 24664 | 22264 | 22205 | 25018.75 |
| CC (1/2 bag/cup) | 20902 | 22773 | 23379 | 23454 | 24025 | 24689 | 24532 | 24381 | 24307 | 22849 | 22503 | 17927 | 22976.75 |

**Table 34. Day 7 BioTek Readout for HIV Experiment**

| HIV Assay Day 7 | Trial 1 | Trial 2 | Trial 3 | Trial 4 | Trial 5 | Trial 6 | Trial 7 | Trial 8 | Trial 9 | Trial 10 | Trial 11 | Trial 12 | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control (CEM-GFP with HIV) | 32647 | 36731 | 37587 | 37028 | 36306 | 37288 | 35178 | 38106 | 37684 | 34616 | 31746 | 31324 | 35520.08333 |
| EGT (0.1 mmol) | 29845 | 33824 | 34996 | 35188 | 34595 | 32185 | 35924 | 40101 | 43055 | 42192 | 39086 | 33801 | 36232.66667 |
| AP (5 mg/mL) | 32652 | 35374 | 36142 | 36131 | 35858 | 32567 | 32553 | 31567 | 30703 | 30664 | 28430 | 26524 | 32430.41667 |
| CB (1 mg/mL) | 25767 | 26636 | 27315 | 30068 | 28506 | 28182 | 28696 | 29256 | 29357 | 28866 | 29173 | 26650 | 28206 |
| PPC (10 g/cup) | 24486 | 26829 | 27258 | 26866 | 25290 | 26607 | 26309 | 24724 | 24585 | 24477 | 22343 | 22403 | 25181.41667 |
| CC (1/2 bag/cup) | 20512 | 22734 | 23256 | 23318 | 23607 | 24525 | 24601 | 23978 | 23786 | 22723 | 22180 | 17890 | 22759.16667 |

## APPENDIX B

## GRAPHS FOR EACH DAY, DAILY AVERAGES, AND DAILY TRENDS OF CYTOTOXICITY ASSAY READINGS AND HIV EXPERIMENT READINGS

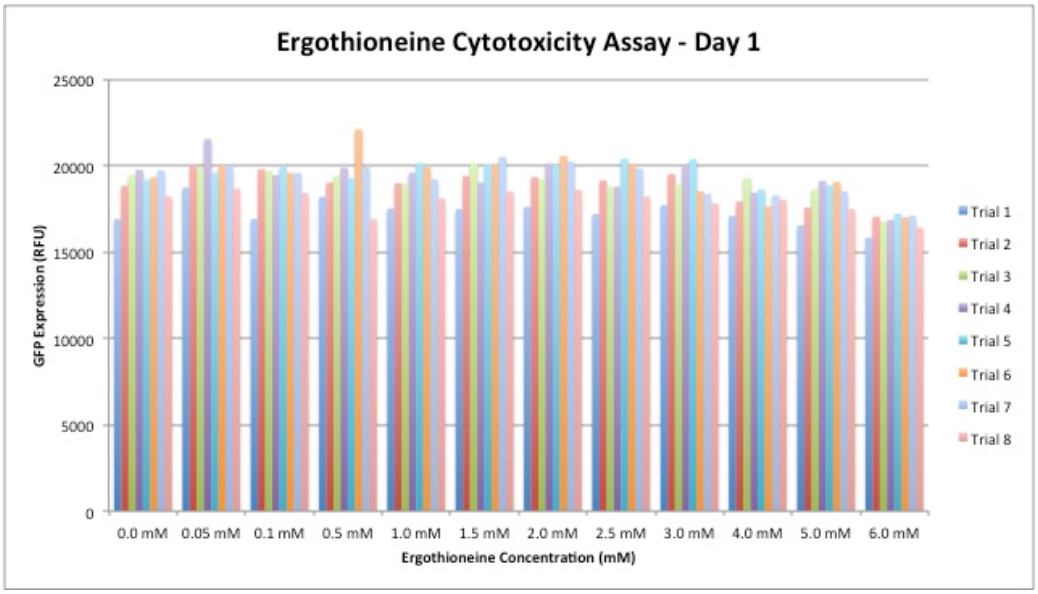


**Figure 10. Day 1 Daily Readings Graph for Ergothioneine Cytotoxicity Assay**

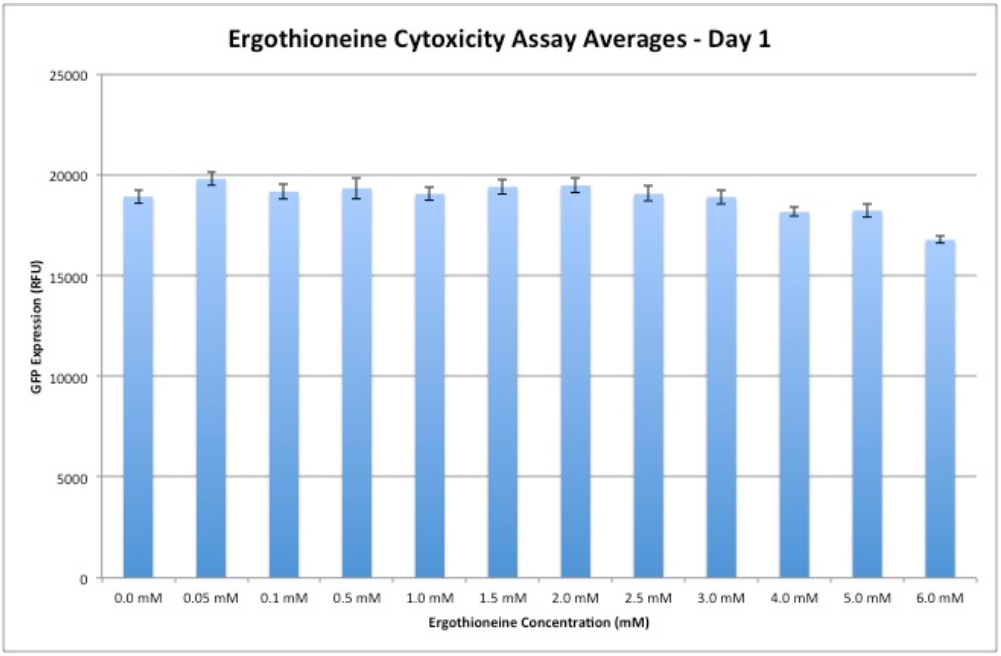


**Figure 11. Day 1 Daily Averages Graph for Ergothioneine Cytotoxicity Assay**

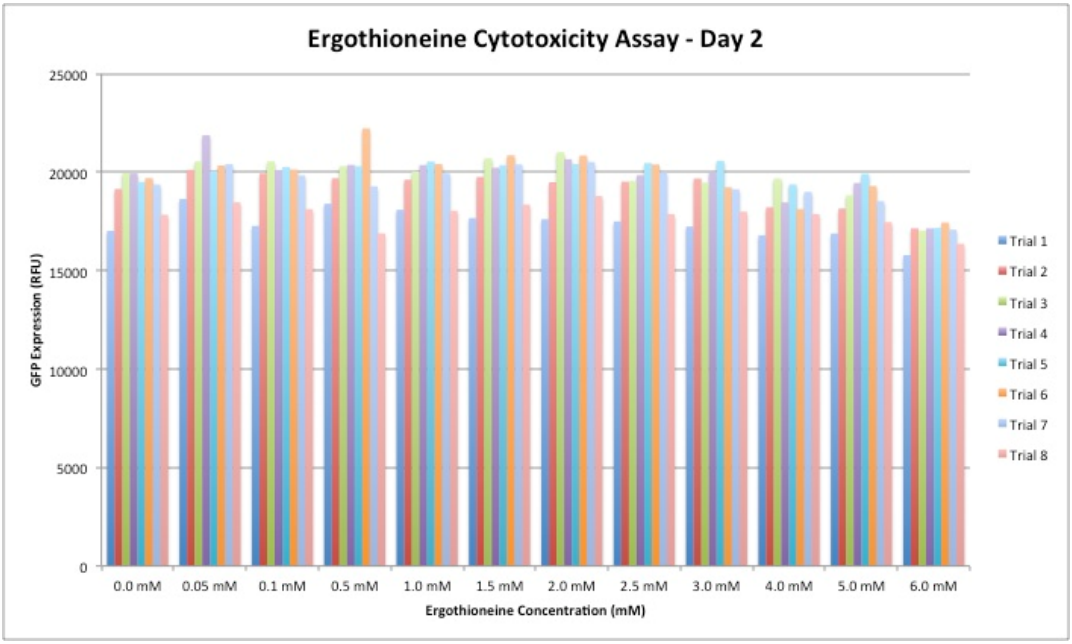


**Figure 12. Day 2 Daily Readings Graph for Ergothioneine Cytotoxicity Assay**

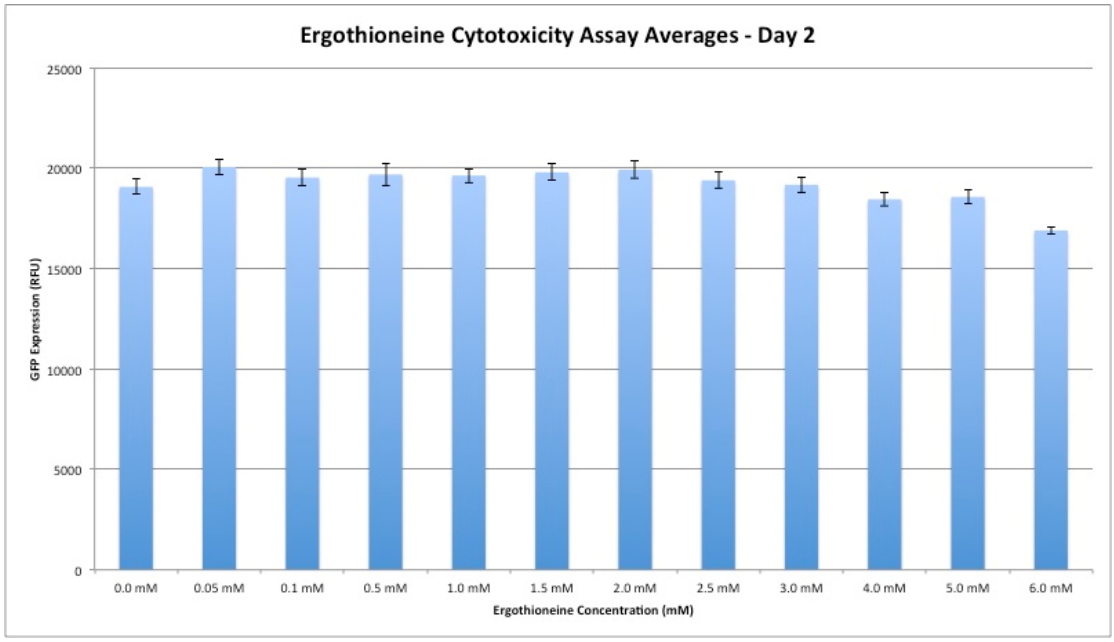


**Figure 13. Day 2 Daily Averages Graph for Ergothioneine Cytotoxicity Assay**

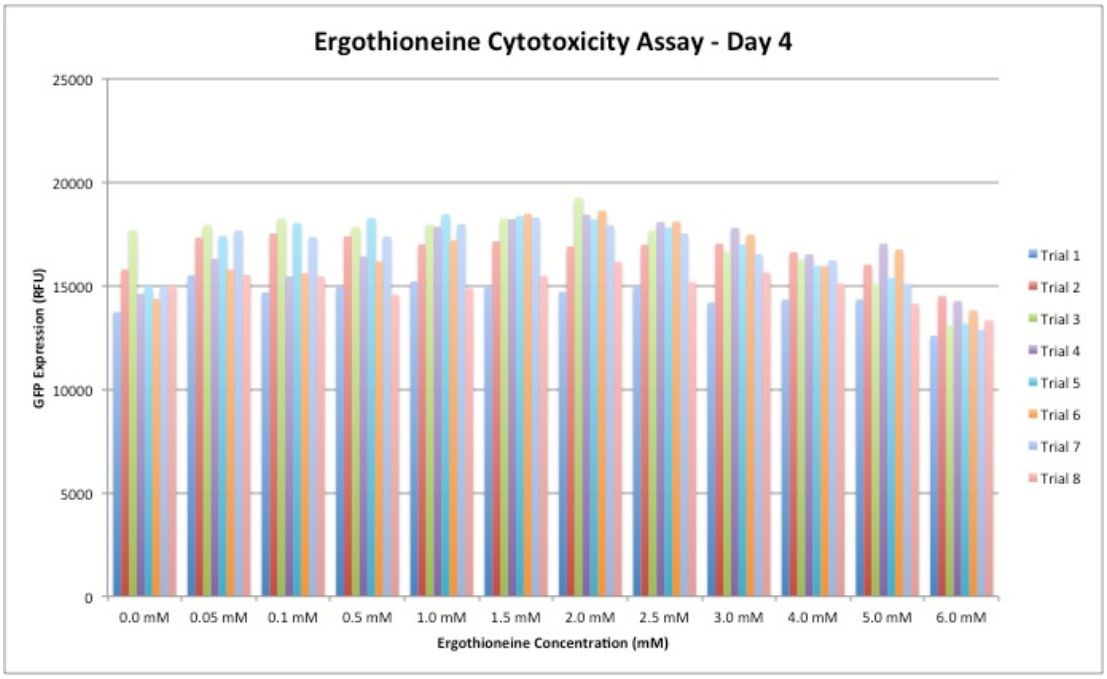


**Figure 14. Day 4 Daily Readings Graph for Ergothioneine Cytotoxicity Assay**

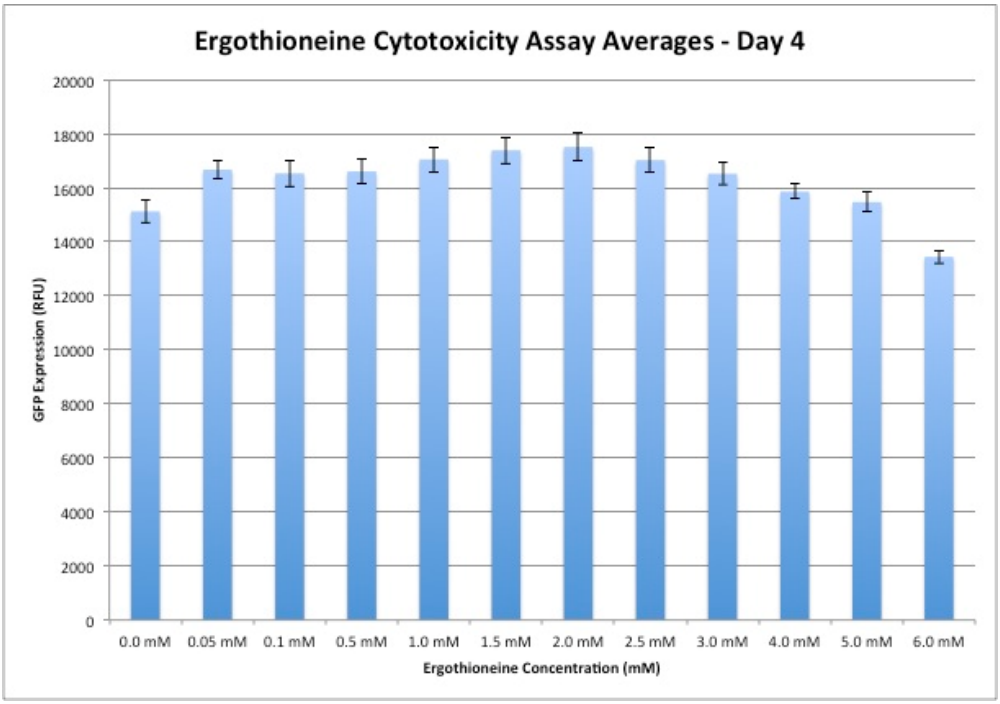


**Figure 15. Day 4 Daily Averages Graph for Ergothioneine Cytotoxicity Assay**

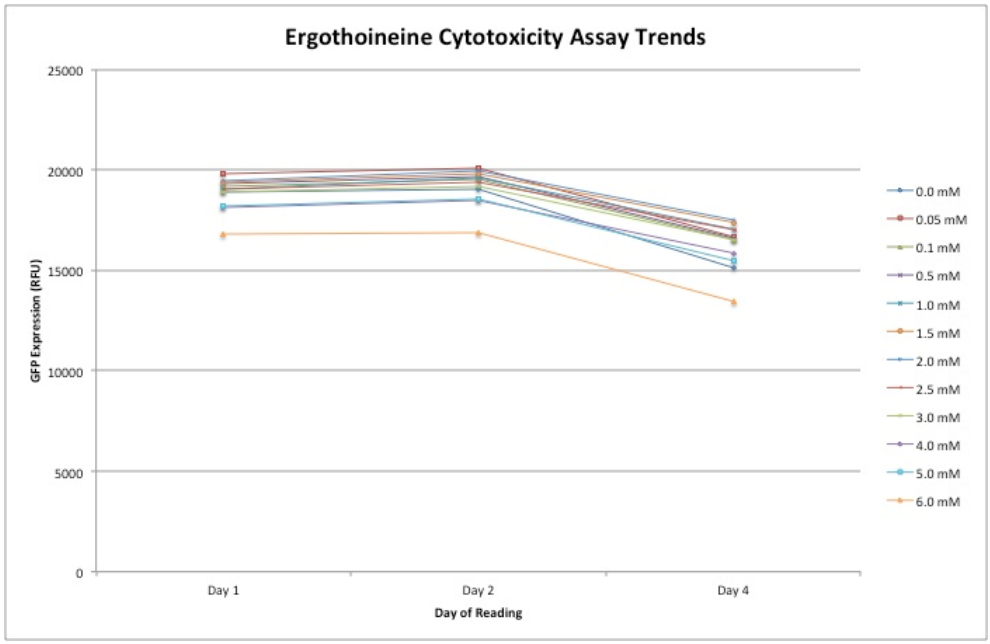


**Figure 16. Daily Trends for Ergothioneine Cytotoxicity Assay**

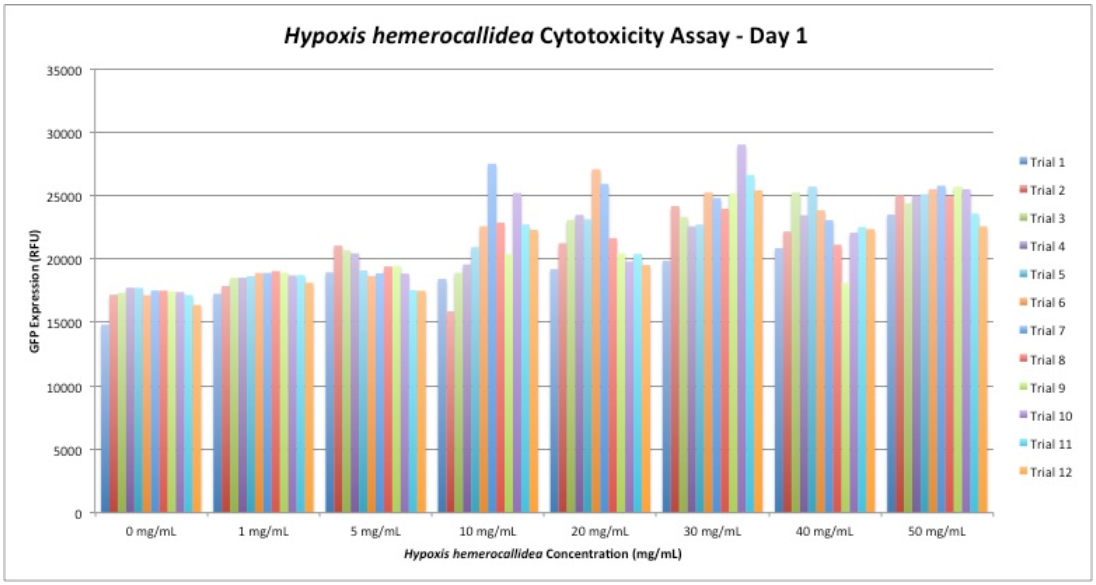


**Figure 17. Day 1 Daily Readings Graph for *Hypoxis hemerocallidea* Cytotoxicity Assay**

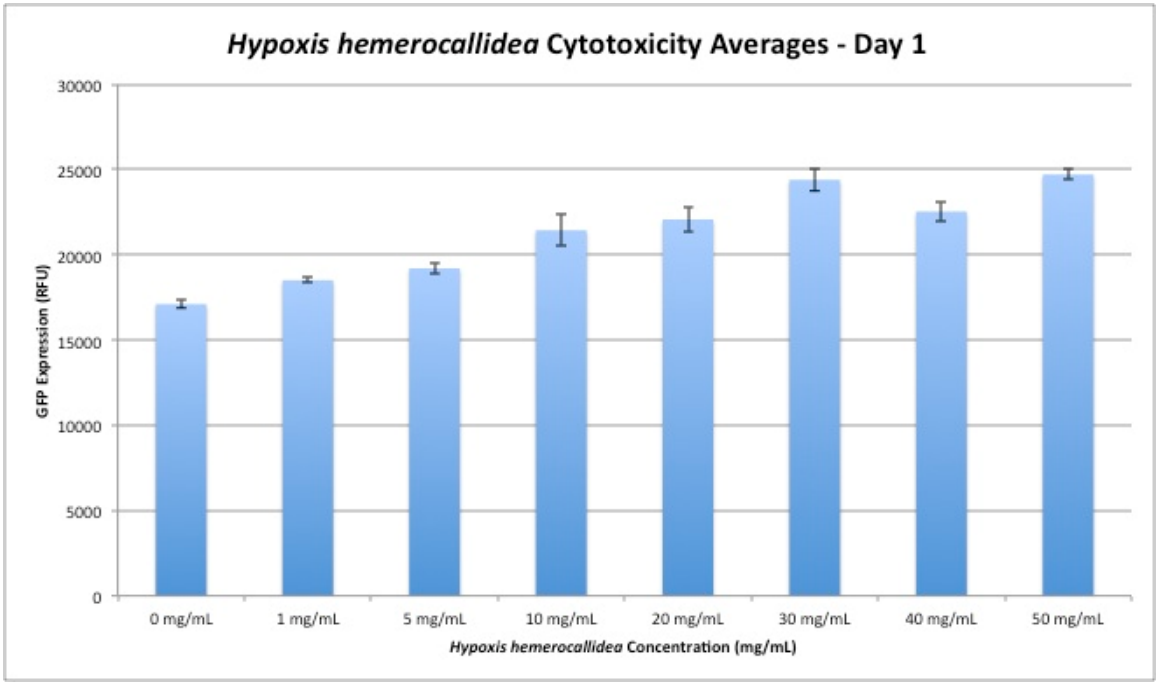


**Figure 18. Day 1 Daily Averages Graph for *Hypoxis hemerocallidea* Cytotoxicity Assay**

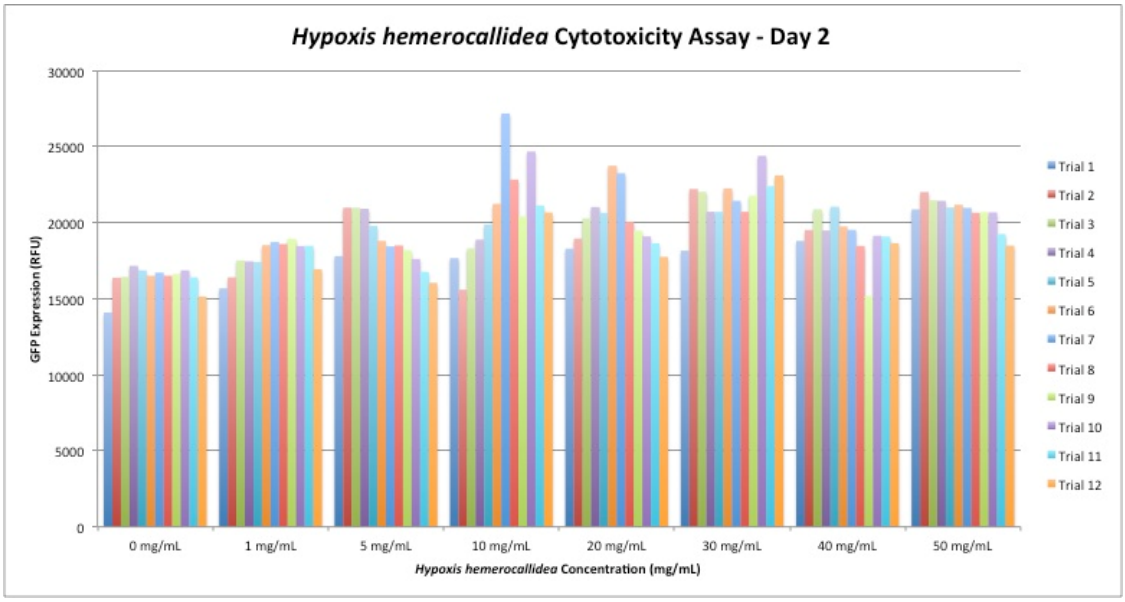


**Figure 19. Day 2 Daily Readings Graph for *Hypoxis hemerocallidea* Cytotoxicity Assay**

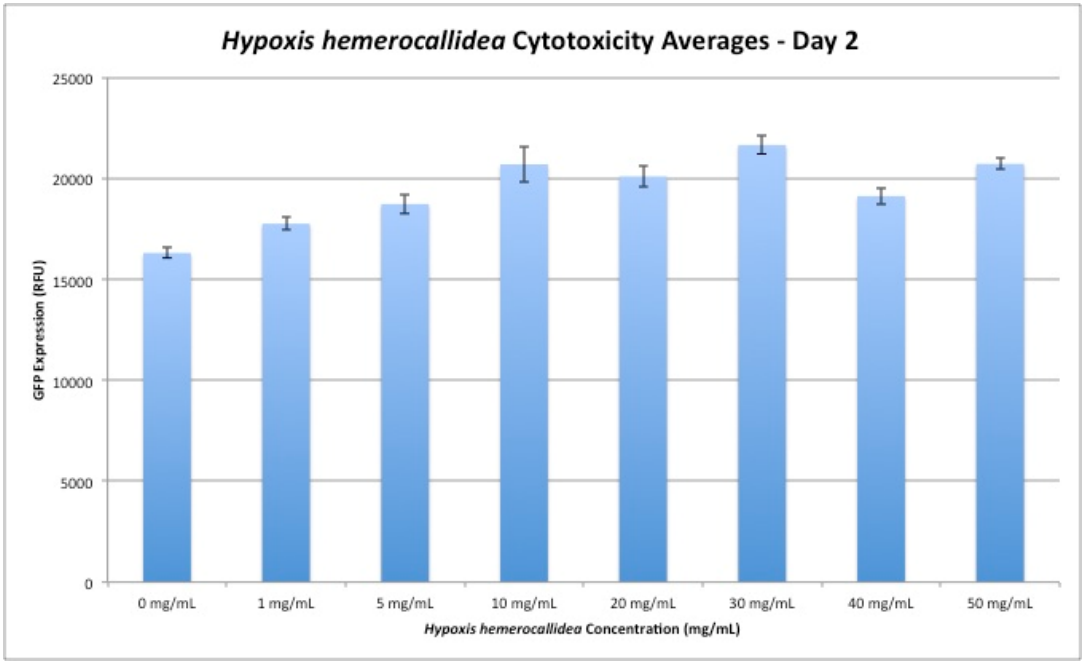


**Figure 20. Day 2 Daily Averages Graph for *Hypoxis hemerocallidea* Cytotoxicity Assay**

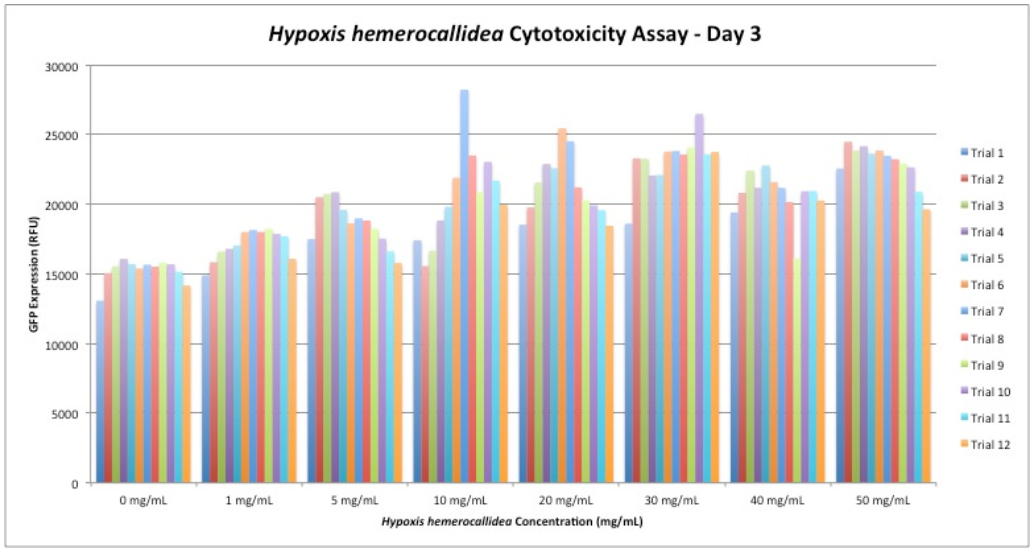


**Figure 21. Day 3 Daily Readings Graph for *Hypoxis hemerocallidea* Cytotoxicity Assay**

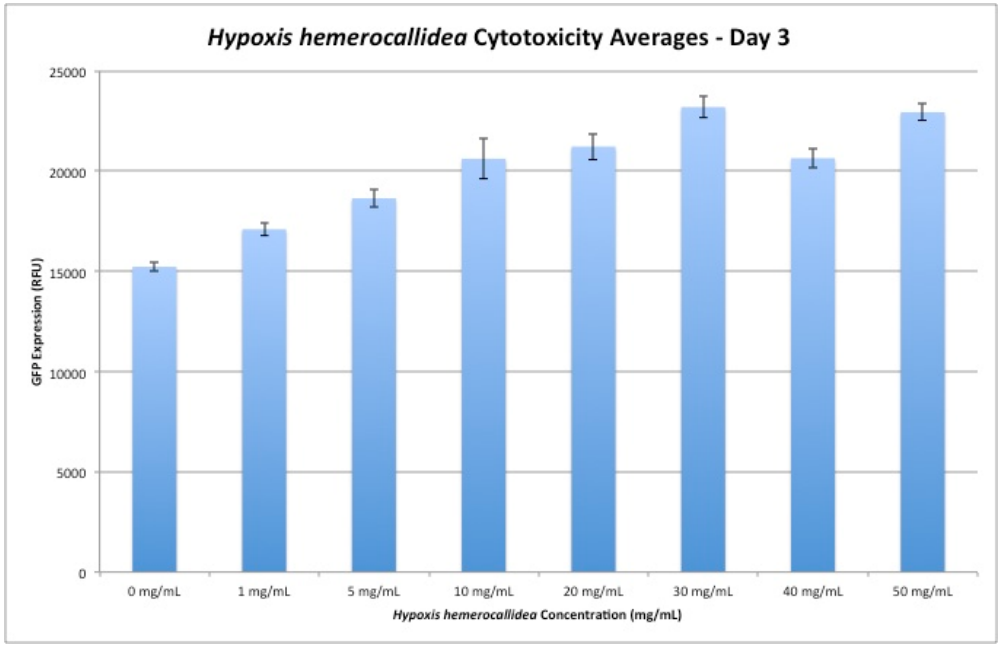


**Figure 22. Day 3 Daily Averages Graph for *Hypoxis hemerocallidea* Cytotoxicity Assay**

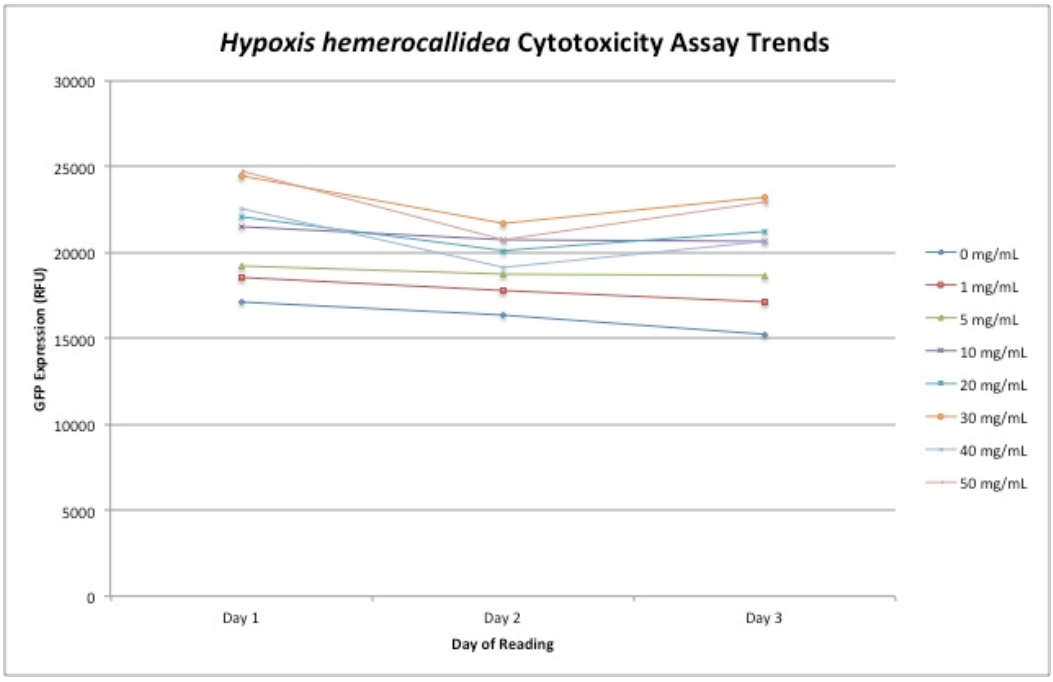


**Figure 23. Daily Trends Graph for *Hypoxis hemerocallidea* Cytotoxicity Assay**

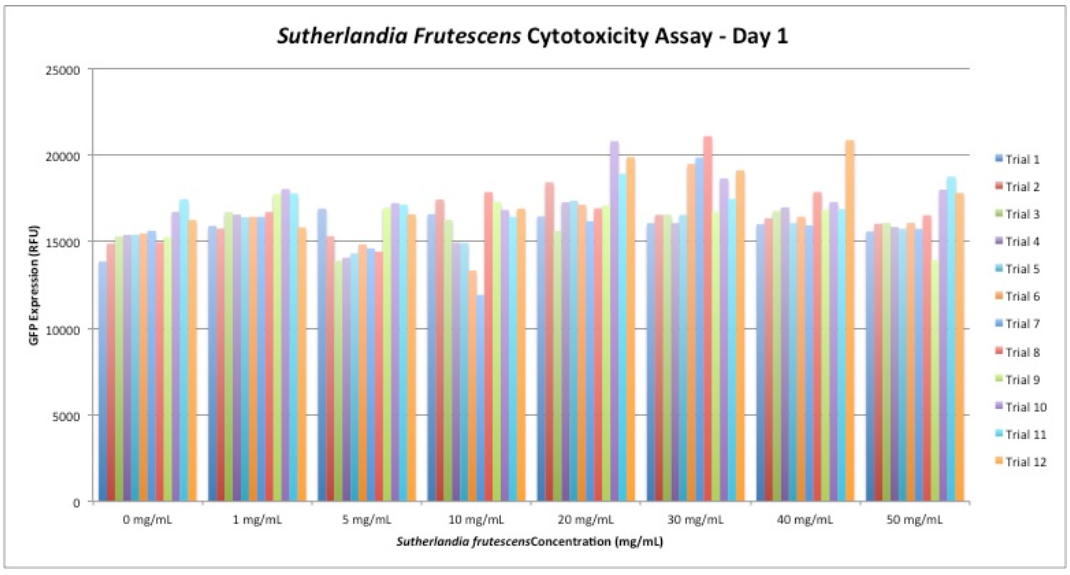


**Figure 24. Day 1 Daily Readings Graph for *Sutherlandia frutescens* Cytotoxicity Assay**

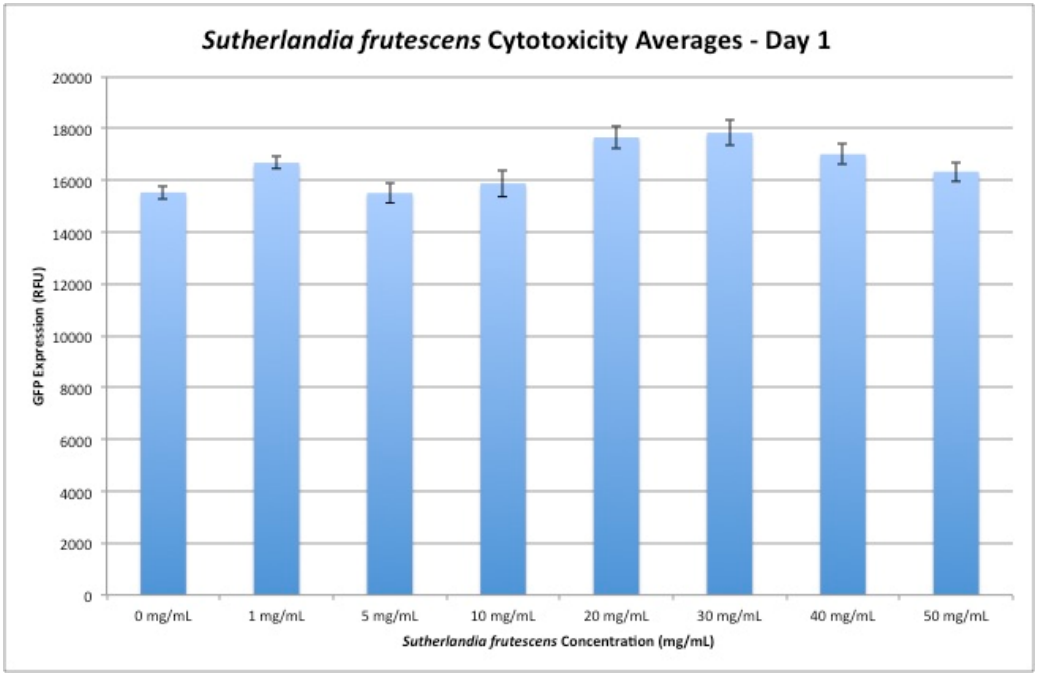


**Figure 25. Day 1 Daily Averages Graph for *Sutherlandia frutescens* Cytotoxicity Assay**

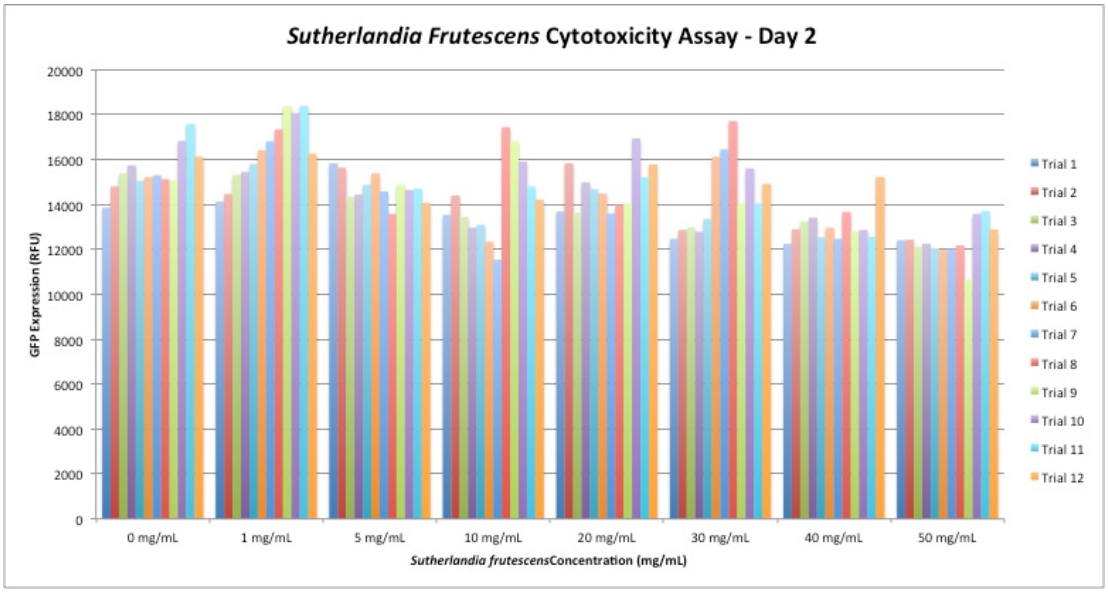


**Figure 26. Day 2 Daily Readings Graph for *Sutherlandia frutescens* Cytotoxicity Assay**

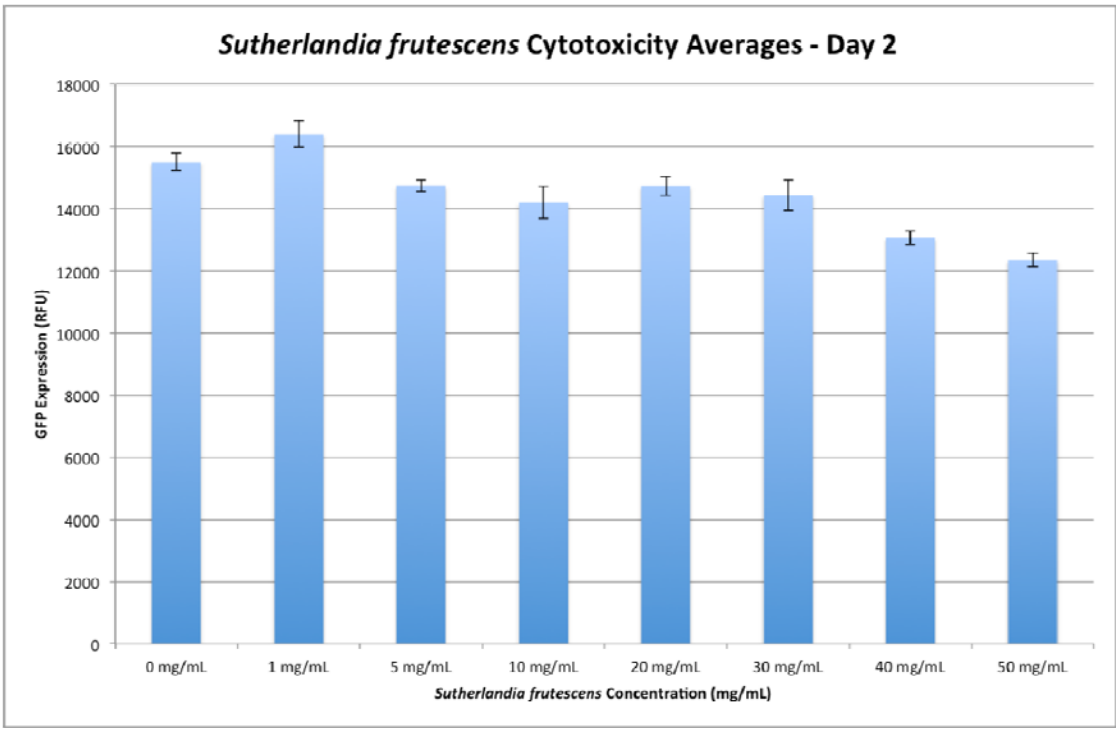


**Figure 27. Day 2 Daily Averages Graph for *Sutherlandia frutescens* Cytotoxicity Assay**

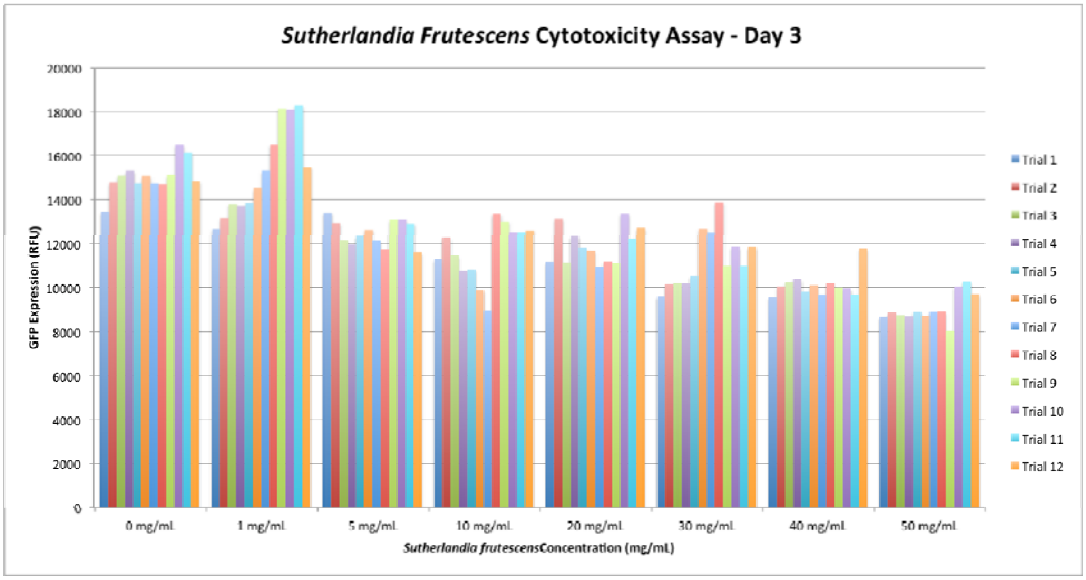


**Figure 28. Day 3 Daily Readings Graph for *Sutherlandia frutescens* Cytotoxicity Assay**

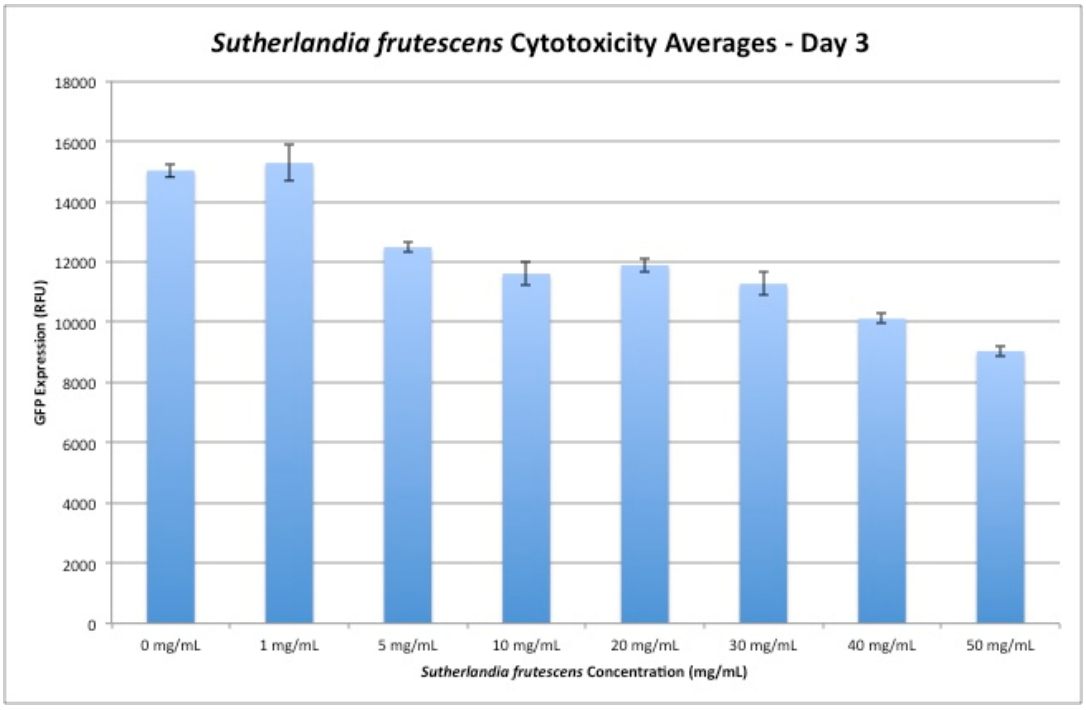


**Figure 29. Day 3 Daily Averages Graph for *Sutherlandia frutescens* Cytotoxicity Assay**

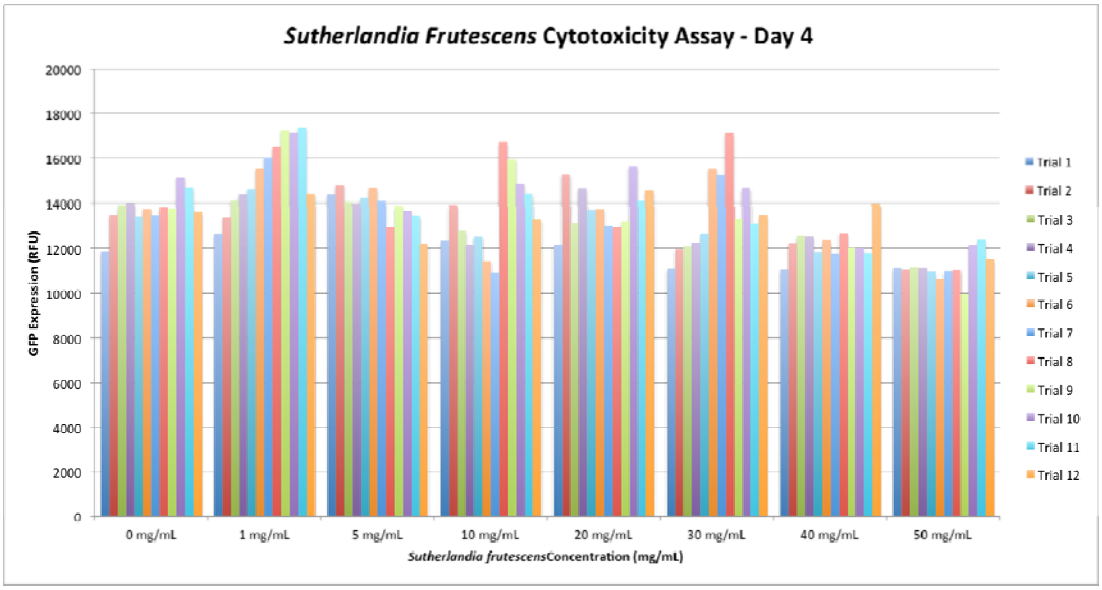


**Figure 30. Day 4 Daily Readings Graph for *Sutherlandia frutescens* Cytotoxicity Assay**

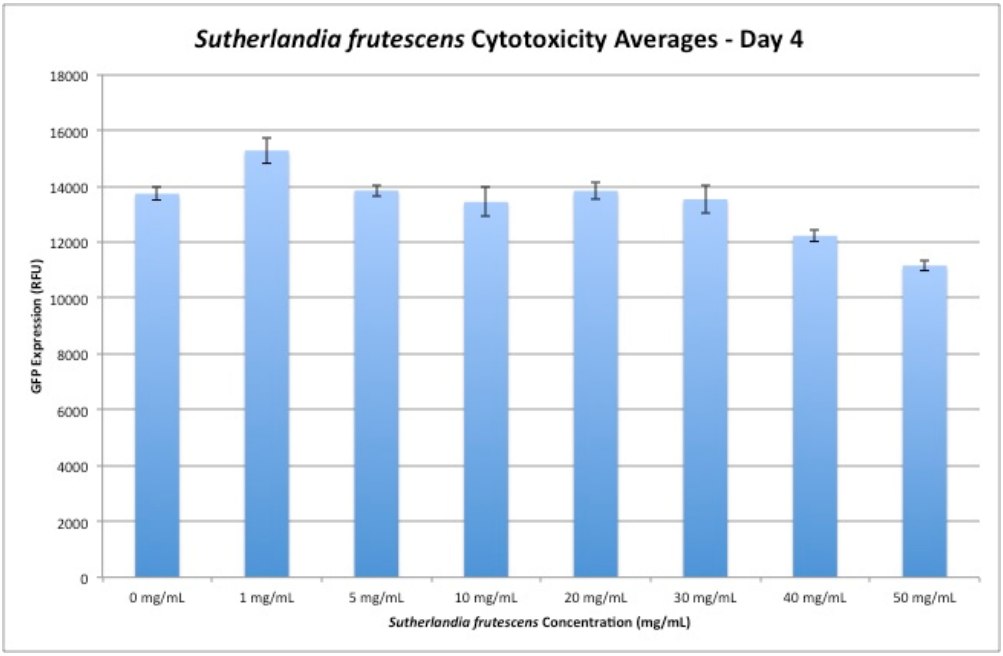


**Figure 31. Day 4 Daily Averages Graph for *Sutherlandia frutescens* Cytotoxicity Assay**

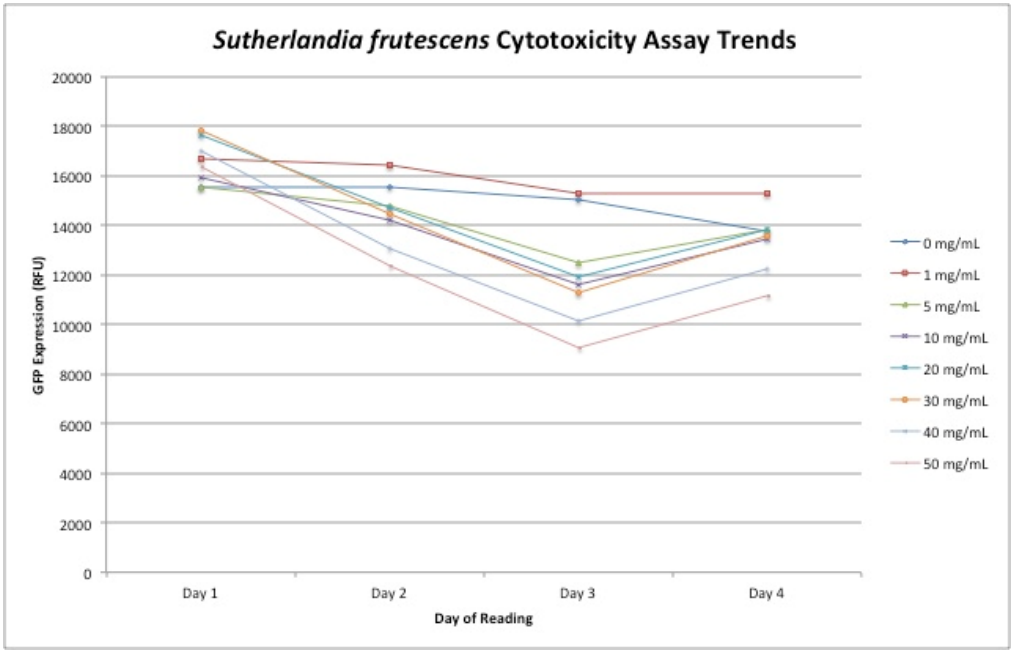


**Figure 32. Daily Trends Graph for *Sutherlandia frutescens* Cytotoxicity Assay**

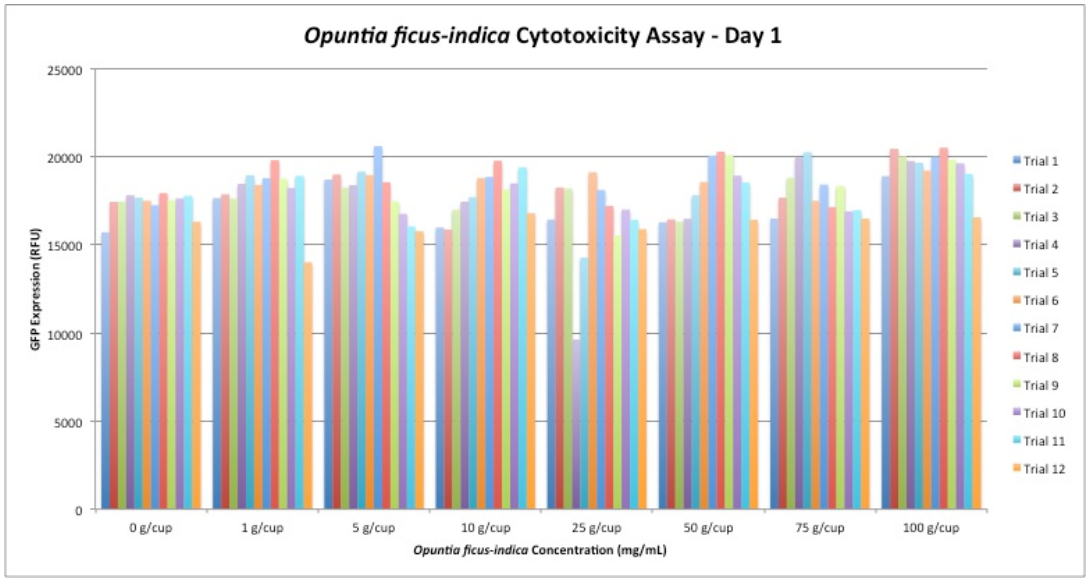


**Figure 33. Day 1 Daily Readings Graph for *Opuntia ficus-indica* Cytotoxicity Assay**

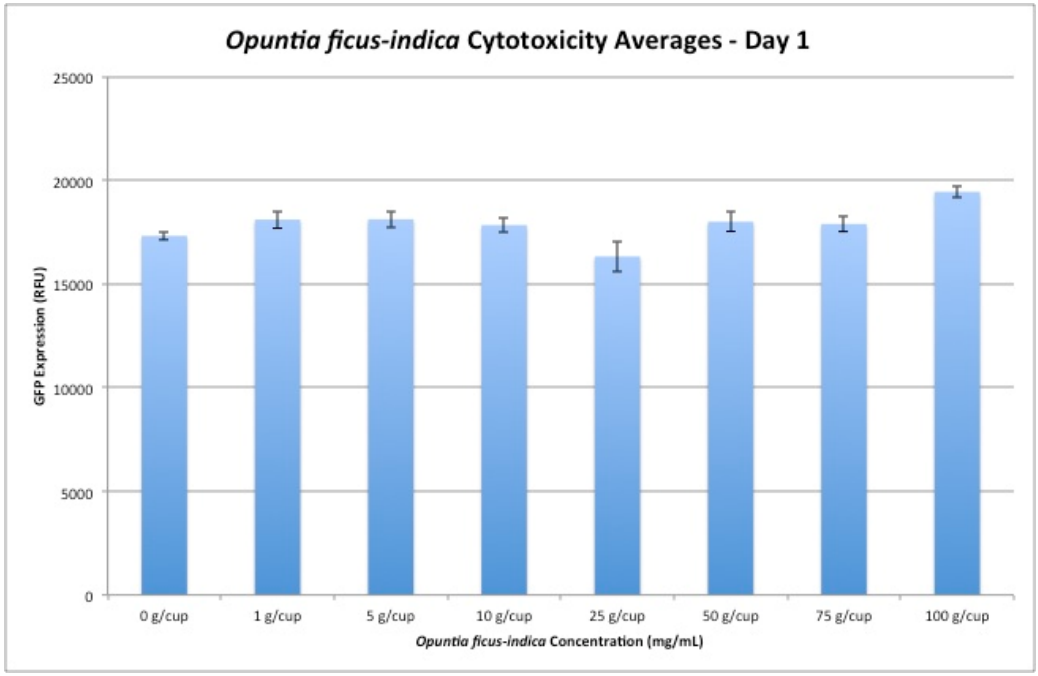


**Figure 34. Day 1 Daily Averages Graph for *Opuntia ficus-indica* Cytotoxicity Assay**

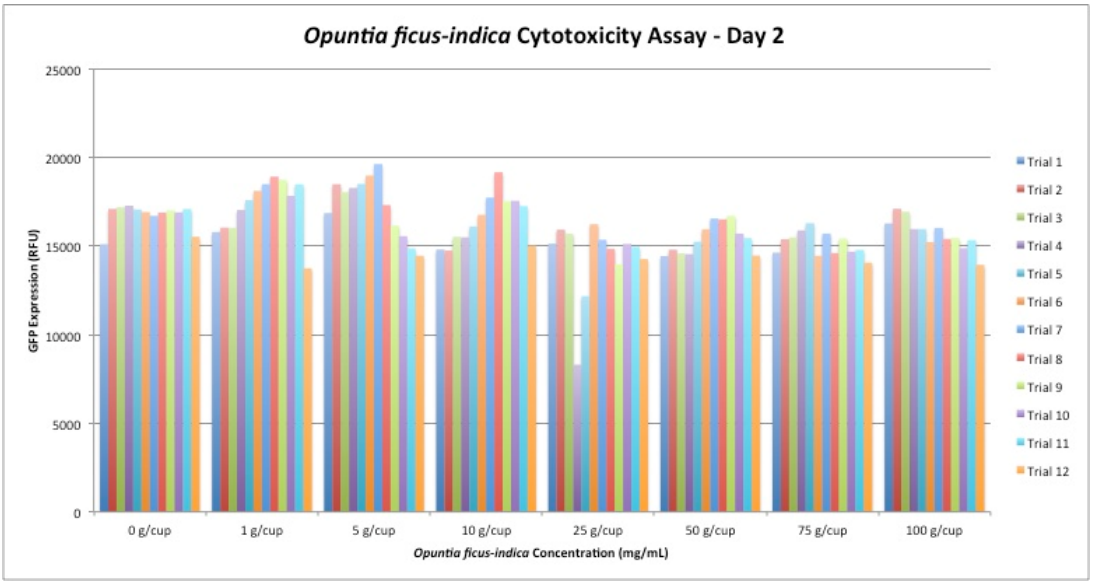


**Figure 35. Day 2 Daily Readings Graph for *Opuntia ficus-indica* Cytotoxicity Assay**

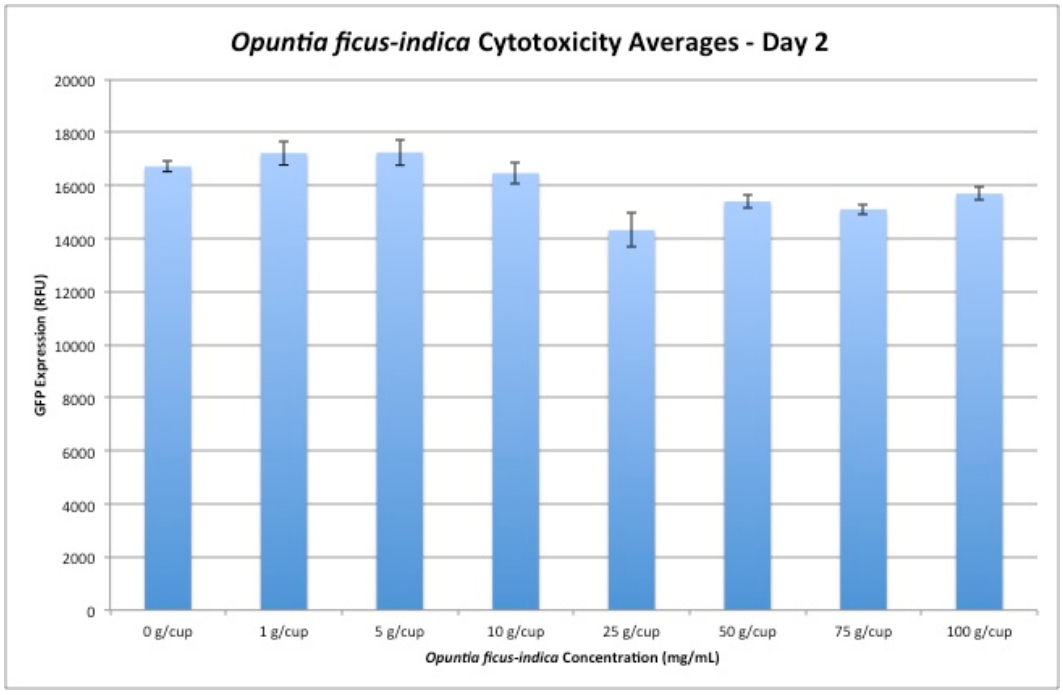


**Figure 36. Day 2 Daily Averages Graph for *Opuntia ficus-indica* Cytotoxicity Assay**

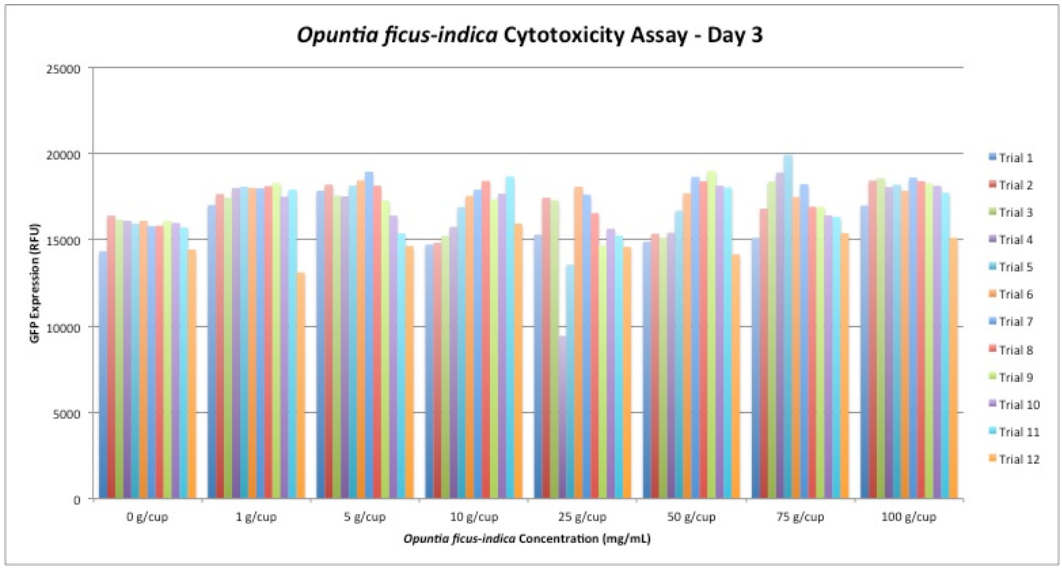


**Figure 37. Day 3 Daily Readings Graph for *Opuntia ficus-indica* Cytotoxicity Assay**

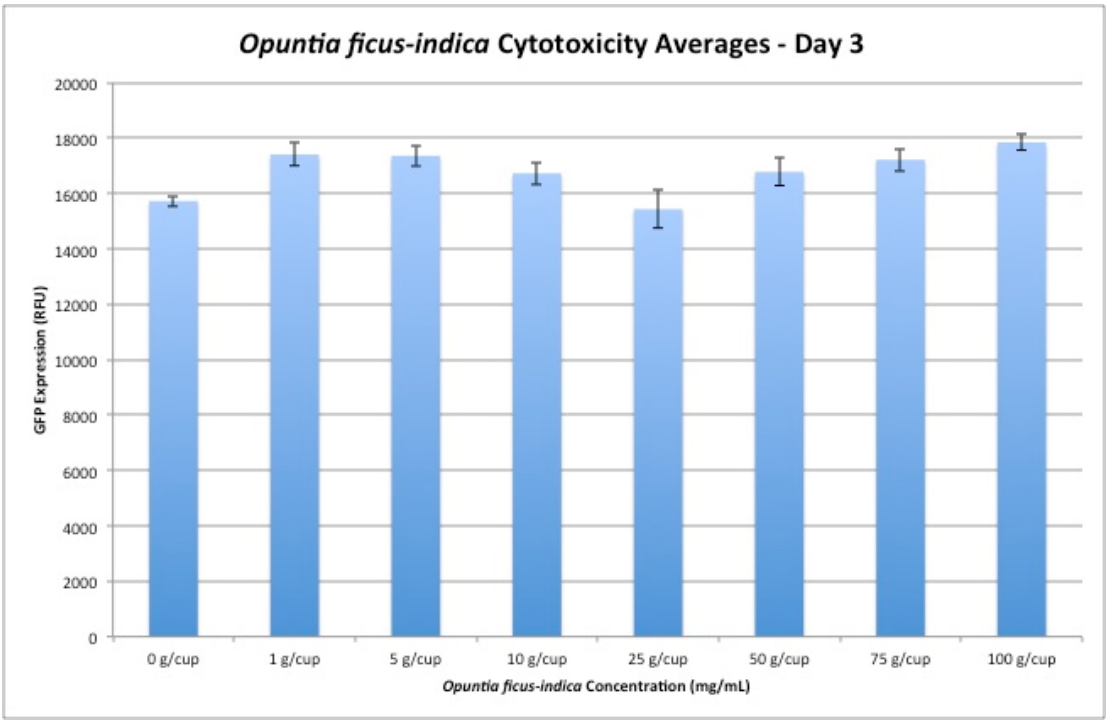


**Figure 38. Day 3 Daily Averages Graph for *Opuntia ficus-indica* Cytotoxicity Assay**

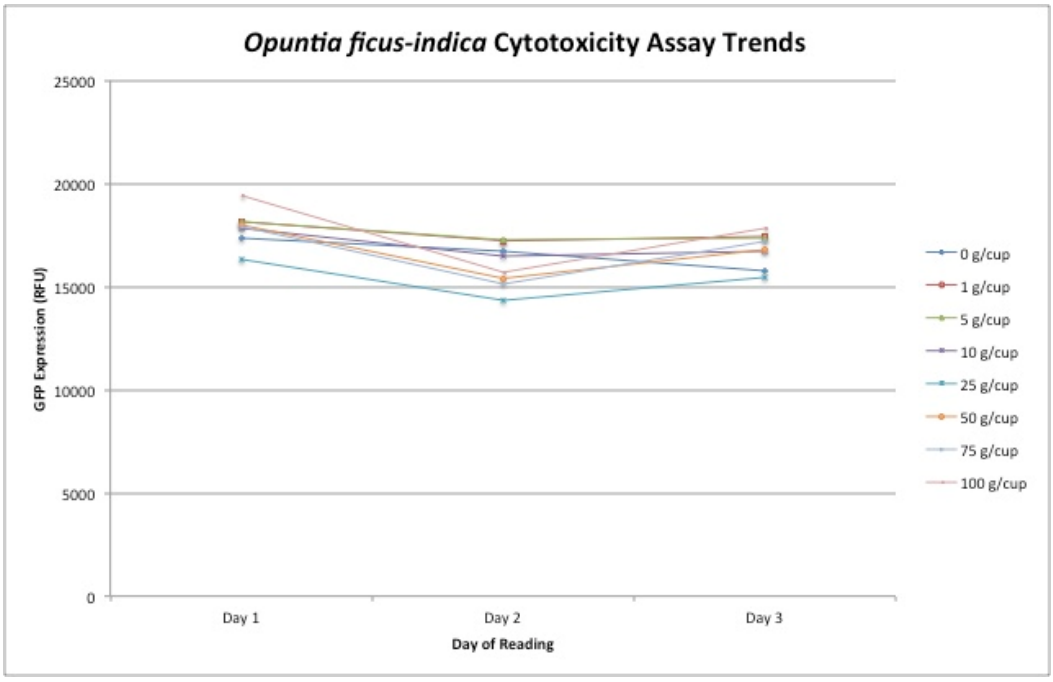


**Figure 39. Daily Trends Graph for *Opuntia ficus-indica* Cytotoxicity Assay**

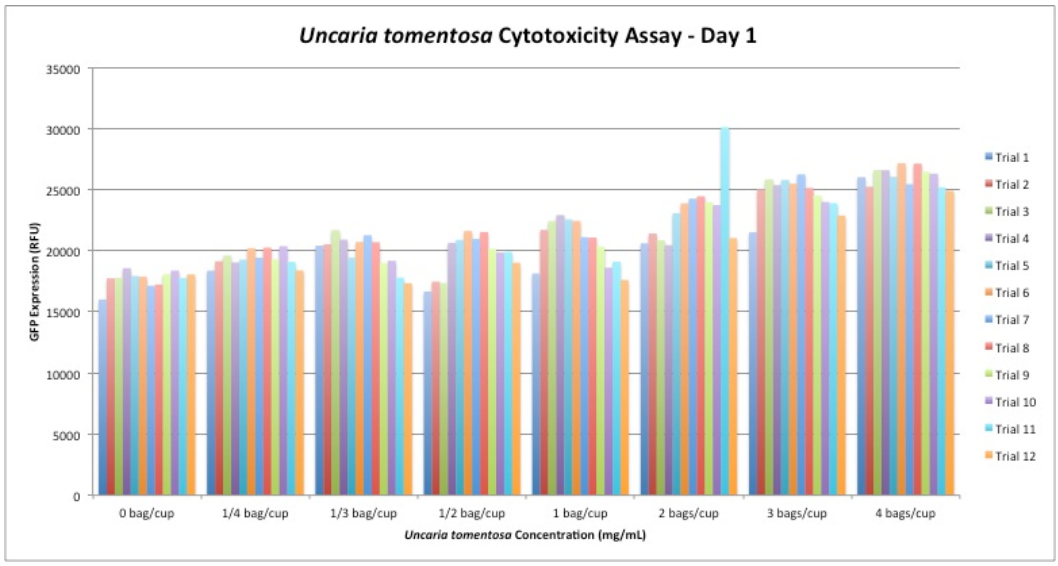


**Figure 40. Day 1 Daily Readings Graph for *Uncaria tomentosa* Cytotoxicity Assay**

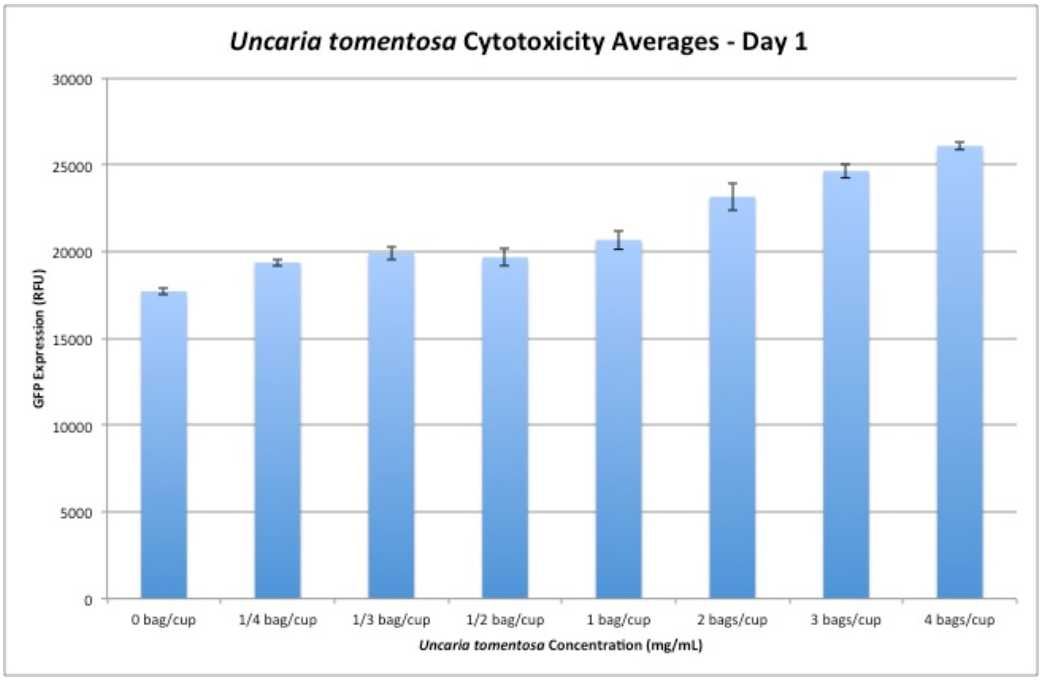


**Figure 41. Day 1 Daily Averages Graph for *Uncaria tomentosa* Cytotoxicity Assay**

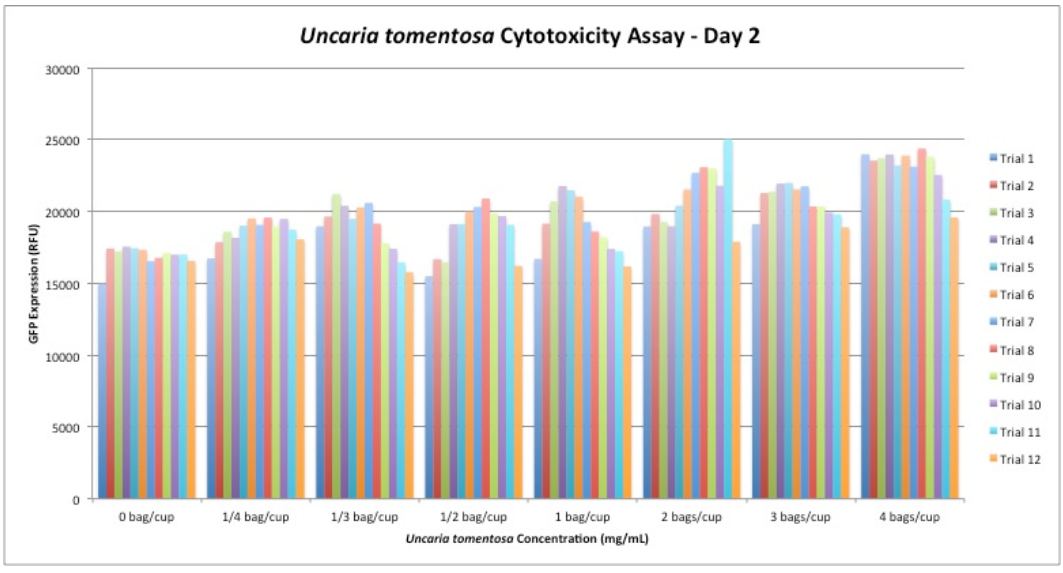


**Figure 42. Day 2 Daily Readings Graph for *Uncaria tomentosa* Cytotoxicity Assay**

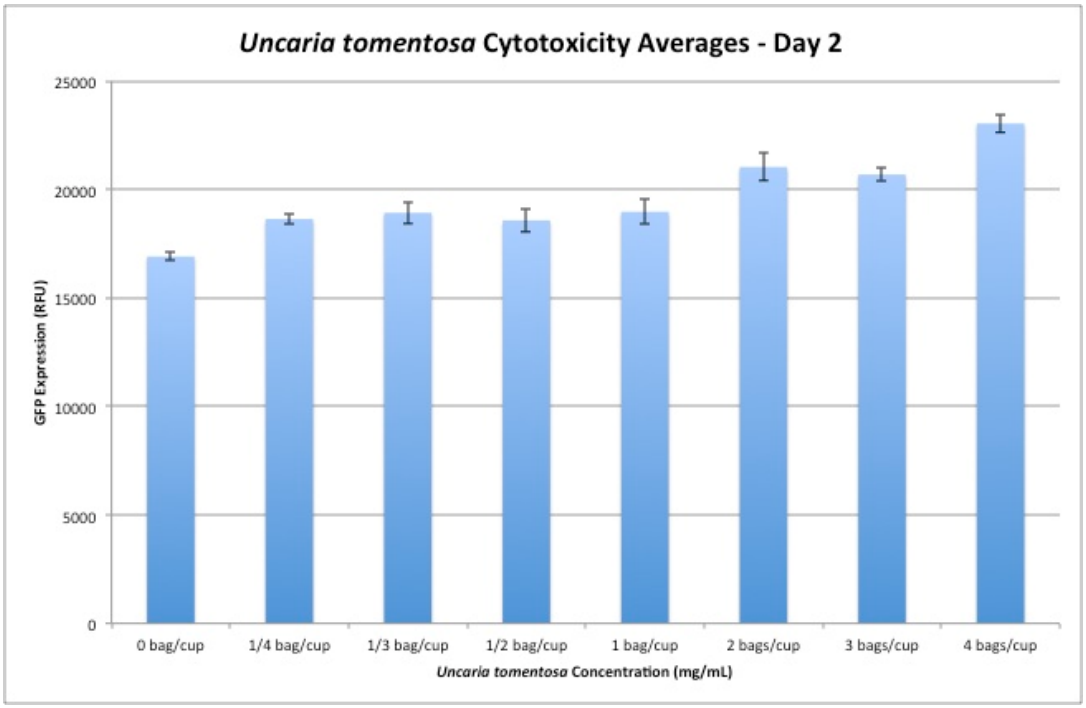


**Figure 43. Day 2 Daily Averages Graph for *Uncaria tomentosa* Cytotoxicity Assay**

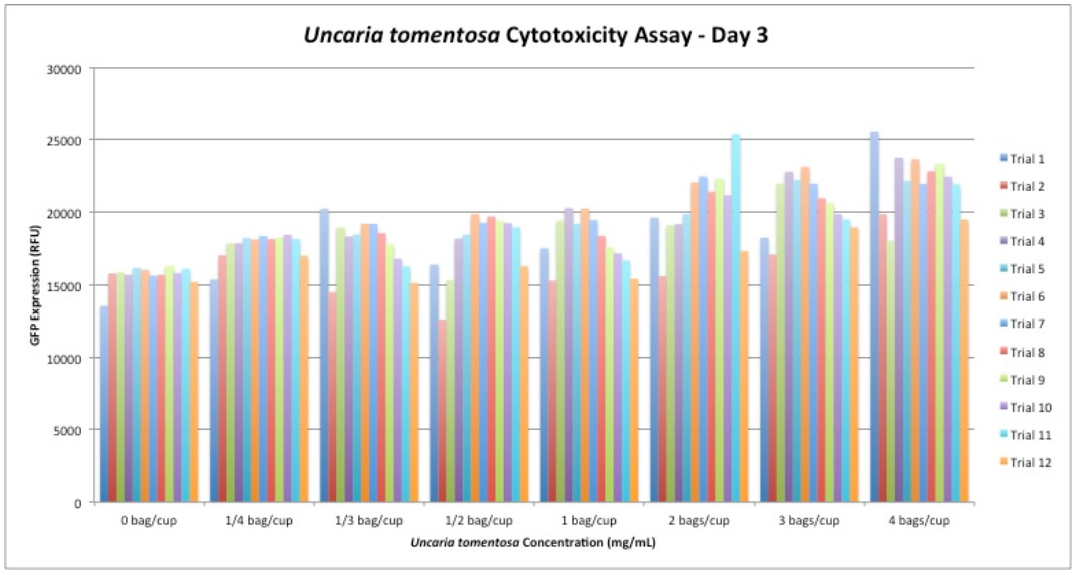


**Figure 44. Day 3 Daily Readings Graph for *Uncaria tomentosa* Cytotoxicity Assay**

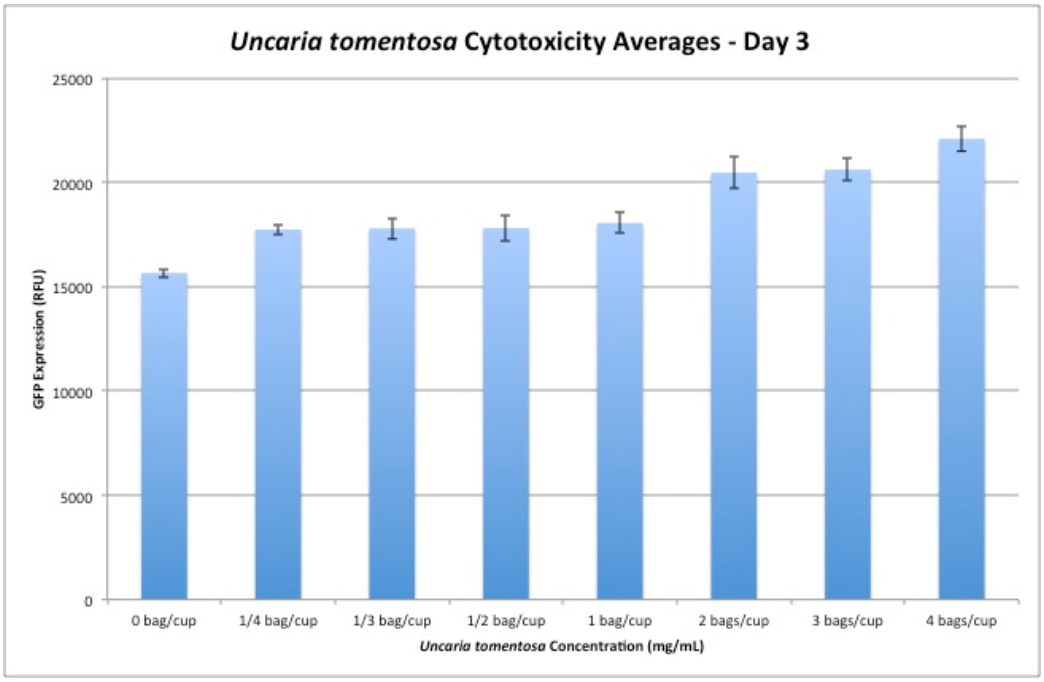


**Figure 45. Day 3 Daily Averages Graph for *Uncaria tomentosa* Cytotoxicity Assay**

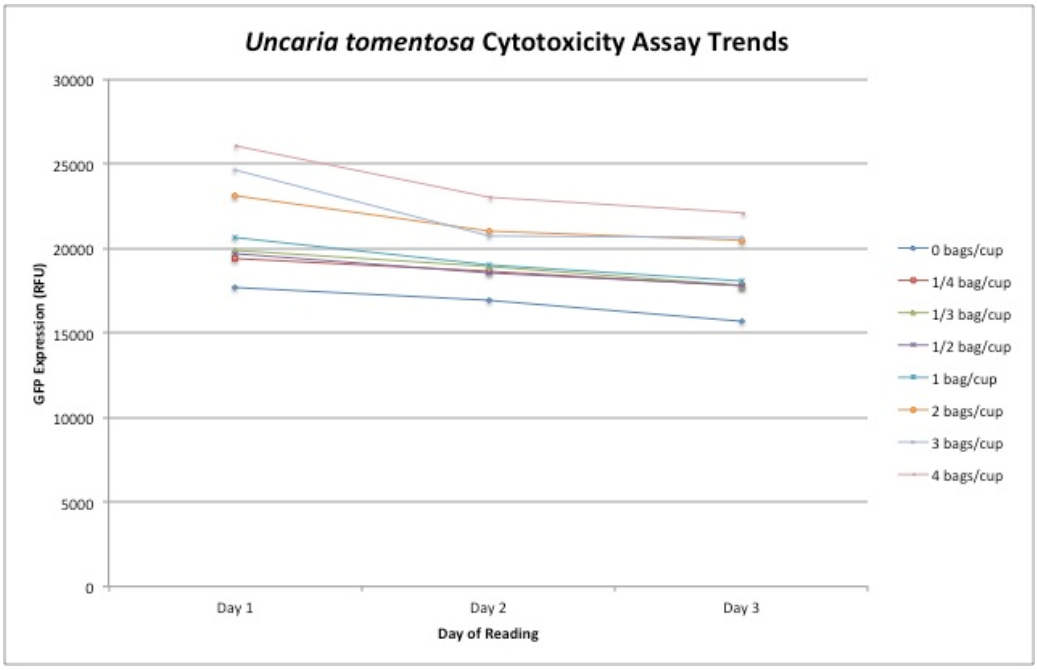


**Figure 46. Daily Trends Graph for *Uncaria tomentosa* Cytotoxicity Assay**

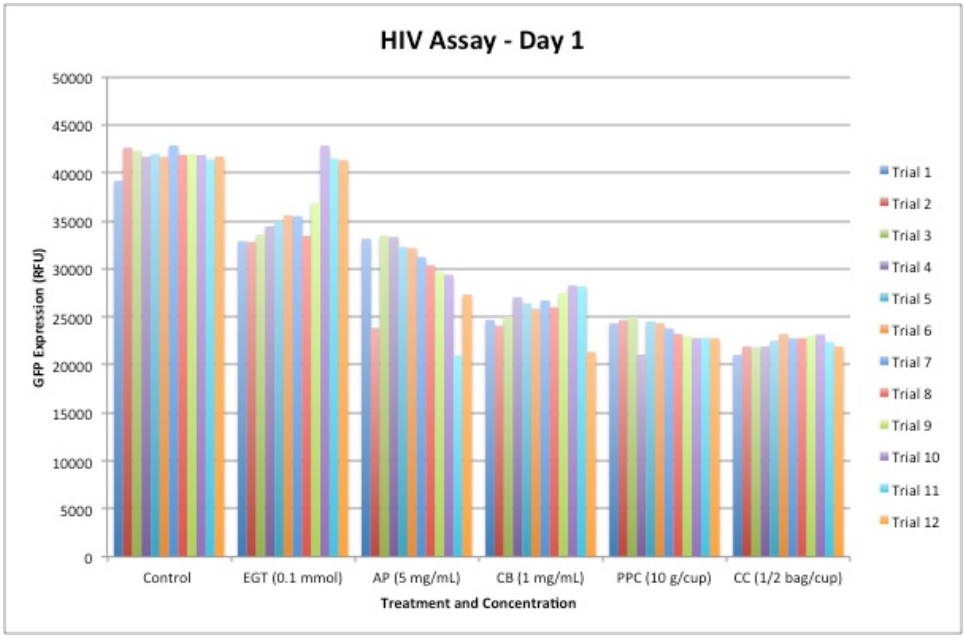


**Figure 47. Day 1 Daily Readings Graph for HIV Experiment**

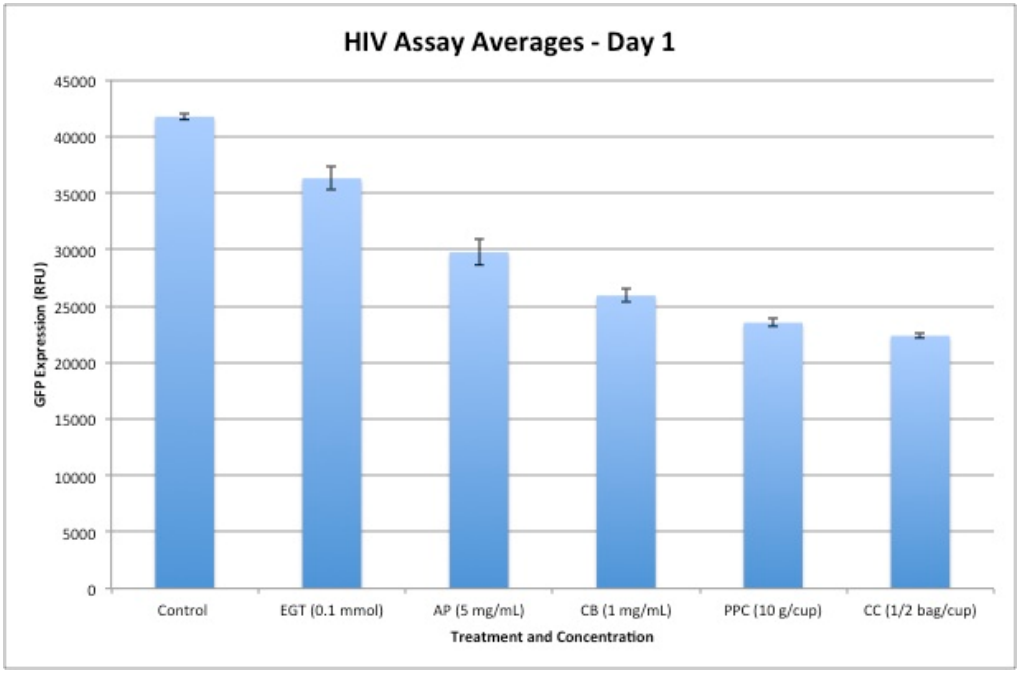


**Figure 48. Day 1 Daily Averages Graph for HIV Experiment**

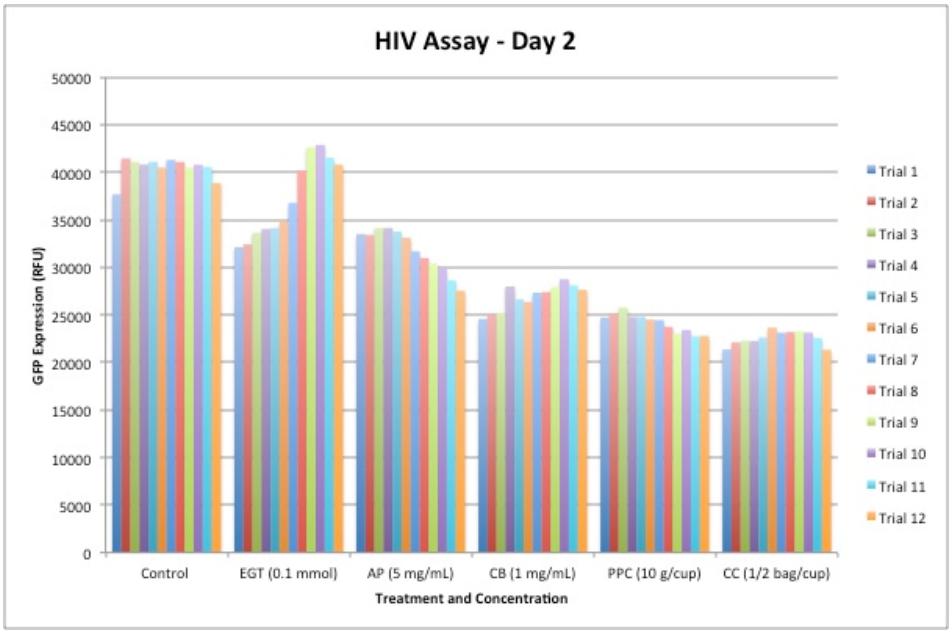


**Figure 49. Day 2 Daily Readings Graph for HIV Experiment**

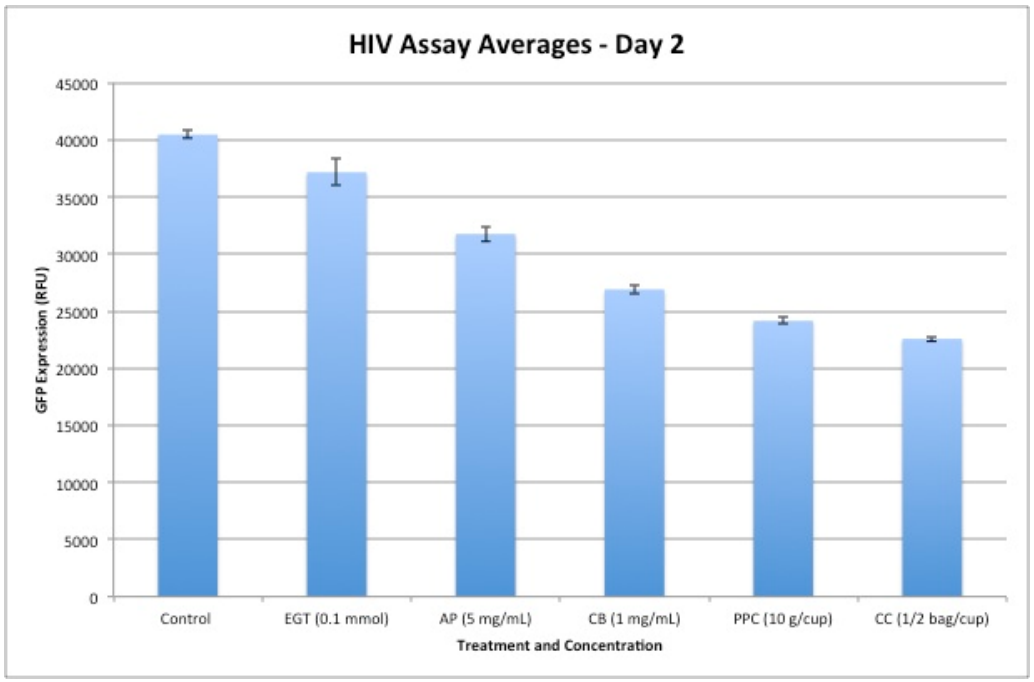


**Figure 50. Day 2 Daily Averages Graph for HIV Experiment**

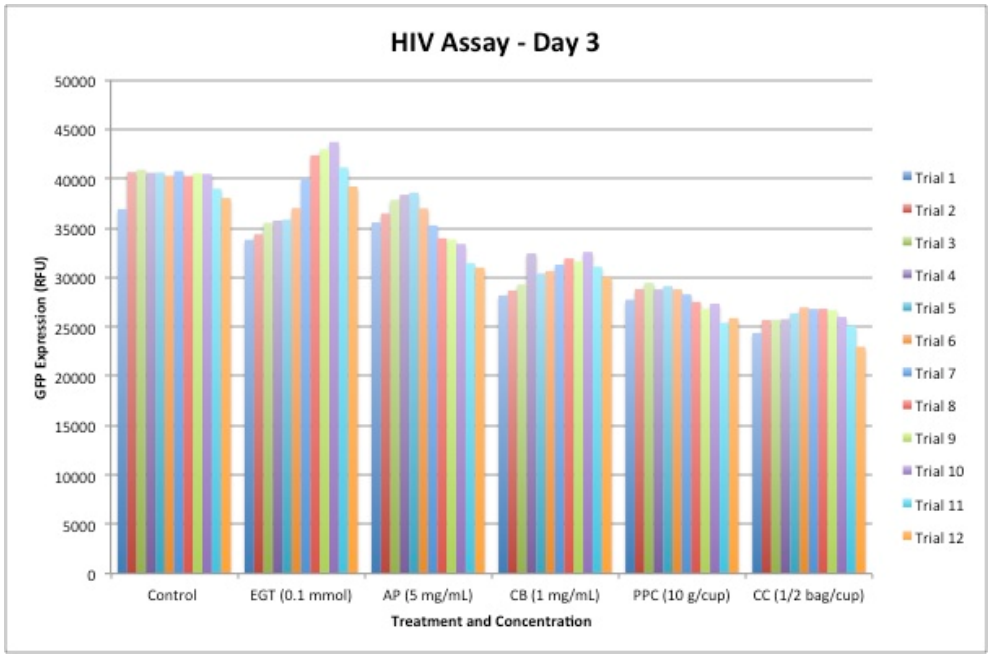


**Figure 51. Day 3 Daily Readings Graph for HIV Experiment**

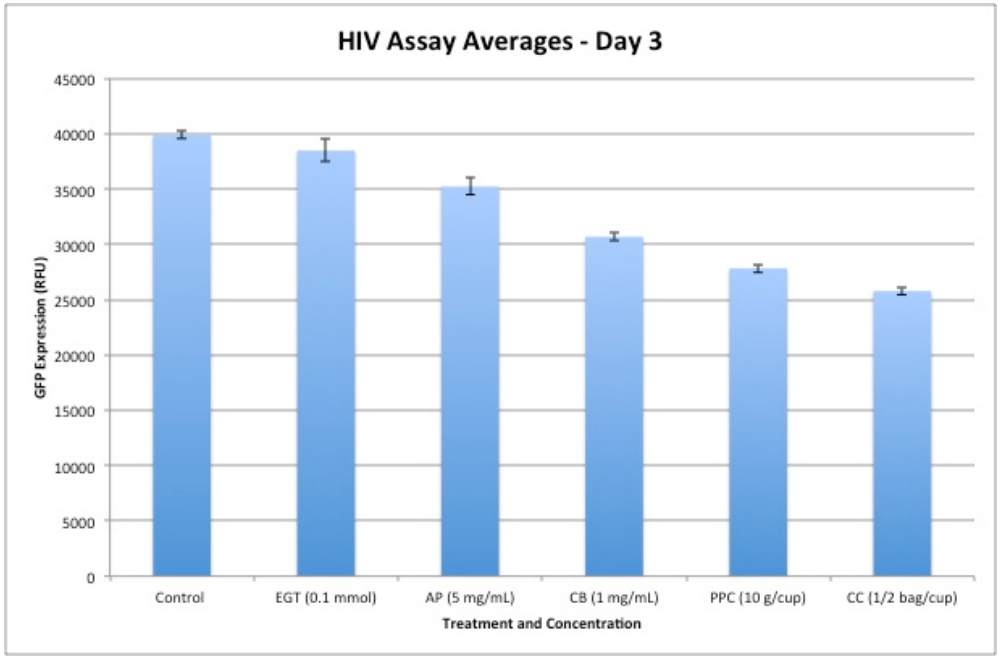


**Figure 52. Day 3 Daily Averages Graph for HIV Experiment**

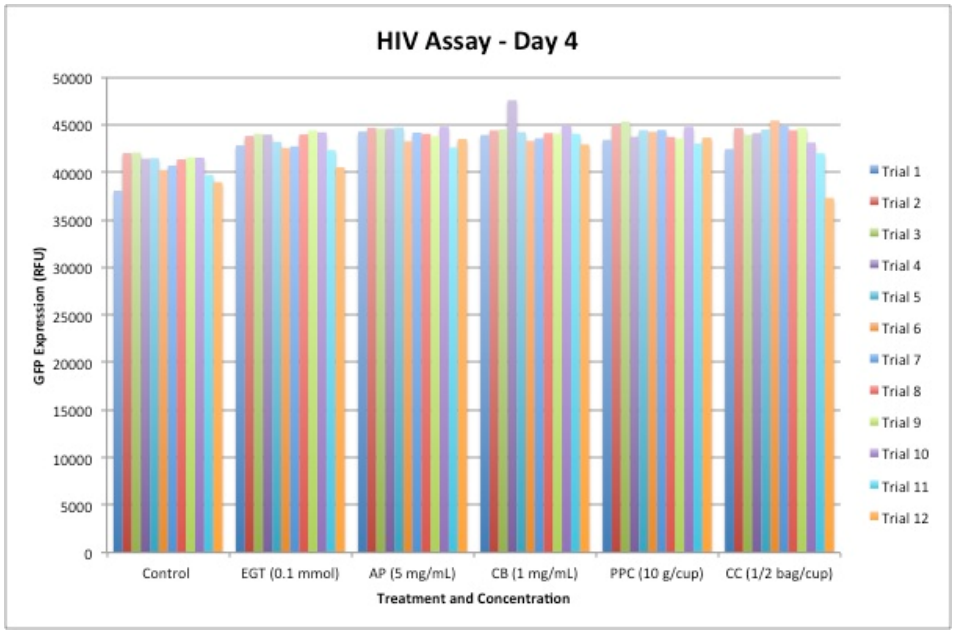


**Figure 53. Day 4 Daily Readings Graph for HIV Experiment**

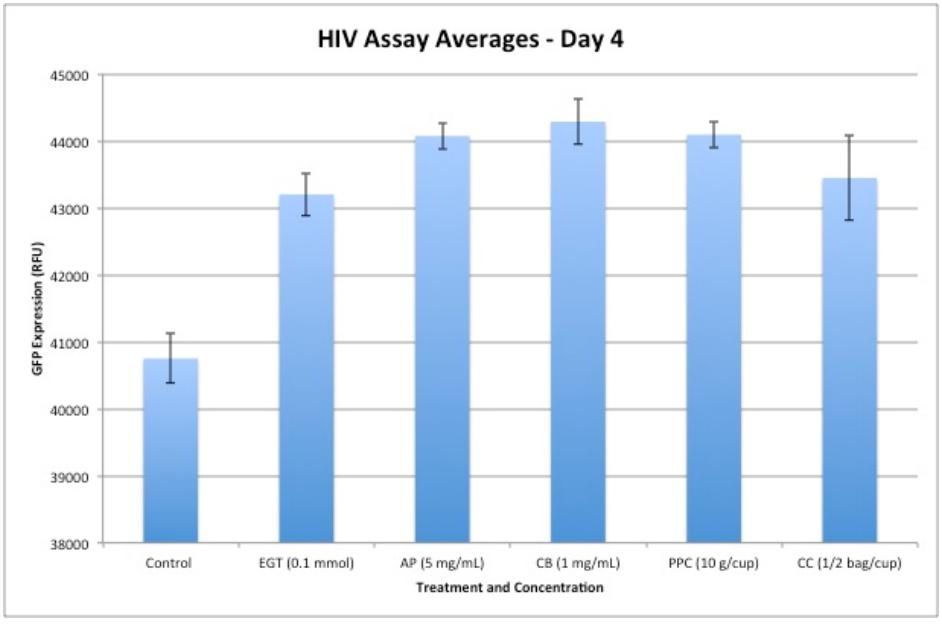


**Figure 54. Day 4 Daily Averages Graph for HIV Experiment**

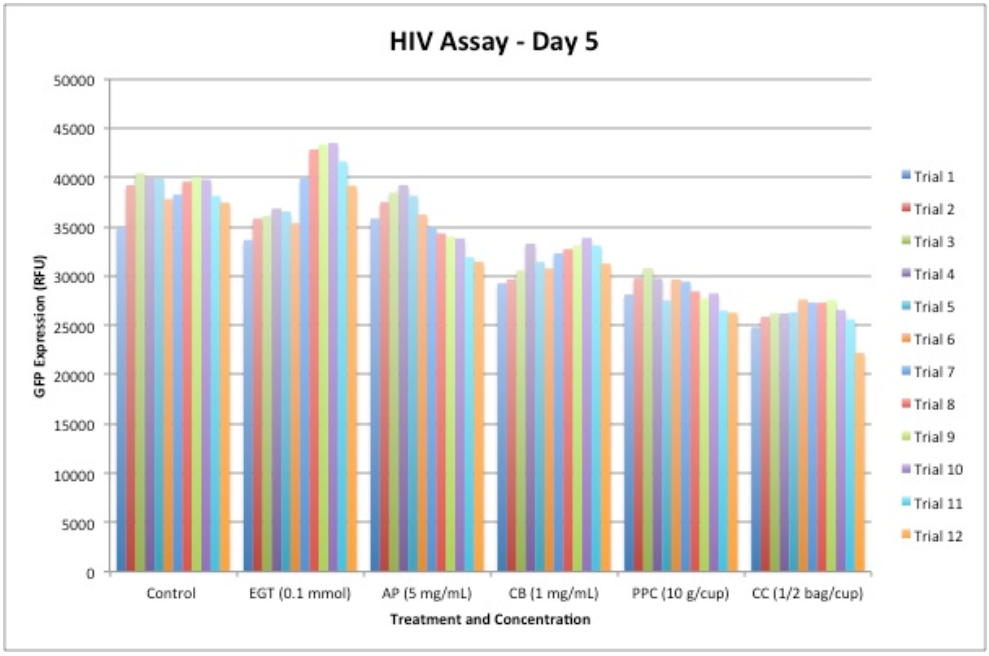


**Figure 55. Day 5 Daily Readings Graph for HIV Experiment**

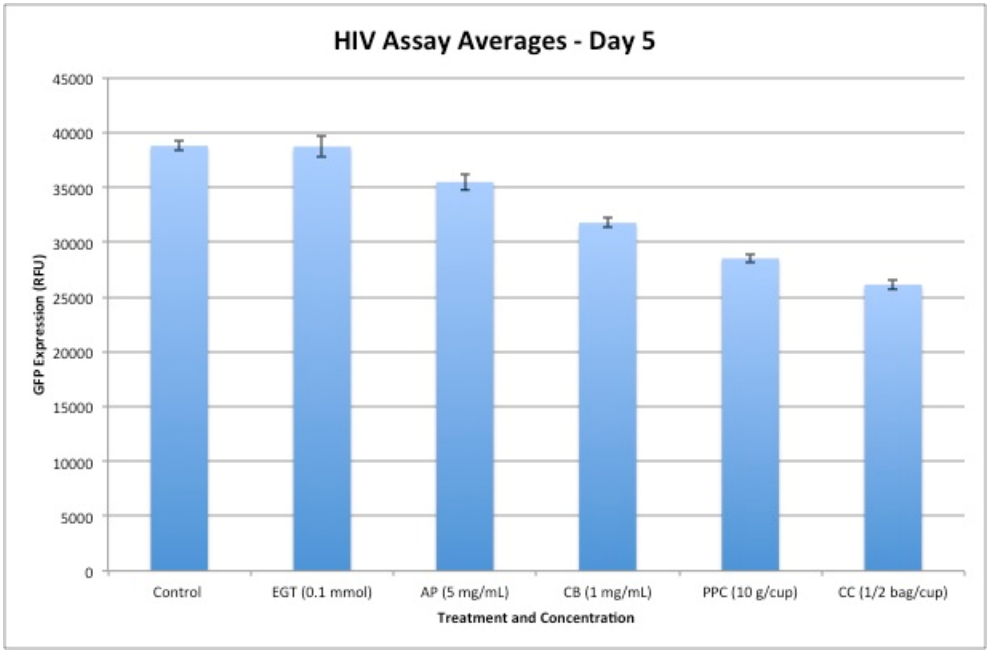


**Figure 56. Day 5 Daily Averages Graph for HIV Experiment**

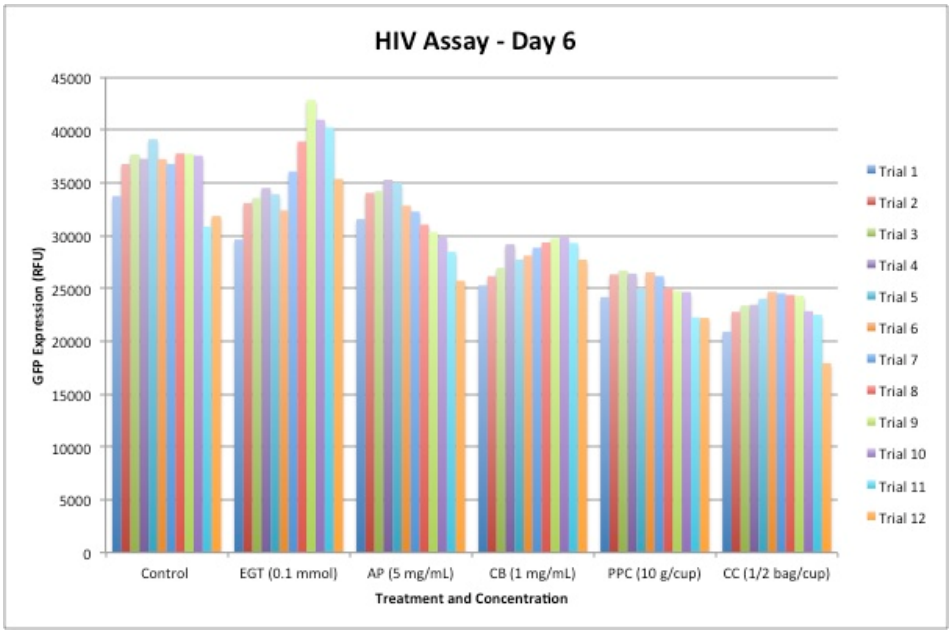


**Figure 57. Day 6 Daily Readings Graph for HIV Experiment**

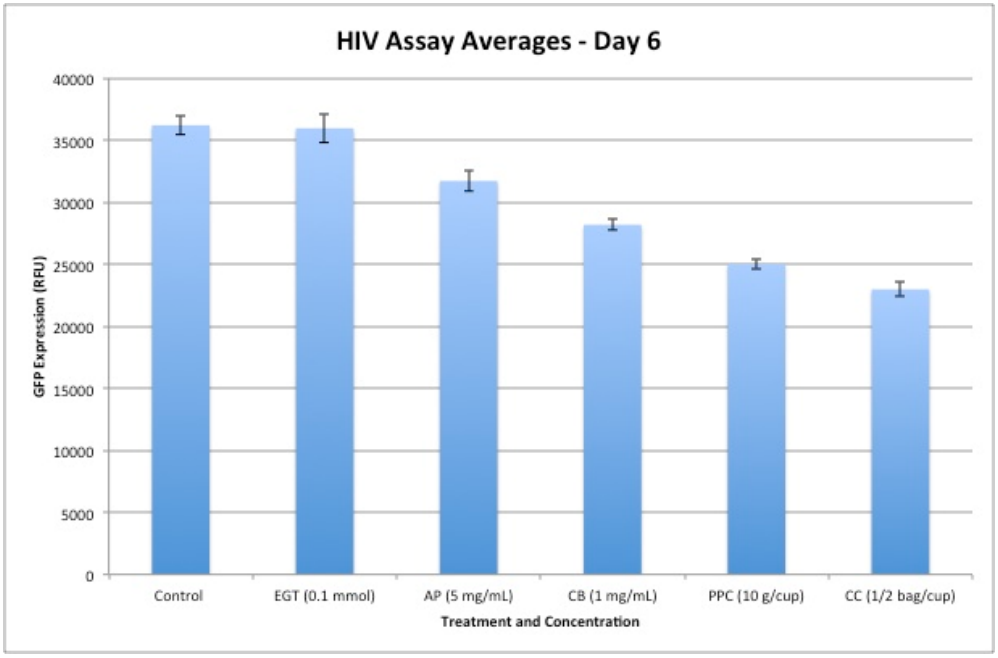


**Figure 58. Day 6 Daily Averages Graph for HIV Experiment**

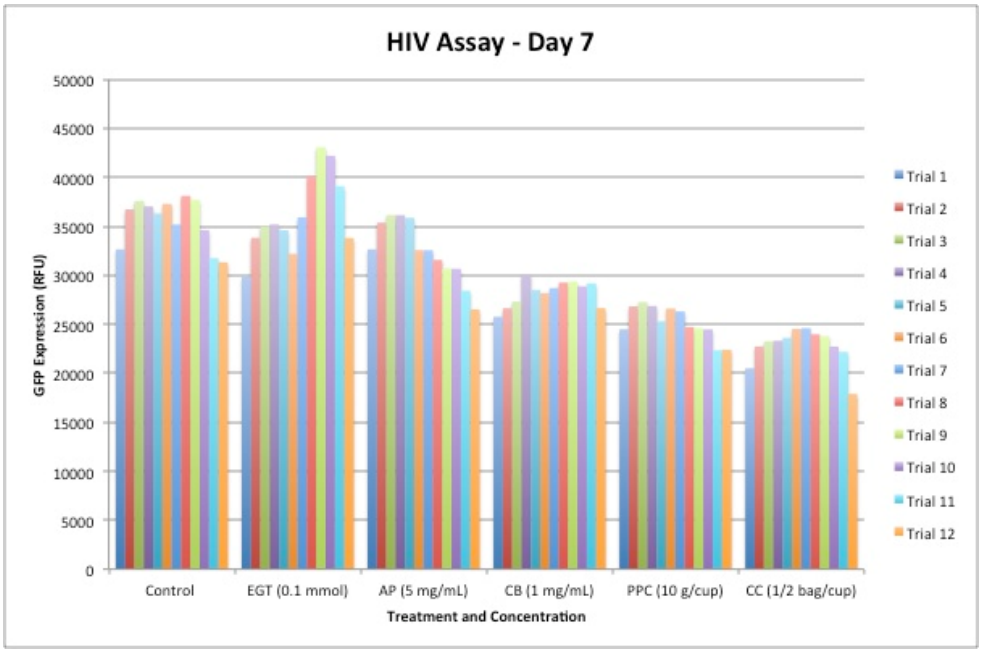


**Figure 59. Day 7 Daily Readings Graph for HIV Experiment**

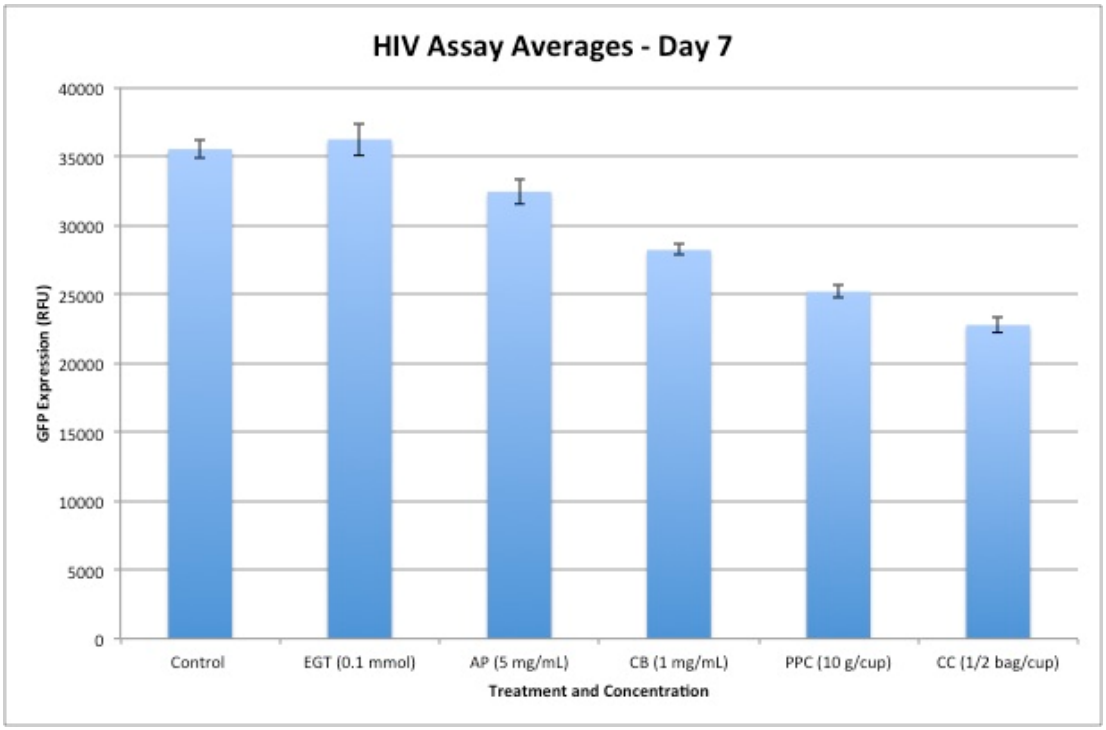


**Figure 60. Day 7 Daily Averages Graph for HIV Experiment**

## APPENDIX C

## PHOTOGRAPHS OF UNINFECTED VERSUS INFECTED CELLS IN BRIGHTFIELD AND GFP IMAGING

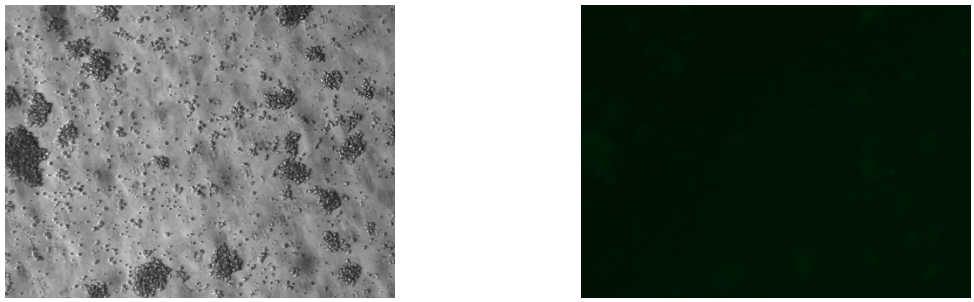

**Figure 61. Untreated, Uninfected CEM-GFP Cells under Brightfield and GFP Lighting at 10x**

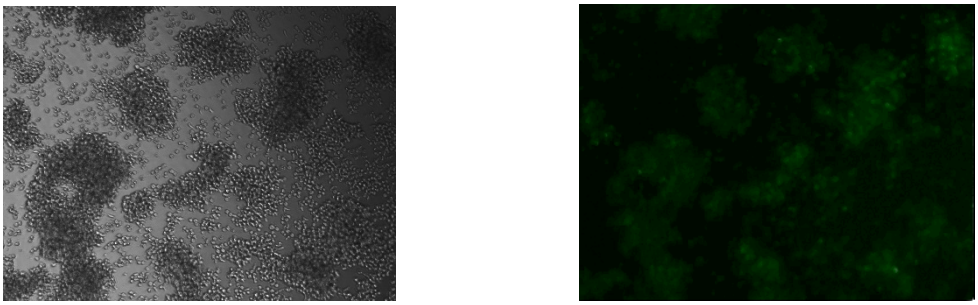

**Figure 62. Untreated, HIV-infected CEM-GFP Cells under Brightfield and GFP Lighting at 10x**