Identification of Tumor Necrosis Factor-α Inhibitors from Traditionally Used Medicinal Plants

By

Meagan E. Garrison

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Thesis Committee:

Dr. Anthony L. Farone (Committee Chair)

Dr. Mary B. Farone

Dr. Stephen M. Wright

This research is dedicated to my loving and supportive husband, encouraging mother, and late stepfather.

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ABSTRACT

The inflammatory response protects our bodies from foreign pathogens. However, when inflammation is uncontrolled it can lead to inflammatory and autoimmune diseases. The drugs currently used as treatment for these diseases, such as non-steroidal anti-inflammatory drugs (NSAIDs), have very serious side effects. There is a need for less toxic, alternative treatments. Traditional Chinese Medicine is becoming more popular in the United States and many plants are being evaluated for their medicinal purposes. In this study, 12 subfractions of one primary extract from a particular Chinese plant, designated 25A, and 44 fractions of a local plant, *Cichorium intybus* (chicory), were screened for cytotoxicity to human macrophage-like cells and the non-toxic concentrations were then assayed for TNF-α inhibitory activity. Fraction 7 of 25A exhibited greater than 90% inhibition of TNF-α while simultaneously having lower than 10% toxicity to THP-1 human macrophage-like cells and was further separated into three pure fractions. 25A-7 F_1 was the only fraction to show greater than 90% TNF-α inhibition. The same process was repeated with the 44 chicory samples from four sources: wild-type, hairy root, and callus cell culture with and without elicitors. The callus cell culture subfractions showed no inhibitory activity. The remaining two sources, wild-type and hairy root, had promising activity with various fractions producing TNF-α inhibition. This research provides potential anti-inflammatory treatment candidates from medicinal plants.

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I. INTRODUCTION

Inflammation and Disease

Inflammation is an important immune response that maintains homeostasis by protecting the body from damage and infection by microbial pathogens such as bacteria, viruses, and parasites. The inflammatory response can result in heat, redness, swelling, pain, and loss of function when activated white blood cells recognize the injury or invasion of microbes into the site. Cytokines are soluble mediators responsible for regulating the inflammatory response, and include chemokines, interleukins, and tumor necrosis factor (Feghali and Wright 1997).

Though inflammation has evolved to protect our bodies, it is also central in numerous autoimmune and inflammatory diseases when uncontrolled and damages the host's tissues (Elias et al 2008). Examples of these illnesses include inflammatory bowel disease (IBD), such as Crohn's disease (CD) and ulcerative colitis (UC), rheumatoid arthritis (RA), fibromyalgia, systemic lupus erythematous (SLE), and Parkinson's disease. Inflammatory responses are becoming increasingly important due to their association with the connection between innate immunity and diseases (Woo et al 2015).

The increasing global incidence of inflammatory diseases, specifically CD and UC, over time can be seen in Fig 1. The prevalence of IBD has been significantly increasing in developed countries in the past decade.

The second highest prevalence of IBD was recorded in North America at 249 per 100,000 persons diagnosed with UC and 319 per 100,000 persons diagnosed with CD, suggesting that these conditions are emerging as diseases as associated with developed countries (Molodecky et al 2012).

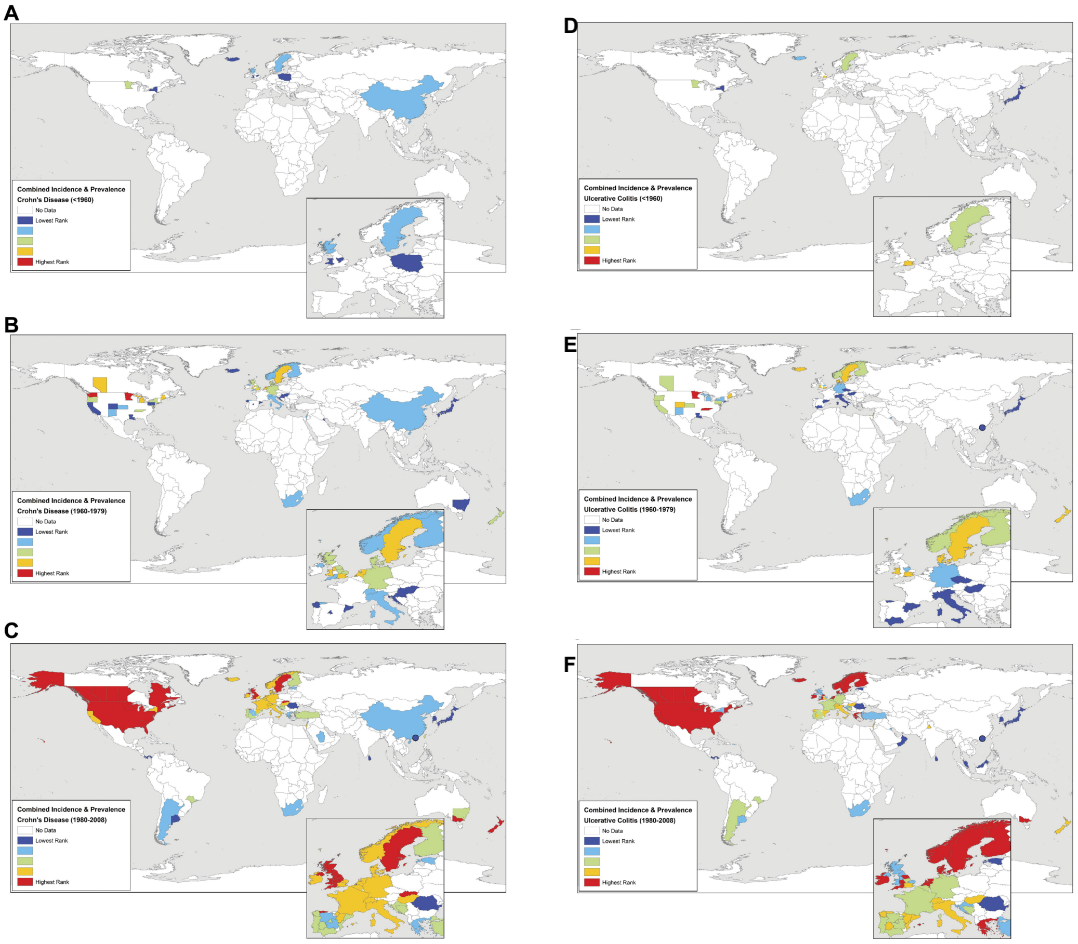


Figure 1A-F. Incidence of Irritable Bowel Diseases (IBD) Globally (Molodecky et al 2012).
The global incidence rates of IBD, specifically CD, for countries reporting data A) prior to 1960, B) between 1960 and 1979, and C) after 1980. The countries reporting data also include incidence of UC D) prior to 1960, E) between 1960 and 1979, and E) after 1980. The countries in red represent the highest rank of incidence, while the countries in dark blue represent the lowest.

In the United States, a patient suffering from CD, for example, can have direct medical costs adding up to approximately $18,000. This cost includes hospitalizations, diagnostic testing, and treatments.

The cause of many inflammatory diseases is unknown, leading to difficulty in the development of specific treatments. The serious tissue destruction and organ failure in patients suffering from these diseases are due to the chronic, uncontrolled inflammation, therefore much attention is focused on developing effective anti-inflammatory regimens. Current anti-inflammatory treatments are limited by serious side effects and mostly consist of steroids and non-steroidal anti-inflammatory drugs (NSAIDs). According to the Arthritis, Rheumatism, and Aging Medical Information System, the adverse effects of NSAIDs may be associated with more than 100,000 hospitalizations and 16,000 deaths each year (Singh and Ramey 1998). Corticosteroids are less commonly prescribed for inflammatory diseases than NSAIDs. These drugs are only prescribed in circumstances in which the benefit will outweigh the risk, and are also prescribed at the lowest dosage and shortest duration possible. Previous studies have determined that corticosteroids may lead to the up-regulation of genes that induce inflammation, as well as inflammatory-induced depression (Brooks et al 2016).

The most frequently prescribed drugs for acute inflammatory conditions are NSAIDs; however, like corticosteroids, these treatments are not prescribed long-term (Abdel-Tawab et al 2009). For severe cases, such as Crohn's disease and SLE, studies have shown that blocking the inflammatory mediator, tumor-necrosis factor-α (TNF-α), is the most effective method to control inflammation (Thomson et al 2012). Tumor necrosis factor-α is a small protein released primarily by activated macrophages, as well as other

immune and endothelial cells, which promotes various biological processes, including cell differentiation, proliferation, or death to amplify the inflammatory process, specifically triggered by bacterial lipopolysaccharide released during a bacterial infection with the subsequent inflammatory response often resulting in sepsis.

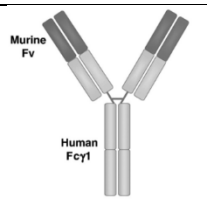
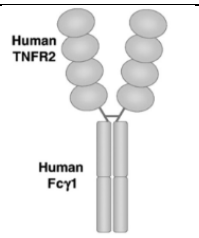
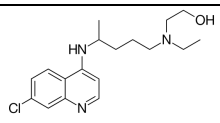
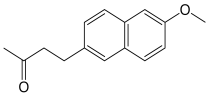
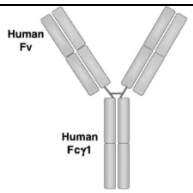
As shown in Table 1, the commonly prescribed drugs approved by the FDA to treat inflammatory diseases are TNF inhibitors. Tumor necrosis factor inhibiting drugs bind to the protein, therefore blocking the binding to the molecule TNFR receptor cell, which suppresses inflammation. Currently, the U.S. Food and Drug Administration (FDA) has approved several TNF-α inhibitors, such as Infliximab, Etanercept, and Adalimumab, for only a few selected diseases because severe side effects have been associated with these therapies (Rodgers et al 2011). The side effects can include increased risk of infections, stomach ulcers, osteoporosis, cancer, stroke, and the worsening of previously acquired medical conditions (Maroon et al 2010).

Aside from the adverse side effects, the cost of the treatments in the United States can be problematic as well. Infliximab and Adalimumab, for example, are the most expensive treatments currently available for IBD, ranging from $2,000-$4,000 per treatment. These drug treatments have lowered the prevalence of hospitalization among CD patients; however, the cost of the treatment itself has more than doubled the average cost for a patient with CD (Park and Bass 2010).

According to the FDA, NSAIDs and corticosteroids should only be taken exactly as prescribed, at the lowest dosage possible, and for the shortest time needed. Unfortunately, inflammatory diseases are lifelong illnesses and therefore require lifelong

treatment. Alternative TNF-α inhibitors for the treatment of inflammatory diseases with less toxic side effects on human cells are needed.

Table 1. Common Drugs Used to Treat Specific Inflammatory Diseases

Disease	Drug	Structure	Mechanism	Side effects
CD	Infliximab		Prevents TNF-α from binding to receptor	Infection, reactivation of hepatitis B and tuberculosis, drug-induced lupus
RA	Etanercept		Prevents TNF-α from binding to receptor	Fatal infections, tuberculosis, fatal blood disorders
SLE	Hydroxychloroquine		Decreases Toll-like receptor signaling, therefore reducing dendritic cell activation	Change in vision, stomach pain, nausea, vomiting
IBD	Nabumetone		Inhibits cyclooxygenase, therefore blocking COX-2 activity	Bleeding in stomach and intestines, risk of heart attack and stroke, increases skin sensitivity to sun
UC	Adalimumab		Prevents TNF-α from binding to receptor	Increased risk of infection, lymphoma, and leukemia, heart failure

Plants Utilized in this Study

Traditional medicinal plants have been used for thousands of years and continue to grow in popularity worldwide. Approximately 25% of new applications received by the FDA are for botanical investigational new drugs (INDs) that originate from herbs used in Traditional Chinese Medicine (TCM) (Qihe et al 2013). One of the most widely known botanical drugs is artemisinin, which is used to treat malaria and was derived from the Chinese herb *qinghao*. Traditional medicine offers an interesting and predominantly unexplored source for the derivation of new therapies.

The Tennessee Center for Botanical Medicinal Research (TCMBR) at MTSU has access to a collection of traditionally used medicinal plants, ranging from anti-cancer, anti-microbial, anti-viral, and anti-inflammatory activity, which have been obtained through the Guangxi Botanical Gardens in China (Cong et al 2014). In this study, a Chinese plant extract designated 25A and a locally sourced plant, *Cichorium intybus,* were investigated as naturally originated alternatives to inhibit TNF-α as a method of treating inflammatory diseases. The extracts from each plant are referred to in this research as specified in Table 2.

Table 2. Plants Utilized in this Study

Plant	Alternative Name(s)	Source	Solvent
Chinese plant extract	25A	Guangxi Botanical Gardens Nanning, China	Petroleum ether
Cichorium intybus (four sources)	Chicory CIW, CICC. CIHR, CIE	Johnson City, TN	Ethyl acetate

Traditional Chinese Medicine Plant

The 25A plant material was collected from Nanning, in Guangxi Province, China. The fruit of this plant is edible while the roots and ripe stems are often used medicinally in TCM. For centuries, local natives have used this particular species to treat rheumatalgia, arthralgia, and traumatic injuries (Fang and Qin 2002). Cytotoxic and antioxidant activity have been determined in previous studies, though the traditional uses have yet to be scientifically tested and confirmed (Aung et al 2016). Previous phytochemical studies have isolated compounds such as terpenoid indole alkaloids, sesquiterpenes, and various glycosides (Cong et al 2014). Terpenoids are the largest family of naturally occurring products with over 30,000 compounds collectively, and are also known to have numerous biological and physiological functions (El-Sayed and Verpoorte 2007). Alkaloids in general are a large class of secondary metabolites and contain heterocyclic nitrogen. This nitrogenous ring gives alkaloids properties that make them pharmacologically active (Verpoorte et al 1997). Terpenoid indole alkaloids are specific compounds often found in plants, which can be used medicinally. The petroleum ether fraction of the plant extract designated as 25A was used in this study.

Locally Sourced Plant

Cichorium intybus, commonly known as chicory, is a woody perennial herb in the family Asteraceae and is widely distributed in Europe and Asia, however the chicory extracts in this study were sourced from Tennessee. Though it is a well-known coffee substitute, chicory has also been widely used to treat ailments including diabetes and wounds (Judzentiene and Budiene 2008). Previous studies have discovered that the main

contents of phytochemical importance are found in the root (Bais and Ravishankar 2001). The roots of *C. intybus* have also previously been identified as containing sesquiterpene lactones, such as lactucin and lactucopicrin, which are light sensitive (Bischoff et al 2004). However, a total of over 100 isolated compounds have been identified from this herb, mostly in the root, for uses such as anti-microbial, anti-helmintic, anti-malarial, anti-inflammatory, analgesic, anti-allergic, and tumor-inhibitory activities (Suresh et al 2005). In this study, 11 subfractions were obtained from four sources of chicory found in Johnson City, Tennessee: wild type (CIW), hairy root (CIHR), callus cell culture with no elicitors (CICC), and callus cell culture with elicitors (CIE).

Bioassay-Guided Fractionation (BGF)

Bioassay-Guided Fractionation (BGF) is a technique used to separate active components of a plant extract based on demonstrated chemical properties and biological activity. The biological activity is determined using the aforementioned cytotoxicity and cytokine release assays. The chemical properties and actual separation of compounds is executed using High Performance Liquid Chromatography (HPLC). The physical apparatus consists of a mobile phase reservoir, a solvent delivery system, constant amount of mobile phase, the column, a detector, and an interface. Different compounds will exit the column at varying retention times. Due to its sensitivity, adaptability to accurate quantitation, suitability for separation, and because of the widespread application to industry-important substances, such as proteins, terpenoids, nucleic acids, hydrocarbons, and a variety of inorganic substances, HPLC is the most commonly used analytic separation technique (Skoog et al 1998).

Cell Culture for Determining Macrophage Function

The human monocyte cell line, THP-1, is an established cell line for modeling TNF-α release from activated macrophages during the inflammatory response. Monocyte cell lines are advantageous over tissue macrophages due to ease of acquisition and the ability to adjust the cell number required in experimentation. However, manual differentiation of the monocytes into macrophages is necessary when using the cell line. Previous studies have determined the effectiveness of the monocytic cell lines and the differentiation of monocytes into macrophages using phorbol myristate acetate to activate Protein Kinase C, which is involved in monocyte differentiation. Though there are advantages and disadvantages to using monocyte cell lines versus tissue macrophages, the cell lines are more efficient for researching macrophage function and have therefore been established as a model for these experiments (Daigneault et al 2010). The standard method used to effectively determine macrophage function, such as the release of TNF-α, involves inducing inflammation with the addition of the most powerful bacterial virulence factors in terms of pro-inflammatory properties, lipopolysaccharide (Martich et al 1993).

Thesis Research Goal and Specific Aims

The overarching goal for this thesis project is to screen crude extracts fractionated from plants used in traditional medicine for discovery of novel compounds that suppress the TNF-α response in the lipopolysaccharide (LPS)-stimulated human macrophage-like cell line, THP-1.

The specific aims of this project were as follows:

Specific Aim 1: Identify maximum concentrations of plant extract fractions that are not cytotoxic to the THP-1 cell line used to assay for the TNF-α response.

Specific Aim 2: Quantitate TNF-α suppression to determine fractions with potential TNF-α inhibitory effect.

Specific Aim 3: Perform bioassay-guided fractionation to isolate compounds that inhibit TNF-α activity. Plant extracts that are non-toxic to human macrophages and inhibit TNF-α will be fractionated until a pure, bioactive compound is isolated.

Specific Aim 4: Determine the concentration of the pure compounds that inhibit TNF-α by 50% by performing an IC_{50} dose response.

II. MATERIALS AND METHODS

Plant Extracts

Plant extracts were provided by the MTSU Chemistry Department and included extracts from chicory and primary extract 25A. 25A is an extract from a plant used in TCM that was originally collected from Jinxiu County, Guangxi, China in May 2011 and was identified by Dr. Yimin Zhao at the Guangxi Botanical Garden of Medicinal Plants. Chicory extracts were collected from a field in Johnson City, TN in October 2014. All extracts were dried and re-suspended in dimethylsulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) at a concentration of 10 mg/mL for extracts containing multiple compounds or 10 μM for pure compounds, and stored at 4°C. The working stock concentration of the extracts containing multiple compounds was diluted to 1 mg/mL for experiments.

Tissue Culture and Cell Maintenance

The human monocyte cell line, THP-1 (ATCC, Manassas, VA), was maintained in suspension cultures in 75 cm^2 tissue culture flasks (Corning Inc., Corning, NY) at 37°C and 5% CO_2 in 15 mL of complete growth medium [RPMI 1640 (Sigma-Aldrich Inc., St. Louis, MO) supplemented with 10% complement-inactivated fetal bovine serum (FBS; Atlanta Biologicals, Atlanta, GA) and 1% penicillin/streptomycin (Sigma-Aldrich) solution]. Cells were passaged every 7 days by transferring 3 mL of the cell suspension into 13 mL of complete medium in a new flask to maintain cell cultures.

Cell Differentiation

For cytotoxicity testing as well as TNF-α detection, THP-1 cells were counted by trypan blue exclusion with a hemocytometer using a solution of 0.04% trypan blue (Sigma-Aldrich) in 1X phosphate buffered saline (PBS; ThermoFisher Scientific, Waltham, MA). The cell number was adjusted to $5x10^5$ cells/mL in complete medium and the cells were seeded into 24-well plates at a concentration of $2.5x10^5$ cells/well. Cells were differentiated from nonadherent monocytes into macrophage-like cells using phorbol 12-myristate 13-acetate (PMA; 100 nM, Sigma Aldrich) in complete medium and incubated at 37°C and 5% CO_2 for 48 h prior to treatments. Macrophage differentiation was confirmed by observation with light microscopy.

Alamar Blue Cytotoxicity Assay

Alamar Blue was used to determine cytotoxicity of the plant extracts. In this assay, resazurin is the active compound found in Alamar Blue and is indicative by being reduced due to oxygen consumption through cell metabolism. When cells are healthy and viable, the blue resazurin is reduced to the fluorescent pink resorufin due to NADH being present in metabolically active cells (O'Brien et al 2000).

To determine the cytotoxicity of extracts or pure compounds on differentiated THP-1 cells, cells were pretreated with fractions of 25A or chicory extracts in duplicates for 1 h and then stimulated with LPS (20 ng/mL) for 4 h to induce a TNF-α response. The 12 subfractions of 25A were initially tested at concentrations of 50 µg/mL and 25 µg/mL. Two subfractions remained toxic at 25 µg/mL and were further diluted and screened at 10 µg/mL and 5 µg/mL to reduce toxicity. The 44 subfractions of chicory were initially

tested at 10 µg/mL. Each experiment consisted of three control groups: untreated cells with medium only, dexamethasone (Sigma-Aldrich), a glucocorticoid that is a known suppressor of cytokine responses (Abraham et al 2006), plus LPS-treated cells as a positive control, and LPS treated cells as a reference control. Supernatants from each well were then collected and the supernatant replaced with 1X Alamar Blue reagent (ThermoFisher Scientific) in complete culture medium for 16 h. A SpectraMax M5 fluorescent plate reader (Molecular Devices, Sunnyvale, CA) measured relative fluorescence at a wavelength of 585 nm (excitation 555-570 nM). Measurements were collected using Softmax Pro version 6.3 software (Molecular Devices), and the average relative fluorescence of each sample was compared to the average of the untreated sample to determine percent viability. Fractions with higher than 90% viability were then assayed for inhibition of the inflammatory cytokine TNF-α.

TNF-α ELISA

For cytokine measurement, THP-1 cells were first plated and differentiated as previously described for cytotoxicity testing. Each experiment contained the same three control groups: untreated cells, cells treated with only LPS, and cells treated with dexamethasone plus LPS. The supernatants of treatment groups which were non-toxic and control groups were tested for the concentration of TNF-α present in the samples by human TNF-α enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN) following the manufacturer's protocol. The percentage of TNF-α produced in each fraction was calculated by comparing the amount of TNF-α (pg/mL) produced to that of the LPS control. The amount of TNF-α produced was subtracted from

100 to obtain a percentage of inhibition. The fractions were required to inhibit TNF-α production by a minimum of 50% to be considered for future analysis. Subfractions that exhibted 50% or greater inhibition at lower concentrations were targeted for further fractionation, due to the requirement of less amount of compound in formulating potential drug therapy.

Statistical Analyses

Experiments were conducted in either duplicate or triplicate, as noted, and repeated independently in order to determine statistical significance of the inhibitory activity. A One-Way ANOVA test performed using www.statpages.info to determine the significant difference among samples in each screening of TNF-α inhibition with a confidence interval of 95%. Samples with a *p*-value of less than 0.05 were considered significantly different.

Fractionation of 25A

In this study, flash column chromatography was performed on the initial petroleum ether extract of 25A, which resulted in 12 subfractions. Of the 12 subfractions tested for biological activity using cytotoxicity and cytokine release assays, fraction 7 was chosen to be further fractionated using preparative scale reversed phase HPLC. Fraction 7 was chosen for further fractionation due to its low cytotoxicity to human monocyte-like cells, high inhibition of TNF-α at low concentrations, and its chemical properties.

Further purification of fraction 7 was done using a semi preparative scale Waters 1524 High Performance Liquid Chromatography system coupled with a UV detector.

Chromatographic separation was carried out using a Thermo Hypersil Gold Aq C-18 (250 X 10 mm; 5u) column at a flow rate of 4.78 mL/min. The mobile phase consisted of A (water with 0.1% trifluoroacetic acid) and B (Acetonitrile with 0.1% trifluoroacetic acid) with the following gradient elution: 10%-90% B at 0-35 min and 90%-10% B at 35-37 min. The injection volume was 1 mL and the detector was set at 254 nm. The three peaks collected during the fractionation were at the following time intervals: F_1 (5-10 minutes), F_2 (15-20 minutes), and F_3 (20-28 minutes). The procedure was repeated thrice. The respective fractions were then pooled and concentrated using a Buchi RotaVapor R-200 (ThermoFisher Scientific) rotary evaporator up to a volume of 1 mL. The fractions were further lyophilized to produce powdered forms of the fractions, dissolved in DMSO at a final stock concentration of 10 mg/mL, and stored at -80°C until further use.

Table 3. Masses of 25A-7 Subfractions

Fraction	Empty tube (mg)	Tube with Fraction (mg)	Fraction (mg)
F_1	10442.6	10443.5	0.9
F_2	10431.1	10432.6	1.5
F_3	10332.9	10335.6	2.7

IC_{50} of 25A-7 F_1

The concentration of 25A-7 F_1 that had the ability to inhibit the production of TNF-α in LPS-stimulated THP-1 cells by 50% was determined by performing an IC_{50} dose response. The compound was tested at four concentrations in a single treatment: 100

μg/mL, 50 μg/mL, 25 μg/mL, and 12.5 μg/mL using the same protocol as previous cell treatments, with the exception of the LPS concentration at 1 μg/mL. The supernatants of the non-toxic concentrations were frozen and saved for testing the percent of TNF-α inhibition by the compound using ELISA.

Biological Activity of Fractions and Subfractions

The biological activity of fraction 7, as well as that of the other 11 subfractions of 25A and the 44 subfractions of chicory was determined. The three subfractions of primary plant extract 25A were initially screened at a concentration of 50 μg/mL for cytotoxicity to human macrophages using the Alamar Blue Cell Viability assay and TNF-α inhibitory activity by ELISA, as previously described.

III. RESULTS

Chinese Extract 25A

Cytotoxicity Screening

Cytotoxicity screening of 12 subfractions of plant extract 25A using the THP-1 cell line was completed. Each subfraction was initially screened at 50 μg/mL and 25 μg/mL (Figure 1A-B). Subfractions 4-7 all resulted in < 90% viability of THP-1 cells at 50 μg/mL and subfractions 4 and 6 produced > 90% viability of the cells at 25 μg/mL. Because subfractions 5 and 7 had < 90% viability at both concentrations, these two subfractions were re-screened for cytotoxicity at 10 μg/mL and 5 μg/mL concentrations as shown in Figure 2. Lower concentrations of both subfractions resulted in >90% viability of THP-1 cells. Figure 2 shows the percent viability of cells treated with subfractions at concentrations of 50 μg/mL, percent viability of cells treated with subfractions at concentrations of 25 μg/mL, and percent viability of cells treated with subfractions 5 and 7 at concentrations of 5 and 10 μg/mL.

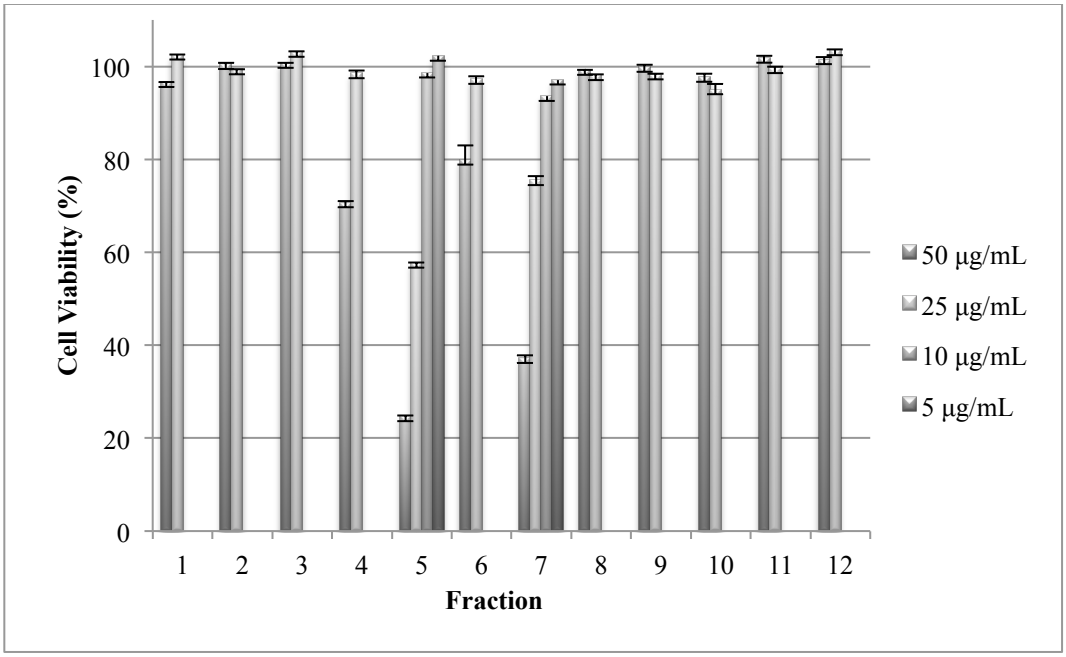


Figure 2. Relative Cell Viability of LPS-Stimulated THP-1 Treated with 25A Subfractions. Subfractions of plant extract 25A were tested for cytotoxicity to PMA-differentiated THP-1 cells. Individual bars represent the average of duplicate wells with standard error of the mean.

TNF-α Inhibition Screening

The 25A subfractions were then tested for inhibition of TNF-α production by LPS-stimulated THP-1 cells using the concentration that produced > 90% viability of the cells. Supernatants from cells treated with nontoxic concentrations of each fraction were tested for TNF-α levels by ELISA. Seven fractions showed greater than 50% inhibition of cytokine activity at 50 μg/mL, and five fractions showed greater than 50% inhibition of cytokine activity at 25 μg/mL. Subfraction 5 was inhibitory at both 10 μg/mL and 5 μg/mL. Subfraction 7 was screened at only 5 μg/mL and showed inhibitory activity, as shown in Figure 3. Fractions demonstrating the greatest inhibition at the lowest concentrations were chosen for further fractionation, which was determined to be Fraction 7.

Among the six fractions (3, 8, 9, 10, 11, 12) of 25A screened at 50 μg/mL, significant differences were shown between the percentages of TNF-α inhibition with *p*-value of 0.0008 in a one-way ANOVA. Among the seven fractions (3, 4, 6, 8, 9, 10, 12) of 25A screened at 25 μg/mL, significant differences were shown with a *p*-value of 0.0000. Lastly, two fractions (5 and 7) of 25A were screened at 5 μg/mL and also showed significant differences between the percentages of TNF-α inhibitions with a *p*-value of 0.0117. Dose response data for cytotoxicity and TNF-α inhibition of the twelve fractions of plant extract 25A are detailed in Appendix A.

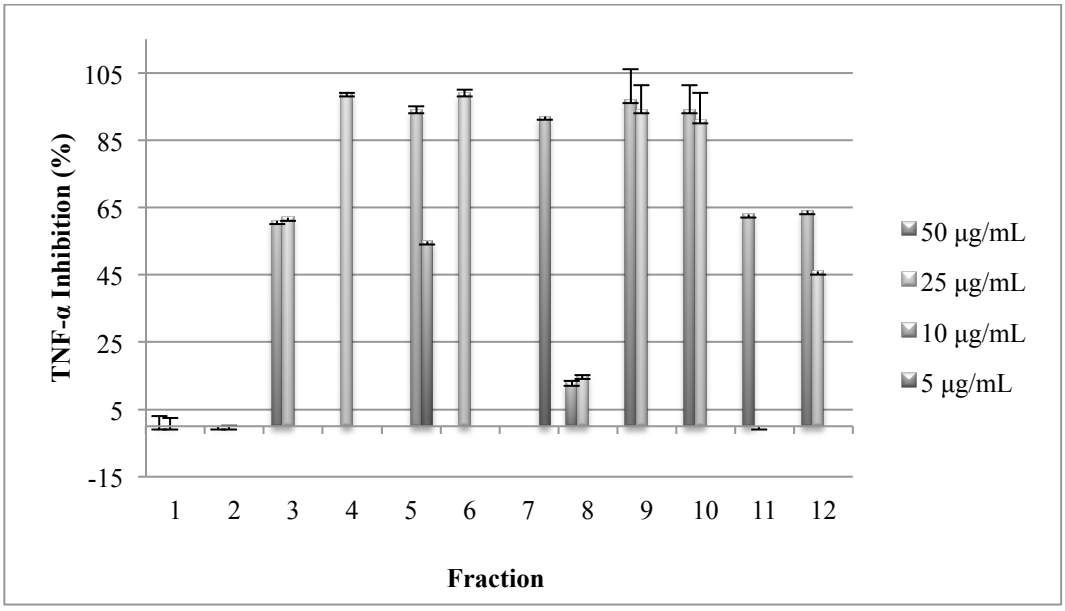


Figure 3. Inhibition of TNF-α Production by Treatment with 25A Subfractions. Inhibition of TNF-α by selected subfractions at 50 µg/mL concentrations (3, 8, 9, 10, 11, 12), inhibition of TNF-α by selected subfractions at 25 µg/mL concentrations (3, 4, 6, 8, 9, 10, 12), and inhibition of TNF-α by subfractions 5 at 10 and 5 µg/mL concentrations, and inhibition of TNF-α by subfraction 7 at 5 µg/m. Individual bars represent the average percent inhibition of duplicate/triplicate wells compared to the control and include standard deviation of the mean.

Chinese Extract 25A-7

Cytotoxicity Screening

Fraction 7 was screened using a dose response for cytotoxicity against THP-1 cell line prior to testing for TNF-α inhibition. The dose response experiment was performed using a 1:2 ratio starting at 10 μg/mL. This subset of screening 25A fraction 7 was performed to determine the validity of the previously recorded viability and TNF-α inhibitory activity prior to fractionation. The dose-response viability of 25A-7 on the monocyte-like cell line showed similar results as previously determined. The two trials depicting the dose response of 25A-7 are shown in Figure 4. Fraction 7 showed >90% viability and therefore was non-toxic to the human THP-1 cells at a concentration of 10 μg/mL and lower, as can be seen in Figure 4.

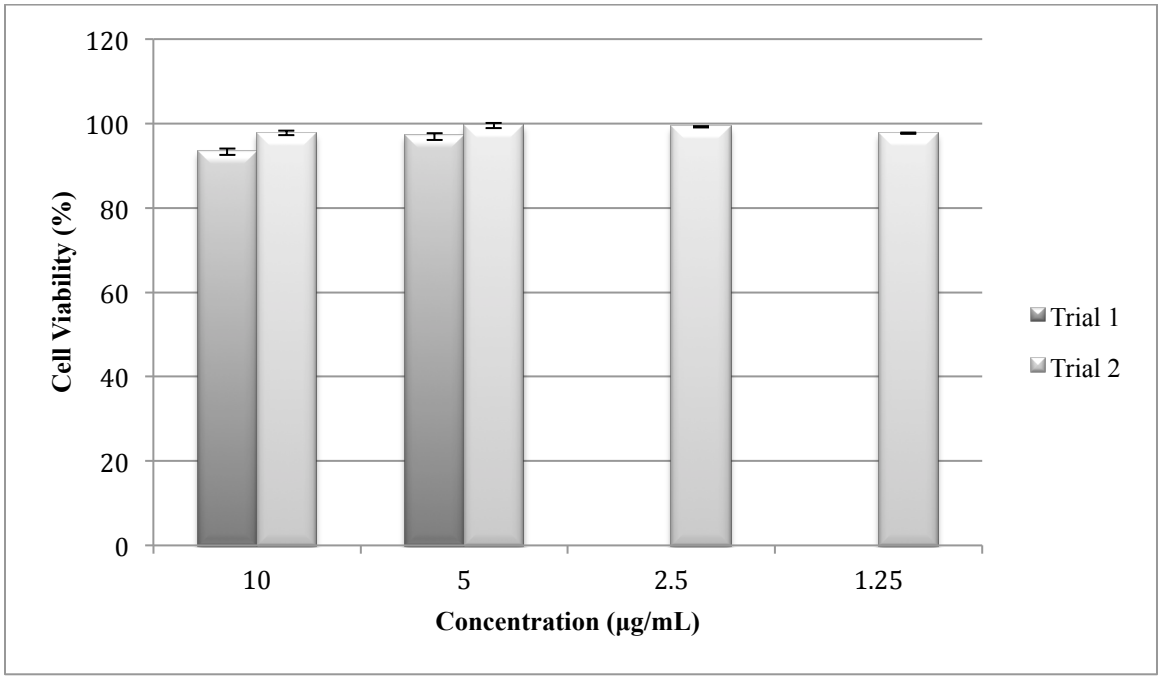


Figure 4. Relative Cell Viability of LPS-Stimulated THP-1 Treated with 25A-7. Plant extract 25A-7 was tested for cytotoxicity to PMA-differentiated THP-1 cells. Individual bars represent the average of triplicate wells with standard error of the mean.

TNF-α Inhibition Screening

Fraction 7 was screened to determine a dose response, as previously described, to confirm TNF-α inhibition prior to fractionation by HPLC. The nontoxic concentrations of 25A-7 were screened for TNF-α inhibition. The 5 μg/mL concentration was repeated. The percentage of inhibition at 5 μg/mL was not significantly different than previously measured. Fraction 7 screened at 10 μg/mL, 2.5 μg/mL, and 1.25 μg/mL also exhibited >50% TNF-α inhibition (Fig 5). Significant differences were not shown among the four concentrations of which 25A-7 was screened for TNF-α inhibition, with a *p*-value of 0.1902 by ANOVA.

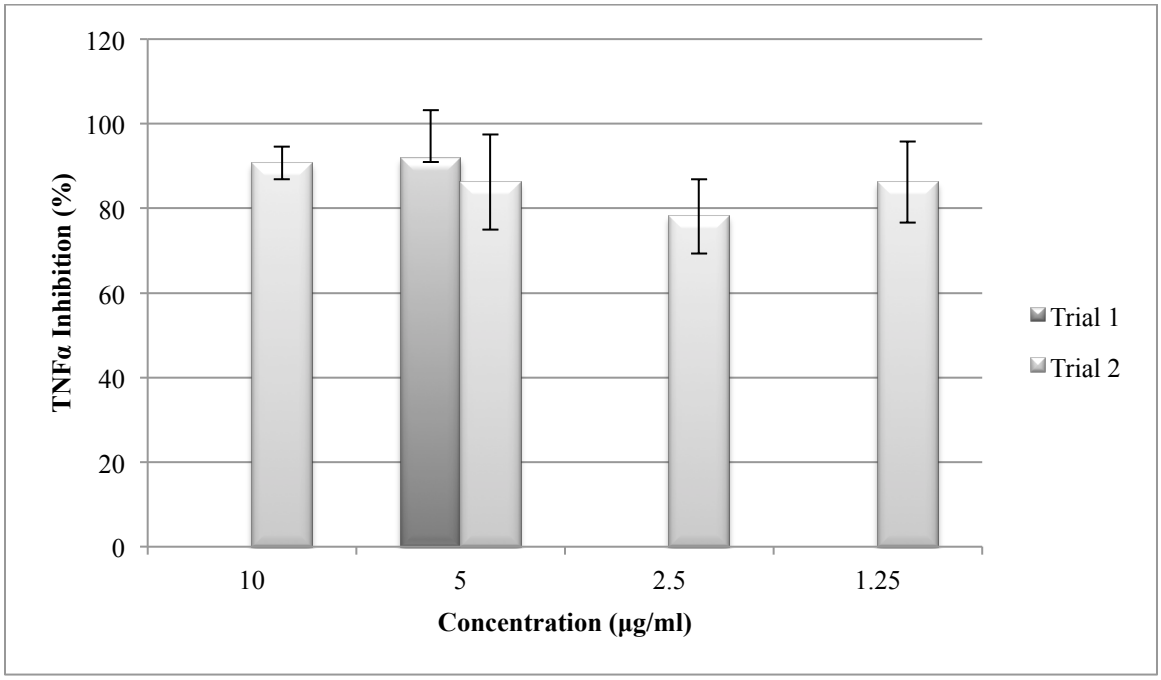


Figure 5. Inhibition of TNF-α Production by Treatment with 25A-7. Inhibition of TNF-α in response to LPS-stimulated THP-1 cells by 25A-7 using a dose response at four concentrations. Individual bars represent the average percent inhibition of duplicate/triplicate wells compared to the control and include standard error of the mean.

High-Performance Liquid Chromatography of 25A-7

Reversed phase HPLC was performed on 25A-7 post-biological determination of TNF-α inhibitory activity. Three peaks were identified and then collected, as shown in Figure 6. Reversed phase HPLC separated the compounds using a mobile phase of a nonpolar and polar solvent, and a stationary phase, which was the column. Fractionation was repeated in order to have duplicates of each collected sample. The resulting three subfractions were designated F_1 (5-10 minutes), F_2, (15-20 minutes), and F_3 (20-28 minutes).

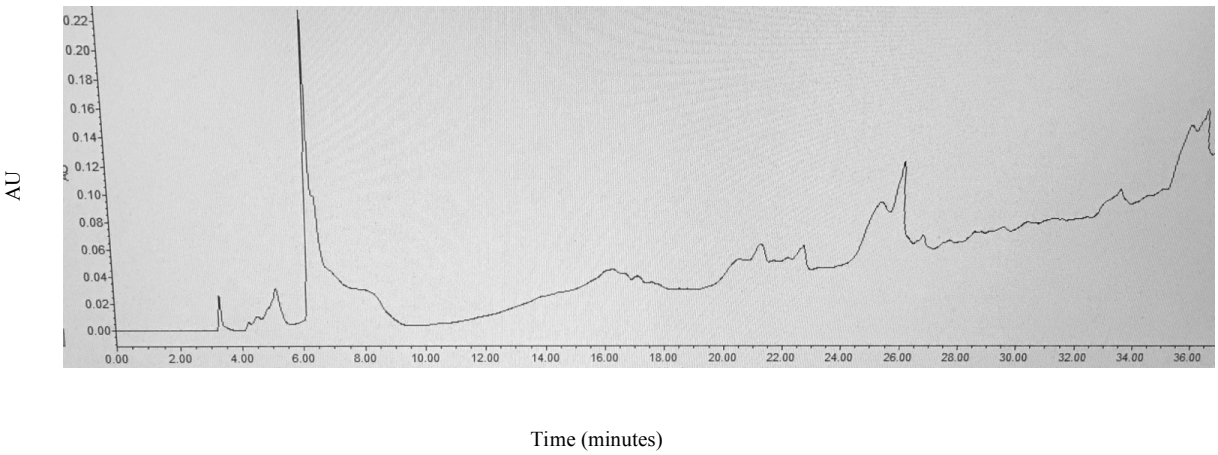


Figure 6. Reversed Phase HPLC of 25A-7. Three peaks were identified and collected at the following time points: 5-10 minutes (F_1), 15-20 minutes (F_2), and 20-28 minutes (F_3).

Chinese Extract 25A-7 Subfractions

Cytotoxicity Screening

Fraction 7 of the primary plant extract 25A was further fractionated using HPLC as previously described. The fractionation resulted in three pure subfractions, referred to as F_1, F_2, and F_3. The three subfractions were screened for biological activity to determine the cytotoxicity of each subfraction on the human monocyte-like cell line and the TNF-α inhibitory activity. Each subfraction was initially screened twice at a concentration of 50 μg/mL in duplicates and then repeated at the same concentration in triplicates. As shown in Figure 7, F_1, F_2, and F_3 were non-toxic to cells at the initial concentration in each experiment with cell viability of >90% for each subfraction.

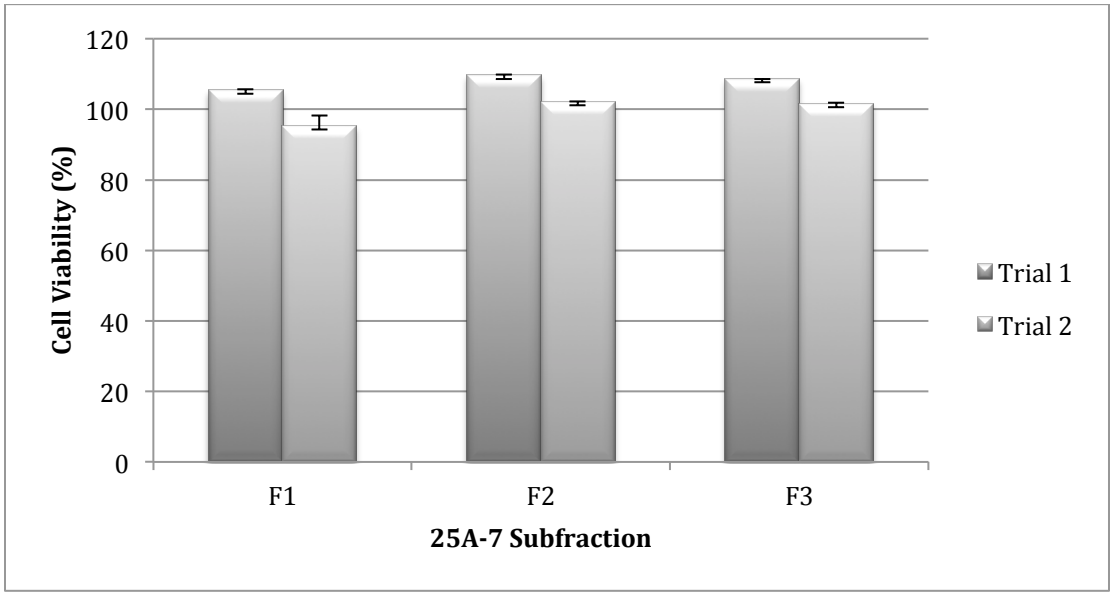


Figure 7. Relative Cell Viability of LPS-Stimulated THP-1 Treated with 25A-7 Subfractions. Subfractions of plant extract 25A-7 were tested for cytotoxicity to PMA-differentiated THP-1 cells. Individual bars represent the average of duplicate wells with standard error of the mean.

TNF-α Inhibition Screening

25A-7 was further separated into F_1, F_2, and F_3 by HPLC. Biological activity was assessed first by determining the cytotoxicity of each subfraction to human macrophages. The non-toxic concentration of each subfraction, which was determined to be 50 μg/mL, was used to test for TNF-α inhibitory activity using ELISA as previously described. In the following experiments, the LPS concentration was increased from 20 ng/mL to 1 μg/mL to ensure sufficient amounts of TNF-α production to enable adequate detection of suppression. The experiments were initially screened in duplicates and then further repeated in triplicate. As shown in Figure 8, F_1 exhibited 50% inhibition, while the remaining subfractions, F_2 and F_3, exhibited <50% inhibition. Among the three subfractions of 25A-7 screened in both duplicate and triplicate, significant differences were shown among the percentages of TNF-α inhibition from each subfraction with a *p*-value of 0.0002 by ANOVA. Dose response data for cytotoxicity and TNF-α inhibition of the three subfractions of 25A-7 are detailed in Appendix B.

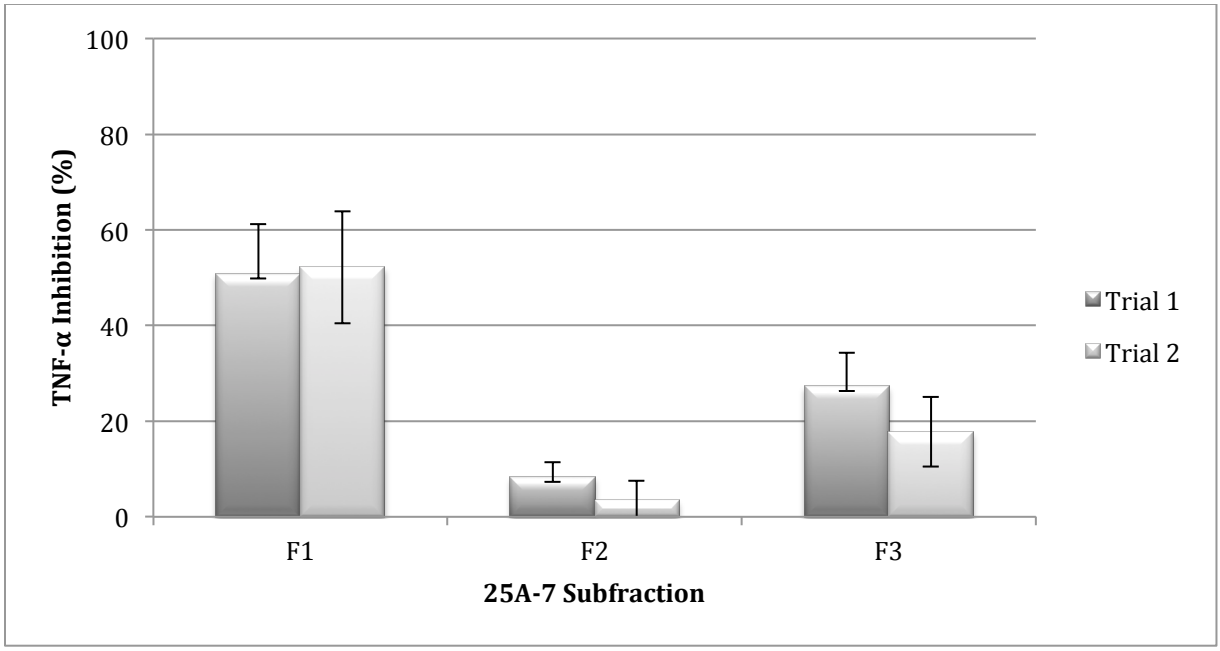


Figure 8. Inhibition of TNF-α Production by Treatment with 25A-7 Subfractions. Inhibition of TNF-α in response to LPS-stimulated THP-1 cells by the more pure fractions of 25A-7 at 50 μg/mL Individual bars represent the average percent inhibition of duplicate/triplicate wells compared to the control and include standard error of the mean.

Preliminary IC_{50} of 25A-7 F_1

The concentration of 25A-7 F_1 on LPS-stimulated (1 μg/mL) THP-1 cells that inhibited TNF-α production by 50% was determined through a dose response of four concentrations. As shown in Figure 9, 100 μg/mL and 50 μg/mL were the concentrations that showed 50% inhibition. The compound at 25 μg/mL actually had higher inhibition at 63%, while the inhibition percentage decreased below 50% at the lower concentration of 12.5 μg/mL. Among the four concentrations of 25A-7 F_1 tested in duplicate, a significant difference was not shown among the percentages of TNF-α inhibition from each concentration with a *p*-value of 0.3853 by ANOVA. Dose response data for cytotoxicity and TNF-α inhibition is detailed in Appendix C.

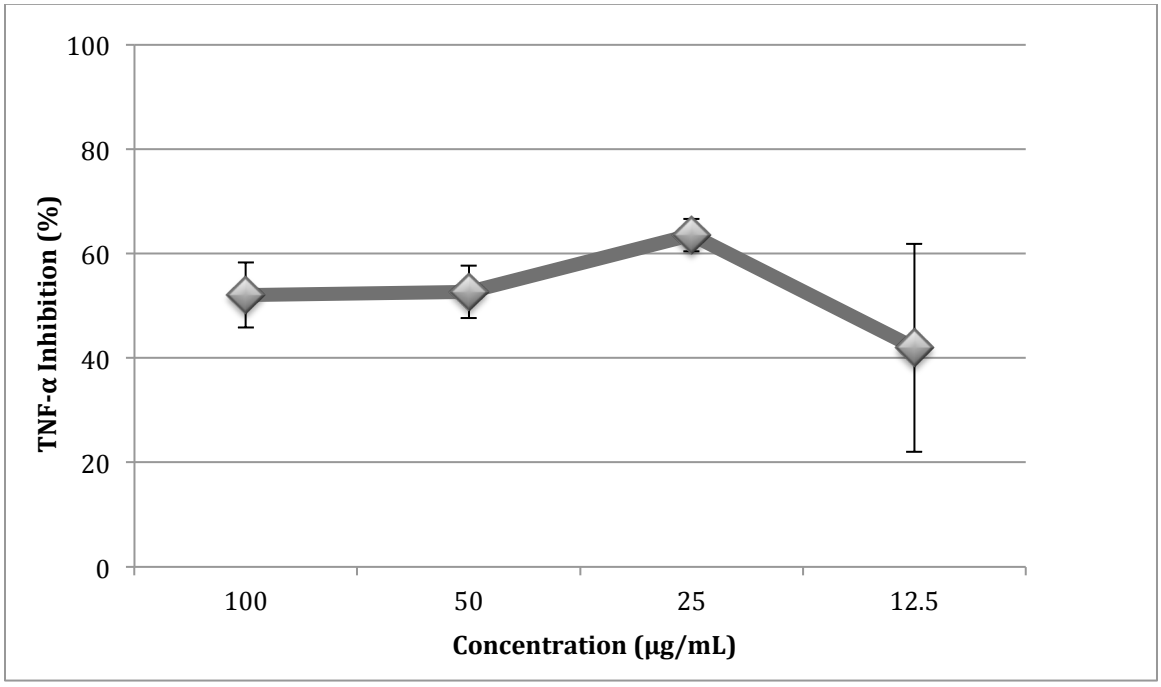


Figure 9. Dose Response of LPS-Stimulated THP-1 by Treatment with 25A-7 F_1. Inhibition of TNF-α in response to LPS-stimulated THP-1 cells by the more pure compound 25A-7 F_1 at four concentrations: 100 µg/mL, 50 µg/mL, 25 µg/mL, and 12.5 µg/mL. Individual bars represent the average percent inhibition of duplicate wells compared to the control and include standard error of the mean.

Chicory (*Cichorium intybus*)

Cytotoxicity Screening

Cytotoxicity screening of 44 subfractions from four sources of chicory extracts using the THP-1 cell line has been completed. The sources are denoted as the following: wild-type as CIW (Fig 10), hairy root as CIHR (Fig 11), callus cell culture with no elicitors as CICC (Fig 12), and callus cell culture with elicitors as CIE (Fig 13). Each subfraction was initially screened at 10 μg/mL. Three subfractions, CIW 4, CIW 10, and CIHR 6, were toxic to human macrophages at 10 μg/mL with <90% viability, and were therefore further diluted and repeated at 5 μg/mL. The three subfractions were non-toxic at the lower concentration and were used to continue with TNF-α inhibition experiments.

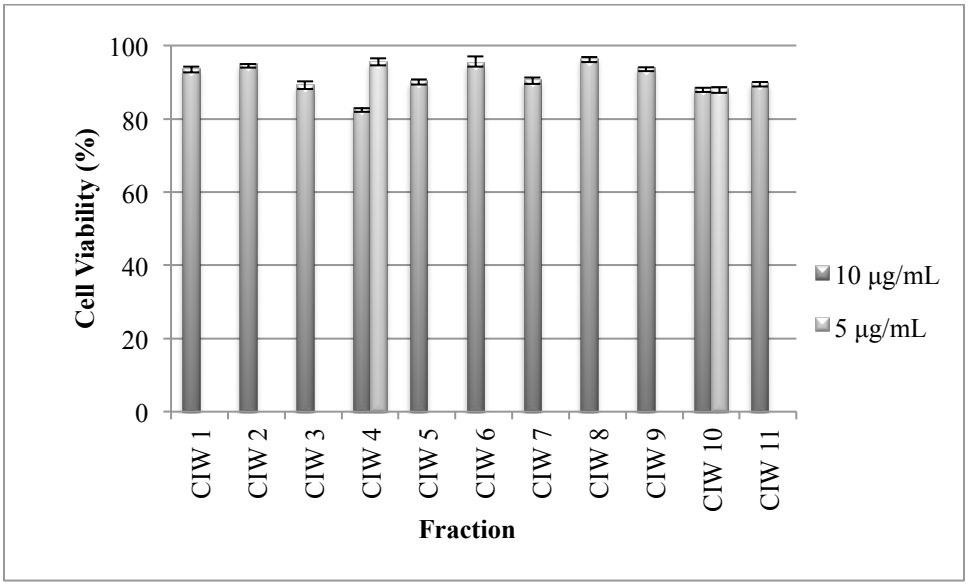


Figure 10. Relative Cell Viability of LPS-Stimulated THP-1 Treated with Wild-Type Chicory Subfractions. Subfractions of wild-type *C. intybus* (CIW) were tested for cytotoxicity to PMA-differentiated THP-1 cells. Individual bars represent the average of duplicate wells with standard error of the mean.

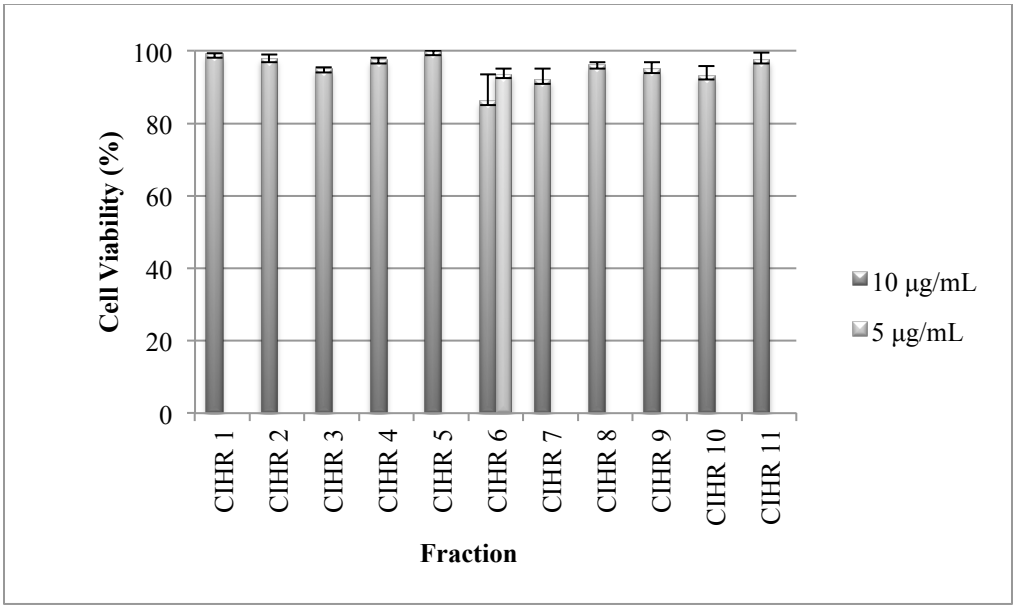


Figure 11. Relative Cell Viabiliy of LPS-Stimulated THP-1 Treated with Hairy Root Chicory Subfractions. Subfractions of hairy root *C. intybus* (CIHR) were tested for cytotoxicity to PMA-differentiated THP-1 cells. Individual bars represent the average of duplicate wells with standard error of the mean.

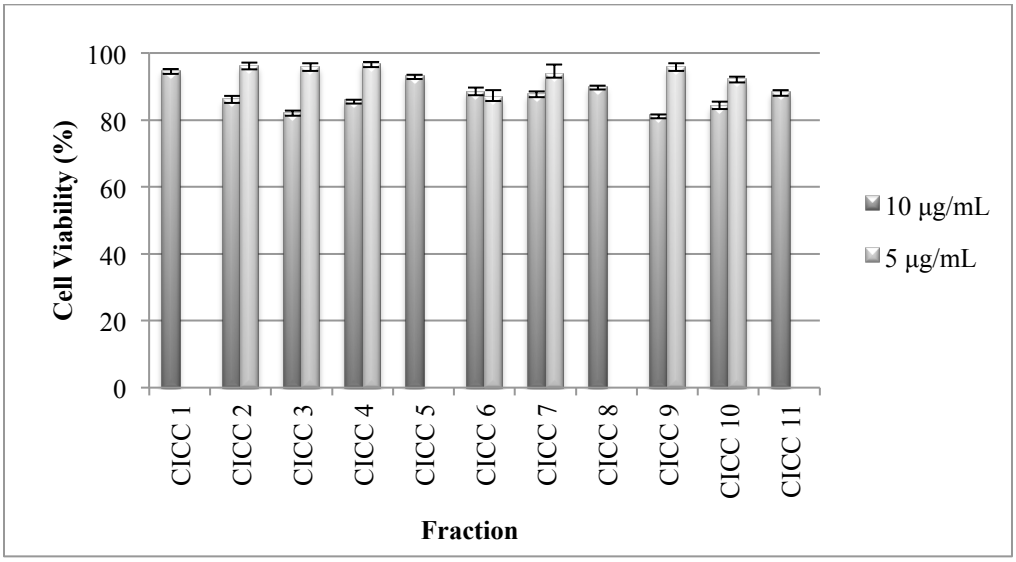


Figure 12. Relative Cell Viability of LPS-Stimulated THP-1 Treated with Callus Cell Culture Chicory Subfractions. Subfractions of callus cell culture *C. intybus* (CICC) were tested for cytotoxicity to PMA-differentiated THP-1 cells. Individual bars represent the average of duplicate wells with standard error of the mean.

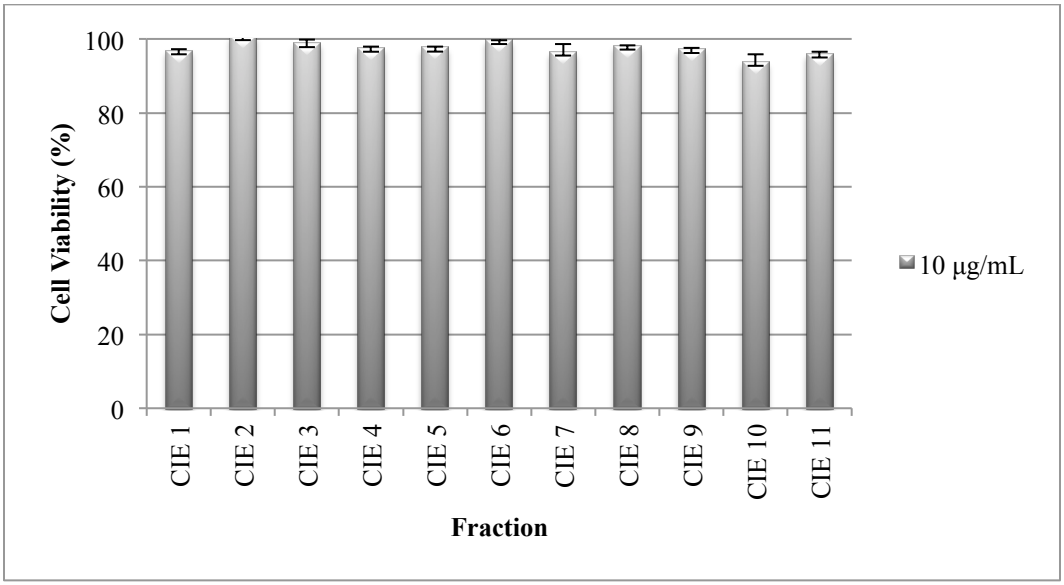


Figure 13. Relative Cell Viability of LPS-Stimulated THP-1 Treated with Callus Cell Culture with Elicitors Chicory Subfractions. Subfractions of callus cell culture with elicitors *C. intybus* (CIE) were tested for cytotoxicity to PMA-differentiated THP-1 cells. Individual bars represent the average of duplicate wells with standard error of the mean.

TNF-α Inhibition Screening

The chicory subfractions were then tested for the ability to inhibit TNF-α production by LPS-stimulated THP-1 cells. Supernatants from cells treated with nontoxic concentrations of each fraction were tested for TNF-α levels (pg/mL) by ELISA. Multiple trials of the various chicory samples with promising inhibitory activity were performed and are indicated in the figure legends of Figures 14-17 by multiple bars at single concentrations of one fraction. Individual bars represent the average percent inhibition of duplicate/triplicate wells compared to the control and include standard error of the mean.

CIW 4 and CIW 8 (Fig 14A-B), as well as CIHR 4 and CIHR 5 (Fig 15), exhibited >50% inhibition of TNF-α production. The CICC and CIE subfractions did not result in any inhibition of >50%. As can been seen in Figure 16, six fractions of CICC did exhibit minimal amounts of inhibition, but failed to reach the standard of at least 50% inhibition required for further experimentation. CIE displayed TNF-α stimulatory activity when the cells were treated with the subfractions and stimulated with LPS, as shown in Figure 17.

Among the 10 CIW fractions screened in trial 1, significant differences between the percentages of TNF-α inhibition were shown with a *p*-value of 0.0036 by ANOVA. Significant differences were also exhibited in trials 2 with *p*-values of 0.0211. Trial 3 included only two fractions: CIW 4 and 5, and the percentages of TNF-α inhibition were drastically reduced and there was not a significant difference between TNF-α inhibition of the two fractions with a *p*-value of 0.1146.

Among the 10 CIHR fractions screened in trial 1 and 5 CIHR fractions screened in trial 2, significant differences between the percentages of inhibition were shown with *p*-values of 0.0009 and 0.0204, respectively, by ANOVA. Trial 3 included only four fractions and no significant difference between the percentages of inhibition were exhibited with a *p*-value of 0.5605. Statistical analyses were not performed on fractions from CICC and CIE due to the lack of TNF-α inhibition exhibited by these sources of chicory. Dose response data for cytotoxicity and TNF-α inhibition of wild-type and hairy root chicory subfractions are detailed in Appendices D and E, respectively.

Chicory extracts were not further fractionated, though potential candidates have been preliminarily identified for future characterization.

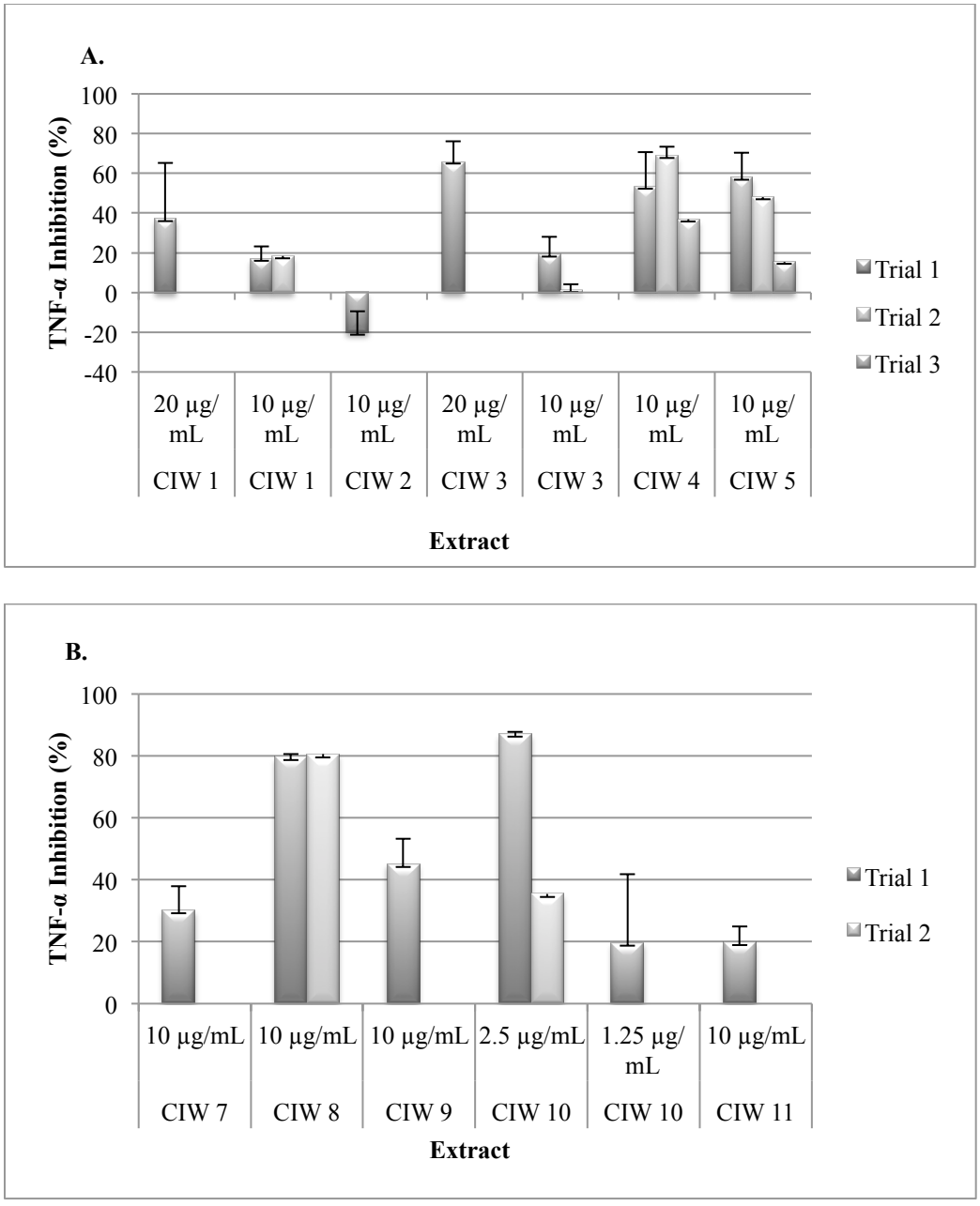


Figure 14. Inhibition of TNF-α Production by Treatment with Wild-Type Chicory Subfractions. Inhibition of TNF-α in response to LPS-stimulated THP-1 cells by selected wild-type *C. intybus* (CIW) subfractions at 10 µg/mL or lower concentrations. A) CIW subfractions 1-5, and B) CIW subfractions 7-11. Individual bars represent the average percent inhibition of duplicate/triplicate wells compared to the control and include standard error of the mean.

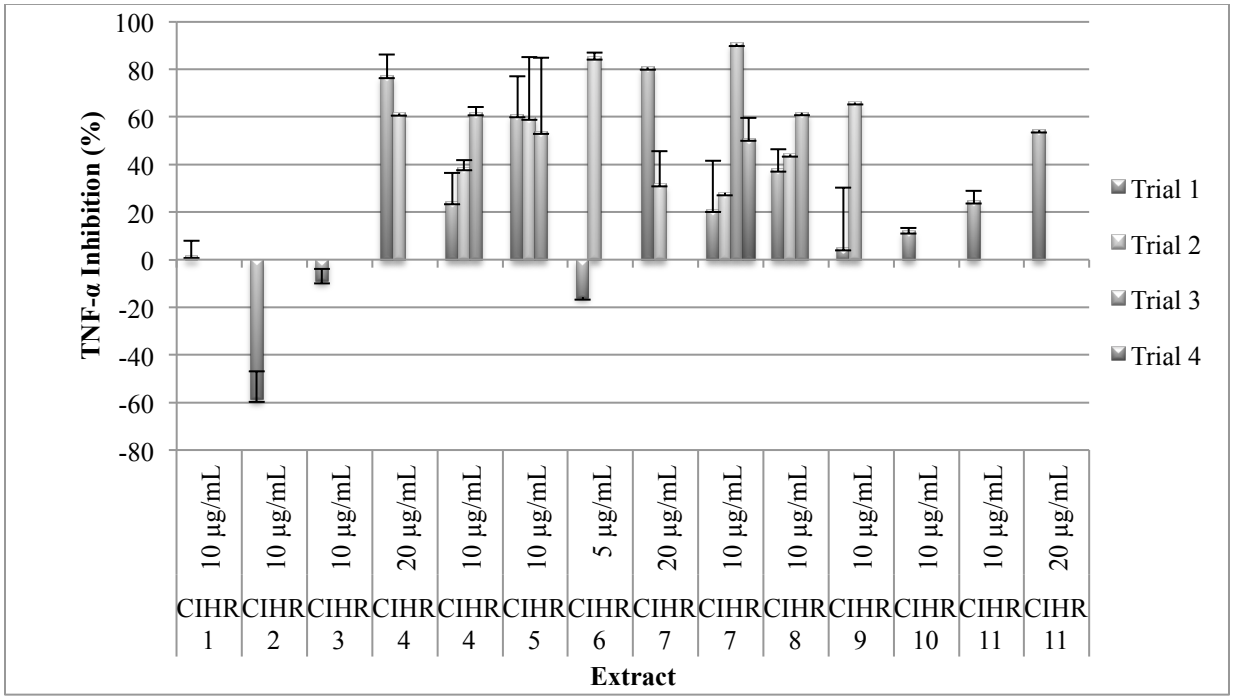


Figure 15. Inhibition of TNF-α Production by Treatment with Hairy Root Chicory Subfractions. Inhibition of TNF-α in response to LPS-stimulated THP-1 cells by selected subfractions of hairy root *C. intybus* (CIHR) at 20 µg/mL or lower concentrations. Individual bars represent the average percent inhibition of duplicate/triplicate wells compared to the control and include standard deviation of the mean.

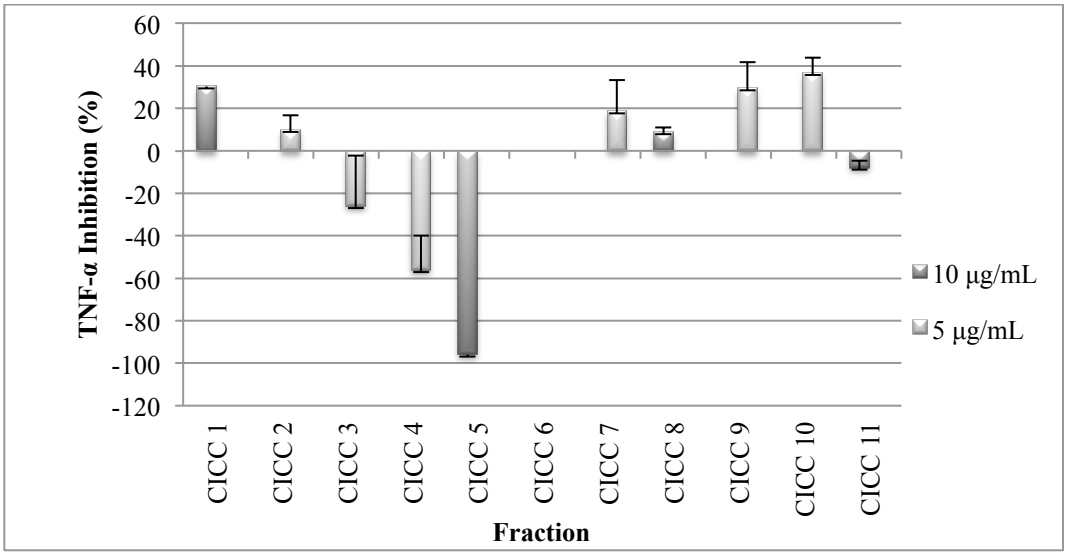


Figure 16. Inhibition of TNF-α Production by Treatment with Callus Cell Culture Chicory Subfractions. Inhibition of TNF-α in response to LPS-stimulated THP-1 cells by selected subfractions of callus cell culture *C. intybus* (CICC) at 10 µg/mL and 5 µg/mL. Individual bars represent the average percent inhibition of duplicate/triplicate wells compared to the control and include standard error of the mean.

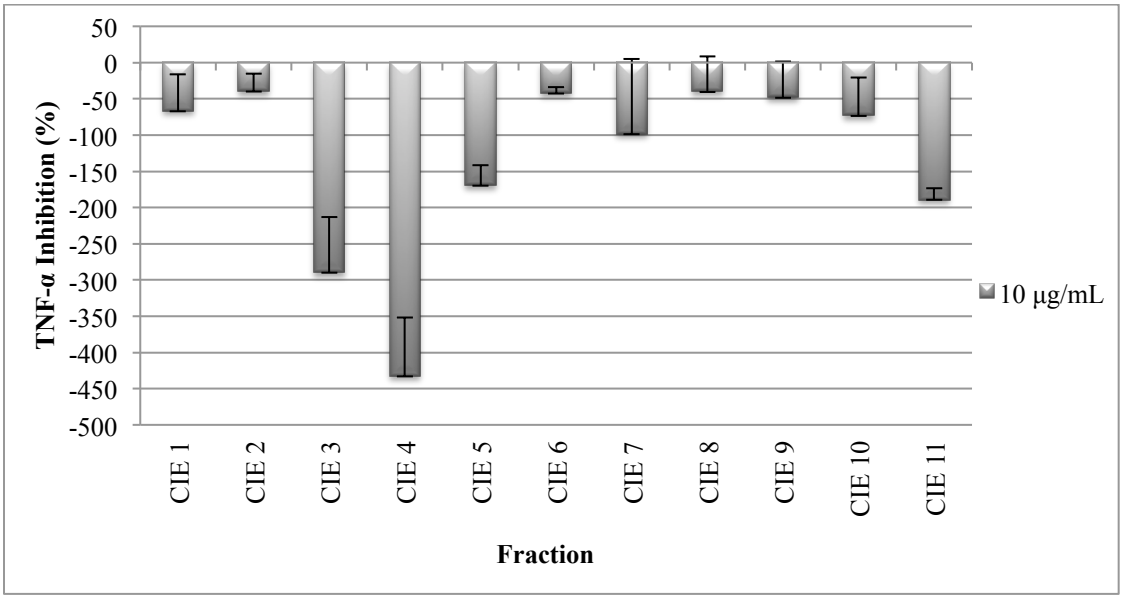


Figure 17. Inhibition of TNF-α Production by Treatment with Callus Cell Culture with Elicitors Chicory Subfractions. Inhibition of TNF-α in response to LPS-stimulated THP-1 cells by selected subfractions of callus cell culture with elicitors *C. intybus* (CIE) at 10 µg/mL. Individual bars represent the average percent inhibition of duplicate/triplicate wells compared to the control and include standard error of the mean.

IV. DISCUSSION

The inflammatory response is essential in fighting infections caused by bacteria, viruses, and parasites. This response leads to the release of chemical mediators, such as pro-inflammatory cytokines and chemokines, resulting in dilation of the blood vessels to allow for increased blood flow and activation of the complement cascade. The cascade of events following inflammation is necessary, though when uncontrolled, can be damaging to the host. Inflammatory diseases arise from uncontrolled inflammation, resulting in the body's immune system attacking itself. There are currently FDA approved medications to treat inflammatory diseases, however the drugs are often toxic and cause severe side effects as well. The need for less toxic alternatives has been a focus of the pharmaceutical research community. Traditional Chinese Medicine is being intensely studied scientifically for potential drug alternatives to an array of illnesses (Manheimer et al 2009). MTSU has a partnership with the Guangxi Botanical Gardens in China and has received a large quantity of plant material to test for potential medicinal purposes. In this study, a plant extract designated as 25A was tested for the ability of compounds in the extract to inhibit macrophage release of TNF-α when stimulated with LPS, a known inflammatory bacterial component, to determine its potential as an anti-inflammatory drug.

The 12 subfractions of the plant, noted during the experiments as 25A 1-12, were initially screened for cytotoxicity with multiple active compounds still detected at concentrations of 50 μg/ml and 25 μg/ml. As shown in Figure 2, fractions 4, 5, 6, and 7 produced less than 90% viability in the human macrophage cell line THP-1 at 50 μg/ml,

fractions 5 and 7 remained toxic at 25 μg/ml and were therefore further tested at 10 μg/ml and 5 μg/ml. Fractions 5 and 7 were non-toxic at the lower concentrations. The cytotoxicity screening was the starting point in this experiment, to determine which fractions would be tested for TNF-α inhibition and at which concentrations. Fraction 7 was re-tested at concentrations of 10 μg/ml, 5 μg/ml, as well as 2.5 μg/ml and 1.25 μg/ml for cytotoxic effects. The results shown in Figure 2 indicate that fraction 7 is non-toxic to human macrophage-like cells at concentrations below 10 μg/ml.

The fractions at non-toxic concentrations were further screened for TNF-α inhibition using ELISA. The criteria in this research for extracts to be further fractionated included greater than 90% viability and greater than 50% TNF-α inhibition. As shown in Figure 3, fractions 3, 9, 10, 11, and 12 showed greater than 50% inhibition at 50 μg/ml. These fractions met the minimum requirements for further fractionation, however, the concentration of 50 μg/mL was taken into account. Fractions that can inhibit TNF-α at lower concentrations are preferred, due to less material being required to produce the drug. Higher potency at a lower concentration is ideal in drug manufacturing.

At 25 μg/ml, fractions 3, 4, 6, 9, and 10 showed greater than 50% inhibition as shown in Figure 3. These fractions had a greater potential for further fractionation due to being active at a lower concentration. However, for this study, fractions 5 and 7 were the best choices for fractionation. Fraction 5 showed greater than 50% inhibition at 10 μg/ml, while fraction 7 showed equal activity at 5 μg/ml. Taking into account the various factors, including dosage and chemical properties, the decision was made to further separate fraction 7 by bioassay-guided fractionation. The dose response of 25A-7 can be

seen in Figures 4 and 5, which show that each concentration (1.25 - 10 μg/mL) is non-toxic and also inhibits TNF-α by at least 50%.

Fraction 7 of 25A was then further separated into three subfractions with the assistance of Raj Ghosh and the facilities in the MTSU Chemistry Department, using reversed phase HPLC. Fractions F_1, F_2, and F_3 (Fig 6) were screened for bioactivity, including cytotoxicity and TNF-α inhibition, as previously performed on the 25A subfractions. As shown in Figure 7, all three fractions were not cytotoxic in the human THP-1 cell line at a concentration of 50 μg/mL, with greater than 90% cell viability. The 50 μg/mL concentration was used to determine the percentage of TNF-α inhibition by ELISA. Figure 8 shows two trials with duplicate and triplicate experiments, respectively. 25A-7 F_1 was shown to be the subfraction of 25A-7 that contributed to the inhibition of TNF-α, with an average of 50% inhibition in each trial.

The preliminary IC_{50} of 25A-7 F_1 was performed to determine the inhibition for the TNF-α response. Though the initial screening at 50 μg/mL exhibited that F_1 was inhibiting at approximately 50%, the IC_{50} was performed to determine whether the activity increased at a higher concentration or maintained or lost activity at lower concentrations. Figure 9 shows that 25A-7 F_1 remained non-toxic at higher and lower concentrations (12.5 - 100 μg/mL). However, TNF-α inhibitory activity began to decrease at 12.5 μg/mL with only 40% inhibition. This is informative in order to understand that this compound must be used at a dosage higher than 12.5 μg/mL to be an effective anti-inflammatory treatment; however, the IC_{50} needs to be repeated using a dose response ranging between six concentrations.

Early drug discovery must meet certain criteria before the developmental meeting with the FDA. The active component must first be identified and confirmed as a hit using biological activity assays. Selecting the candidate also requires that the active component demonstrates *in vivo* activity, has an acceptable safety margin of toxicity in rodents, as well as has manufacturing feasibility and acceptable drug interaction profile. The FDA then approves clinical trials in human subjects (Hughes 2012). Currently, the active component in plant extract 25A has been identified and confirmed using biological assays.

The 44 chicory extracts were initially screened for cytotoxicity at a concentration of 10 μg/mL. The lower concentration of chicory (compared with 50 μg/mL of plant 25A fractions) was chosen based on the numerous samples and need for efficient screening. Samples that showed approximately 50% inhibition were further screened at higher concentrations. As shown in Figures 10 and 11, only three fractions were toxic to human macrophages at a concentration of 10 μg/mL. CIW 4, CIW 10, and CIHR 6 were non-toxic at a lower concentration of 5 μg/mL. Similarly to the experimentation with plant 25A fractions, the non-toxic concentrations of each chicory extract was then screened for >50% TNF-α inhibitory activity using ELISA. Testing of several of the fractions was repeated due components in chicory possibly degrading over time or due to an older cell line that was no longer responding to the LPS stimulation. Figures 14-17 display the inhibition of each fraction from each chicory source at the non-toxic concentrations. Multiple data points indicate that the fraction was repeated at least once. CIW 8 and CIHR 5 at 10 μg/mL were chosen as two potential candidates due to the consistency of inhibiting TNF-α by >50% in each repeated experiment, respectively. As can be seen in

the data in Figures 14-17, only two of the four chicory sources exhibited significant amounts of TNF-α inhibitory activity, the wild-type and the hairy root sources. The callus cell culture sources, with and without elicitors, either showed no activity or increased the production of TNF-α. Synthetic elicitors, such as the elicitors added to the callus cell culture chicory subfractions (CIE) are drug-like molecules that are responsible for plant defense, including protection of crops from diseases and immunity defense (Bektas and Eulgem 2015).

Chicory is not presently being further fractionated; however, more research needs to be conducted using the multiple potential anti-inflammatory targets discovered. This is especially important considering the plant is indigenous to areas of Tennessee. The results are promising and will be beneficial in future research.

The most important future directions of this research involve further experimentation of the isolated compound of plant extract 25A and proper characterization of the compound using Nuclear Magnetic Resonance (NMR). The future directions of this compound also include further biological testing, such as against other cytokines, as well as eventually testing in an animal model.

Inflammatory diseases are a serious concern in the United States, with 1.6 million Americans suffering from IBD alone and the cost and side effects of treatment exponentially rising. Traditionally used medicinal plants offer an exciting alternative or supplement to drug therapy treatments and 25A-7 F_1 has extremely promising potential.

LITERATURE CITED

Abdel-Tawab M, Zettl H, Schubert-Zsilavecz M. 2009. Nonsteroidal anti-inflammatory drugs: a critical review on current concepts applied to reduce gastrointestinal toxicity. Curr Med Chem. 16: 2042-63.

Abraham SM, Lawrence T, Kleman A, Warden P, Medghalchi M, Tuckermann J, Saklatvala J, Clark AR. 2006. Anti-inflammatory effects of dexamethasone are partly dependent on induction of dual specificity phosphatase 1. J Exp Med. 203: 1883-1889.

Aung HT, Sein MM, Aye MM, Thu ZM. 2016. A review of traditional medicinal plants from Kachin State, Northern Myanmar. Nat Prod Commu. 11 (3): 353-364.

Bais HP, Ravishankar GA. 2001. *Cichorium intybus* L. – cultivation, processing, utility, value addition and biotechnology, with an emphasis on current status and future prospects. J Sci Food Agri. 81 (5): 467-484.

Bektas Y, Eulgem T. 2015. Synthetic plant defense elicitors. Front Plant Sci. 5:804.

Bischoff TA, Kelley CJ, Karchesy Y, Laurantos M, Nguyen-Dinh P, Arefi AG. 2004. Antimalarial activity of Lactucin and Lactucopricrin: sesquiterpene lactones isolated from *Cichoirum intybus* L. J Ethnopharmacol. 95 (2-3): 455-457.

Brooks KA, Lawson MA, Smith RA, Janda TM, Kelley KW, McCusker RH. 2016. Interactions between inflammatory mediators and corticosteroids regulate transcription of genes within the Kynurenine Pathway in the mouse hippocampus. J Neuroinflamm. 13:98.

Cong HJ, Zhao Q, Zhang SW, Wei JJ, Wang WQ, Xuan LJ. 2014. Terpenoid indole alkaloids from *Mappianthus iodoides* Hand.-Mazz. Phytochemistry 100: 76-85.

Daigneault M, Preston JA, Marriott HM, Whyte MKB, Dockrell DH. 2010. The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages. Pub Lib Sci. 5(1): 8668

Elias K, Laurence A, Davidson TS, Stephens G, Kahno Y, Shevach EM, O'Shea JJ. 2008. Retinoic acid inhibits Th17 polarization and enhances FoxP3 expression through a Stat-3/Stat-5 independent signaling pathway. Blood 111:1013-1020.

El-Sayed M, Verpoorte R. 2007. Catharanthus terpenoid indole alkaloids: biosynthesis and regulation. Phytochem Rev. 6(2): 277-305.

Fang D, Qin DH. 2002. Two new species of Adandra and Mappianthus from Guangxi. Acta Bot Yunn. 24: 709-711.

Feghali CA, Wright TM. 1997. Cytokines in acute and chronic inflammation. Front Biosci. 2: 12-26.

Hughes M, Inglese J, Kurt A, Andalibi A, Patton L, Austin C, Baltezor M, Beckloff M, Sittampalam S, Weingarten M, Weir S. 2012. Early drug discovery and development guidelines: for academic researchers, collaborators, and start-up companies. NCBI Bookshelf. [cited 2017 February 21]. Available from: http://cores.utah.edu.

Judzentiene A and Budiene, J. 2008. Volatile constituents from aerial parts and roots of *Cichorium intybus* L. (chicory) grown in Lithiuania. Chemija 19: 25-28.

Manheimer E, Wieland S, Kimbrough E, Cheng K, Berman BM. 2009. Evidence from the Cochrane Collaboration for Traditional Chinese Medicine therapies. J Altern Complement Med. 15(9): 1001-1014.

Maroon JC, Bost JW, Maroon A. 2010. Natural anti-inflammatory agents for pain relief. Surg Neurol Int. 1:80.

Martich GD, Boujoukos AJ, Suffredini AF. 1993. Response of man to endotoxin. J Immunobio. 187(3-5): 403-416.

Molodecky NA, Soon IS, Rabi DM, Ghali WA, Ferris M, Chernoff G, Benchimol EI, Panaccione R, Ghosh S, Barkema HW, Kaplan GG. 2012. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. J Gastroenterol. 142(1): 46-52.

O'Brien J, Wilson I, Orton T, Pognan F. 2000. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur J Biochem. 267 (17): 5421-5426.

Park KT, Bass D. 2010. Inflammatory bowel disease – attributable costs and cost effective strategies in the United States: A review. Inflamm Bowel Dis. 17 (7): 1603-1609.

Qihe X, Bauer R, Hendry B, Fan, TP, Zhao Z, Duez P, Simmonds MS, Witt CM, Lu A, Robinson N, Guo D, Hylands PJ. 2013. The quest for modernisation of Traditional Chinese Medicine. BMC Complement Altern Med. 13:132.

Rodgers M, Epstein D, Bojke L, Yang H, Craig D, Fonseca T, Myers L, Bruce I, Chalmers R, Bujkiewicz S, Lai M, Cooper N, Abrams K, Spiegelhalter D, Sutton A, Sculpher M, Woolacott N. 2011. Etanercept, infliximab and adalimumab for the treatment of psoriatic arthritis: a systematic review and economic evaluation. NIHR Health Technology Assessment programme: Executive Summaries.

Singh G and Ramey D. 1998. NSAID induced gastrointestinal complications: the ARAMIS perspective. J Rheumatol. 51(Suppl.): 8-16.

Skoog D, Holler J, Nieman T. 1998. Principles of Instrumental Analysis. Thompson Learning, Inc. 5: 673-697, 725-766.

Suresh B, Sherkhane PD, Kale S, Eapen S, Ravishankar A. 2005. Uptake and degradation of DDT by hair root cultures of *Cichorium intybus* and *Brassica juncea*. Chemosphere 61 (9): 1288-1292.

Thomson ABR, Gupta M, and Freeman HJ. 2012. Use of the tumor necrosis factor blockers for Crohn's disease. World J Gastroenterol. 18: 4823–4854.

U.S. Food and Drug Administration (FDA), Center for Drug Evaluation and Research. Medication guide for non-steroidal anti-inflammatory drugs (NSAIDs). August 2007. Retrieved December 23, 2016, from http://www.fda.gov/downloads/Drugs/DrugSafety/ucm089162.pdf.

Verpoorte R, van der Heijden R, Moreno PRH. 1997. Biosynthesis of terpenoid indole alkaloids in *Catharanthus roseus* cells. Academic Press 49: 221-299.

Woo JH, Lee JH, Kim H, Park SJ, Joe E, Jou I. 2015. Control of inflammatory responses: a new paradigm for the treatment of chronic neuronal diseases. Exp Neurobiol. 24: 95-102.

APPENDICES

APPENDIX A. Cell Viability and TNF-α Inhibition of 25A Subfractions

25A Fraction	Concentration (µg/mL)	Cell Viability (%)*	Cell Viability SEM	TNF-α Inhibition (%)	TNF-α Inhibition SEM
1	50 µg/mL	96.577	0.075	0	N/A
	25 µg/mL	102.485	0.026	0	N/A
2	50 µg/mL	100.442	0.274	0	N/A
	25 µg/mL	99.311	0.099	0	N/A
3	50 µg/mL	100.699	0.014	61	3.11
	25 µg/mL	103.075	0.157	62	2.53
4	50 µg/mL	70.698	0.39	N/A	N/A
	25 µg/mL	98.445	0.707	99	0.11
5	50 µg/mL	24.659	0.248	N/A	N/A
	25 µg/mL	57.632	0.139	N/A	N/A
	10 µg/mL	98.625	0.954	94	1.14
	5 µg/mL	102.226	0.148	55	3.35
6	50 µg/mL	79.819	3.253	N/A	N/A
	25 µg/mL	97.247	0.642	99	0.04
7	50 µg/mL	37.18	0.613	N/A	N/A
	25 µg/mL	75.424	0.944	N/A	N/A
	10 µg/mL	93.575	0.419	N/A	N/A
	5 µg/mL	97.117	2.356	92	2.25
8	50 µg/mL	99.192	0.13	13	7.12
	25 µg/mL	98.061	0.192	15	0.92
9	50 µg/mL	99.835	0.553	97	7.12
	25 µg/mL	98.217	0.257	94	1.85
10	50 µg/mL	97.692	0.705	94	0.39
	25 µg/mL	94.956	1.278	91	0.24
11	50 µg/mL	101.803	0.525	63	9.07
	25 µg/mL	99.554	0.435	0	7.3
12	50 µg/mL	101.348	0.611	64	7.3
	25 µg/mL	103.395	0.228	46	8

*

* Values greater than 100% demonstrate higher cell count than the control

APPENDIX B. Cell Viability and TNF-α Inhibition of 25A-7 Subfractions

25A-7 Subraction	Concentration (µg/mL)	Cell Viability (%)*	Cell Viability SEM	TNF-α Inhibition (%)	TNF-α Inhibition SEM
F_1	50 µg/mL	105.389	0.339	50.844	10.328
		95.289	2.943	52.211	11.737
F_2	50 µg/mL	109.546	0.328	8.308	3.145
		102.105	0.227	3.477	4.041
F_3	50 µg/mL	108.597	0.031	27.312	6.963
		101.57	0.273	17.810	7.300

*

* Values greater than 100% demonstrate higher cell count than the control

*

APPENDIX C. IC_{50} of 25A-7 F_1

Concentration (µg/mL)	Cell Viability (%)*	Cell Viability SEM	TNF-α Inhibition (%)	TNF-α Inhibition SEM
100	95.451	0.042	52.054	6.214
50	103.857	0.184	52.693	5.045
25	102.714	0.001	63.508	3.085
12.5	101.867	0.025	41.960	19.927

* Values greater than 100% demonstrate higher cell count than the control

APPENDIX D. Cell Viability and TNF-α Inhibition of Wild-Type Chicory Subfractions

Chicory Fraction	Concentration (µg/mL)	Cell Viability (%)*	Cell Viability SEM	TNF-α Inhibition (%)	TNF-α Inhibition SEM
CIW 1	20 µg/mL	102.402	0.359	36.92	28.17
	10 µg/mL	93.649	0.566	16.96	6.37
	10 µg/mL	102.961	0.326	18.21	6.15
CIW 2	10 µg/mL	94.991	0.054	-20.28	10.87
CIW 3	20 µg/mL	101.874	0.411	65.8	10.36
	10 µg/mL	89.085	1.157	19.06	9.12
	10 µg/mL	104.061	0.611	1.26	
CIW 4	10 µg/mL	82.908	0.037	53.09	17.45
	5 µg/mL	95.548	0.908	68.72	3.01
	5 µg/mL	101.691	0.267	36.74	6.00
CIW 5	10 µg/mL	90.34	0.469	57.74	12.57
	10 µg/mL	103.572	0.264	47.93	4.71
	10 µg/mL	102.087	0.017	15.4	8.75
CIW 6	10 µg/mL	95.22	1.78	58.59	23.91
	10 µg/mL	105.864	0.457	Out of range	N/A
CIW 7	10 µg/mL	90.545	0.744	30.17	7.67
	10 µg/mL	100.755	0.578		
CIW 8	10 µg/mL	96.503	0.381	79.67	0.89
	10 µg/mL	99.402	0.044	80.51	7.89
CIW 9	10 µg/mL	93.981	0.167	45.09	8.19
	10 µg/mL	95.933	0.369		
CIW 10	10 µg/mL	88.342	0.063	N/A	N/A
	5 µg/mL	88.098	0.528	N/A	N/A
	2.5 µg/mL			87.17	0.67
	2.5 µg/mL	98.298	0.658	35.45	24.99
	1.25 µg/mL			19.67	22.06
CIW 11	10 µg/mL	89.798	0.255	19.83	5.08

*

* Values greater than 100% demonstrate higher cell count than the control

*

APPENDIX E. Cell Viability and TNF-α Inhibition Hairy Root Chicory Subfractions

Chicory Fraction	Concentration (µg/mL)	Cell Viability (%)*	Cell Viability SEM	TNF-α Inhibition (%)	TNF-α Inhibition SEM
CIHR 1	10 µg/mL	99.057	0.321	1.67	6.39
CIHR 2	10 µg/mL	97.803	1.108	-58.76	11.81
CIHR 3	10 µg/mL	95.013	0.374	-8.97	5.05
CIHR 4	20 µg/mL	102.418	0.561	77.27	8.91
	20 µg/mL	104.183	0.177	61.51	6.97
	10 µg/mL	97.451	0.641	22.28	12.04
	10 µg/mL	100.671	0.077	38.58	5.52
	10 µg/mL	101.617	0.391	61.60	8.66
CIHR 5	10 µg/mL	99.804	0.169	60.84	16.17
	10 µg/mL	103.055	0.022	59.73	17.62
	10 µg/mL	98.657	0.404	53.82	20.13
CIHR 6	10 µg/mL	85.983	7.494	N/A	N/A
	5 µg/mL	93.498	1.579	-15.83	0.27
	5 µg/mL			85.01	45.85
CIHR 7	20 µg/mL	100.262	0.467	80.82	1.12
	20 µg/mL	101.7	0.500	31.71	3.12
	10 µg/mL	91.894	3.24	20.00	20.62
	10 µg/mL	100.115	0.407	28.03	25.28
	10 µg/mL	100.464	0.254	90.76	2.61
	10 µg/mL	99.673	0.444	50.94	8.61
CIHR 8	10 µg/mL	96.046	0.758	37.85	8.67
	10 µg/mL	99.273	0.092	44.29	1.95
	10 µg/mL	97.139	0.143	61.69	30.96
CIHR 9	10 µg/mL	94.838	2.007	4.86	25.38
	10 µg/mL	98.421	1.205	66.16	13.78
CIHR 10	10 µg/mL	93.081	2.657	11.96	1.35
CIHR 11	20 µg/mL	101.435	0.276	54.43	16.94
	10 µg/mL	97.501	2.052	24.59	4.24

* Values greater than 100% demonstrate higher cell count than the control