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Determination of the Active Components of Elderberry Extracts on Immune Function and Tumor Cell Growth

Sahar Rizvi
Indiana University - Purdue University Fort Wayne

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Elliott Blumenthal
Chair

Robert J. Visalli

Vincent M. Maloney

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DETERMINATION OF THE ACTIVE COMPONENTS OF ELDERBERRY EXTRACTS ON IMMUNE FUNCTION AND TUMOR CELL GROWTH

A Thesis

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of

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For my parents, without whose love and encouragement, I could not have come this far.

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ABSTRACT

Rizvi, Sahar. M.S., Purdue University, December 2012. Determination of the Active Components of Elderberry Extracts on Immune Function and Tumor Cell Growth. Major Professor: Elliott Blumenthal.

Present cancer treatments cause the cessation of the body's own natural defenses, resulting in the susceptibility of patients to other illnesses, namely infection, during therapy. Contemporary research must focus on developing treatments to preserve or even augment the body's natural defenses by pursuing naturally-derived elements devoid of such adverse effects. Seeking more natural treatments, *Sambucus Nigra* (elderberry) proves to be an important source of known immune stimulators including anthocyanins, catechins, and tannins, as wells as several known antioxidants and tumor suppressive agents in the form of flavonoids and proanthocyanidins.

Our primary goal was to separate the distinctive components of an elderberry extract using gravity column chromatography and examine their proliferative effects on T lymphocytes as well as their inhibitory effects on the growth of human skin melanoma cells.

Due to the successful effects of crude elderberry extracts on both immune enhancement and tumor suppression, we concluded that there must be specific components within the berry that enables its therapeutic value.

The effects on immune cell stimulation were assessed through the quantitative analysis of spleen cell proliferation, which yielded significantly positive spleen cell stimulation following the addition of and concanavalin A, a known mitogen, as compared to the spleen cells incubated solely with concanavalin A.

The inhibitory effect of the extract fractions was examined on a melanoma cell line. The successful suppression of tumor cell growth by several of the elderberry extract fractions, without mass suppression, was observed. In vivo studies using specific elderberry fractions may validate the use of the resolved components as viable therapeutic agents both in immunotherapy as well as chemotherapy.

INTRODUCTION

Cancer

Cancer is a devastating illness with both somatic and emotional consequences, and when uncontrolled, results in rapid death, making cancer the leading cause of mortality in the United States, second only to cardiovascular disease. According to the *American Cancer Society*, one out of every four deaths in the United States is due to cancer. Furthermore, in 2010 approximately 1,529,560 new cases of cancer were predicted to be diagnosed, and about 569,490 Americans were expected to die of cancer which meant nearly 1,500 people per day. Skin cancer is the most prevalent form, with an estimated over 3.5 million cancers diagnosed in more than two million people in the United States alone. Each year there is a greater incidence of skin cancer than the collective incidence of breast, prostate, lung, and colon cancers. The most malignant and dire form of skin cancer is a melanoma. Every sixty-two minutes, a person dies from melanoma, and in 2010 it was estimated that approximately 11,790 deaths would occur as a result of skin cancer. To understand the destructive consequences of the disease it is important to comprehend the pathological process of the diseased tissue. Cancer is the accelerated production of cells that have ceased to follow their expected reproductive and proliferative functions. These cells may also proceed to invade adjacent tissues contiguous to the atypical growth, and even possibly spread to other organs in the body,

in a process known as metastasis. Exposure to chemical carcinogens, radiation and even some viruses may produce mutations in cells, resulting in unregulated growth patterns. Cancer cells retain the ability to become malignant through mutations in growth control mechanisms of cells, including proto-oncogenes and their corresponding tumor suppressor proteins.

With the promising developments in clinical research and technology, the diagnosis and management of numerous diseases have improved significantly over time. The number of deaths per year related to cancer remains high, but the mortality rate has declined due to the progression and advancements in treatment modalities, allowing a greater chance of possibly surviving this morbid disease.

Role of the Immune System in Tumor Detection and Elimination

The human immune system, which contains a network of cells, tissues, and organs work synchronously together to defend against foreign invaders. The integrity of the immune system is critical in the identification and targeting of mutated cells in the body. The ultimate operative responsibility of the immune system is to differentiate between a "self" cell and a "non-self," or foreign, cell in order to rid the body of external intruders, while maintaining a homeostatic balance of self cells. This ability enables the immune cells to directly attack foreign cells and readily eliminate them through various immunological mechanisms. The immune cells are challenged when they encounter cancerous cells; by acting as a "self" cell, altered cells often go undetected by surveillance mechanisms during the initial phases of tumorigenesis. Once a tumor cell is recognized, cell growth can be halted through the process of necrosis or apoptosis. Necrosis is characterized by

mitochondrial swelling and increased permeability of the plasma membrane, while apoptosis is the process of programmed cell death and involves the breakdown of the cell into membrane-bound components that can be easily removed.

The immune system is categorized into two main subsets, the innate immune system and the acquired immune system. The innate immune system is characterized as the fast-acting, nonspecific portion of the immune system, comprising of the body's natural barriers, phagocytes, natural killer cells, and other molecules involved in the stimulation of other immune responses. In the event of tumorigenesis, natural killer (NK) cells serve a key role in stimulating a cascade of events resulting in tumor cell death. In addition to the activation of other immune cells through the secretion of cytokines and other factors, NK cells primarily attack tumor cells through the release of the cytolytic protein, perforin as well as serine proteases known as granzymes. Perforin generates transmembrane holes in target cells that can function as channels for other defensive proteins to be inserted. Granzymes then invade the target cells, forcing them to undergo apoptosis through the activation of certain caspase molecules. The cytotoxic effects of natural killer cells are highly specific and generally function successfully without harming the normal cells.

Acquired immunity constitutes the portion of the immune system that takes longer to respond and is antigen specific, and forms memory cells to act quickly in any subsequent exposure to the same invader. The acquired immune system further divided into the humoral immune response, governed by the B lymphocytes and the cell-mediated response, mediated by the T lymphocytes. B cells primarily release antibodies in response to exposure to a foreign antigen, and are successful in combating bacteria, bacterial

toxins, and viruses that circulate freely in body fluids, before they enter cells. T cells are responsible for targeting bacteria and viruses that have already invaded host cells and are inaccessible to antibodies, fungal infections, cancer cells, and the rejection of transplanted tissue. Therefore, in the defense against tumor cells, it is the cell-mediated immune function that engages a non-specific, nonclonal reponse in combating these altered cells.

In order for the cytotoxic T (T_c) lymphocytes to take action against tumor cells, the abnormal cells must surface proteins present that can be recognized as foreign. These may be neoantigens or even alterations of existing antigens and can be formed through the modifications in gene expression. The T_c cells then react to tumor cells through the recognition of these surface antigens by triggering apoptosis through one of two pathways. The first pathway entails the release of granules from the cytotoxic cells that in turn activate the appropriate components that will induce apoptosis, while the second pathway involves the union of the Fas ligand on the surface of the T cell with the Fas surface protein on the target cell. This association leads to the activation of the enzymes that can trigger apoptosis through the destruction of the cytoskeleton proteins of the tumor cell.

Tumors may illicit an immune response as apparent through the regression of residual tumors once a primary tumor is removed. The presence of tumor-specific antibodies and T lymphocytes in patients with tumors also confirms an active immune reaction opposing the propagation of malformed cells. There is even an increased prevalence of malignancies in immunodeficient patients including transplant patients, who have increased susceptibility to Kaposi sarcoma and patients with acquired immune

deficiency syndrome, who have a greater propensity to Epstein Barr virus-induced lymphoma.

Cancer cells employ several different methods to evade recognition and destruction by the immune system. Not all cancer cells possess all of the surface markers that are required for complete activation of the cytotoxic T cells. This could include the altered surface antigens, the major histocompatibility complex molecule or even the co-stimulatory surface molecules. Absence of any one of these molecules will result in a lack of reactivity by the T_c cells. In addition to the physical attributes of the tumor cells, themselves, tumor cells have been known to release chemical substances that possess the ability to suppress the responses of the immune system. Melanoma cells in particular secrete several substances, including follistatin, placenta growth factor and vascular endothelial growth factor. Notably, melanoma cells secrete transforming growth factor-beta (TGF-β), which serves an important role in immunosuppression. TGF- β has possesses inhibitory effects on the proliferation of thymocytes, B and T lymphocytes, natural killer cells, monocytes, as well as macrophages, making its release by melanoma cells highly detrimental to the host's reactivity against these tumor cells.

Caspase Activity

Caspases are proteases that possess an integral role in apoptosis, by the physical breakdown of cellular components. Caspase regulation is the central focus in anti-cancer medication due to their profound impact on the apoptotic process. Even when the body mounts a response to carcinogenic activity, the rate of transformation of these cells and the production of mutated progeny considerably outweighs the defense capabilities of the

immune system, and therefore external assistance is mandatory in the form of different therapeutic regimens. There are two primary pathways to activate caspase activity. The first pathway, called activation-induced cell death, which is the most common pathway of apoptosis activation outside the thymus, involves the Fas surface protein and the Fas ligand and usually takes about 2-4 hours. Upon T cell activation, the expression of these surface proteins is up regulated by the T cell. The binding of Fas ligand to Fas causes the recruitment of FADD (Fas-associated protein with death domain) followed by the subsequent binding of procaspase 8. The association of procaspase 8 and FADD causes the proteolytic cleavage of procaspase 8 into its active form, caspase 8, which then initiates a cascading event of proteolysis and ultimately cell death.

Another pathway used in inducing apoptosis, the mitochondrial death pathway, is much longer than the Fas pathway, taking up to 8-10 hours. In this pathway, cytochrome c, which normally inhabits in the inner mitochondrial membrane, escapes into the cytosol which enables it to bind to the protein Apaf-1 (apoptotic protease-activating factor I). This causes Apaf-1 to undergo an ATP-dependent conformational change and oligomerization, which enables its binding to procaspase 9 and the conversion of procaspase 9 to caspase 9. The complex formed here consisting of caspase 9, Apaf-1, and cytochrome c, is termed the apoptosome and it cleaves procaspase 3, generating the active form caspase 3, which leads to more events that result in apoptosis. The mitochondrion also releases AIF (apoptosis inducing factor) which also functions in cell death.

Current Cancer Treatment Options

Through several decades of research, clinical trials, and determination, cancer treatment has evolved from the primitive techniques of cauterizing mutated tissue to several curative choices in cancer intervention. Presently, the most common treatment options for cancer include chemotherapy, radiation therapy, surgery, and post-surgical pharmaceutical therapy.

Unfortunately, the negative repercussions of these treatment modalities may, at times, outweigh their proposed benefits. Depending on the extent of growth and the locality of the tumor or tumors, surgical techniques such as cryosurgery, electrosurgery and laproscopic surgery are often the preliminary therapeutic approach, but may frequently result in bleeding, scarring, and even loss of bone tissue, which can pave way for further complications. Chemotherapeutic agents such as carboplatin, doxorubicin, and imatinib mesylate commonly induce adverse effects such as anemia, bleeding, alopecia, and fertility changes, while radiation therapy may cause negative symptoms like vomiting, physical pain, and fatigue. A common thread amongst all of these therapeutic agents in treating cancer is that, although they serve to target tumor cell death, they do not enhance the body's own defense system. Even more detrimental to the patient, most therapeutic agents used in cancer treatment are immunosuppressant, which creates susceptibility to other illnesses during the attempt of eliminating malignant tissue. The most common diseases acquired by immunocompromised patients are bacterial, viral, and fungal infections. Prophylactic treatments are commonly used amongst cancer patients but involve an increase in medications required in the diet, amplifying the chance of even more side effects. It is highly encouraged that immunodeficient patients avoid contact

with others and maintain limited interaction with their close family and friends to reduce the risk of contracting infection. This can take a mental and physical toll on both the patient and the patient's family as well.

Natural Therapeutic Agents

With scientific advancement and the progression of cancer therapy, researchers must concentrate on regimens that will not disrupt the function of the patient's immune system by pursuing more naturally-derived elements. A more favorable alternative would be to investigate an agent that would preserve or even augment the body's natural defenses. Research on naturally-derived compounds such as turmeric, ginger, and a wide variety of spices and natural foods have confirmed the existence of therapeutic value in these substances. For centuries, Asian folk medicine has incorporated the benefits of turmeric into their herbal remedies. Now, the use of scientific investigative methods has confirmed the therapeutic benefits possessed by curcumin, the active ingredient in turmeric. The assessment of the suppression of a murine histiocytic tumor, AK-5, was assessed when treated with curcumin. Apoptosis analysis revealed the induction of typical chemical features of apoptosis including evidence of apoptotic bodies as well as AK-5 DNA fragmentation involving caspase-3. 80% of the animals that were transplanted with AK-5 cells did not develop fluid in the peritoneal cavity after the curcumin treatment was administered (Khar 1999).

Extensive studies have established the potential preventative effects of ginger root on noncardia gastric adenocarcinoma. The ginger root was tested on 19 different strains of Helicobacter pylori, several of which were of the CagA+ strain. The ability of the

gingerols in ginger to inhibit the growth of the CagA+ strain of H. pylori establishes its ability to prevent tumor manifestation in the gastric tract due to the known positive role of H. pylori in the development of tumors (Mahady 2003).

Notably, saffron has already been established as an agent suitable for its anti-cancer effects. Furthermore, the chemoprotective role of saffron in the prevention of genotoxicity by known anti-tumor drugs was assessed. Saffron was administered to mice prior to their treatment with cisplatin, cyclophosphamide, or mitomycin C. Comet assays reveal the decreased DNA damage induced by the anti-tumor agents as compared to mice not receiving prophylactic treatment with saffron (Premkumar 2006).

Sambucus Nigra

As researchers continue to search nature for therapies that are non-engineered, elderberry emerges as an essential source of several therapeutic agents. Commonly found in North America and Europe, elderberry's primary use lies in wines, jams, and other foods for both flavor and color enhancement. For centuries, this beneficial fruit served as a source of medicinal therapies including treating open wounds, influenza, and even the common cold in European folk medicine (Balch 1990). As a homeopathic remedy, it has proven its effectiveness in numerous ailments and conditions such as constipation and rheumatism (Kilham 2001). Due to its exhibition of curative value, elderberry has ignited interest in its chemopreventative qualities and immune enhancement capabilities, therefore creating an increase in the investigative research surrounding this berry.

Elderberry as an Immune Modulator

On its own, elderberry, has even displayed a significant role in immune modulation via the stimulation of inflammatory cytokine production (Barak 2002). Interleukins 1, 2, and 6 were increased following elderberry administration. Interferon γ and tumor necrosis factor α were also shown to be increased by elderberry, and therefore substantiates the role of elderberry in the augmentation of cytokine production (Burns 2010).

Elderberry as an Anti-Carcinogenic

Elderberry was shown to serve as an inhibitory of the initiation and promotion stages of carcinogenesis. Both the phenolic components (quercetin, proanthocyanidins, etc,) and the nonphenolic compounds (sesquiterpenes, phytosterols, etc.) were active participants in the strong induction of quinone reductase (anti-initiation) and inhibition of cyclooxygenase-2 (anti-promotion). (Thole 2006). In the combination of several different berry extracts known as *Optiberry*, elderberry and other bacciferous fruits proved to be powerful antioxidants and anti-carcinogens (Bagchi 2004).

Elderberry as an Anti-Viral

The anti-influenza activity of elderberry extract was assessed in an *in vitro* assay, with human influenza A virus particles. The flavonoids in the elderberry extract prevent the entry of viruses into the host cell, thereby inhibiting successful viral infection (Roschek 2009) In a separate study, it was found that patients suffering from influenza-

like symptoms that received 15 mL of elderberry daily, recovered an average of 4 days faster than patients taking a placebo (Zakay-Rones 2004).

Sambucol

Due to the beneficial effects of elderberry, a standardized extract of elderberry has been produced in the form of Sambucol. Studies analyzing the effects of Sambucol have confirmed the anti-viral effects of elderberry. Sambucol proved 99% effective against the H5N1 virus and even neutralized its infectivity in cell cultures (Patton 2006). In two separate studies, Sambucol displayed its chemotherapeutic value in the recovery from influenza. During an outbreak of influenza B, improvement of symptoms was seen sooner as compared to control groups (Zakay-Rones 1995). It decreased the duration of flu-like symptoms in another double-blind, placebo-controlled study (Erling 2004).

The therapeutic activity of elderberry is correlated with its high content of antioxidants and tumor suppressive agents in the form of flavonoids and proanthocyanidins. For a summary of the compounds found in elderberry with their known benefits, see Table A1. In the treatment of cancer, the goal is not only to remove the cancerous tissue, but to augment the body's natural capacity to prevent and combat mutation in the body. As technology advances and new treatments are developed, one must investigate the possibilities of merging natural, immune-enhancing products with the innovated techniques and products. The more natural a product is, the less adverse effects it can have on the body.

Currently, experimental data has proven its role as an antiviral as well as a possible role in the treatment of diabetes through its ability to induce increased glucose

uptake by tissues, open wounds, rheumatoid arthritis, sore throat, influenza, anti-carcinogenic, anti-angiogenic, and antioxidant, even as anti-viral.

In recent years, the therapeutic performance of elderberry in medicinal treatment has been proven against heart attacks, stroke, and even tumor cell proliferation. Based on these suggestions, we sought to discover the exact components of the elderberry that are effect against both tumor cells proliferation as well as their inductive effects on the immunological cells.

Aim

The primary goal for this research endeavor was to isolate the active components of the elderberry extract, which result in immune enhancement and tumor suppression. Once these components were isolated, their proliferative effects on murine cells were assessed and compared to those of a known mitogen, concanavalin A (con A), which has displayed a proficiency in stimulating the proliferation of T lymphocytes. In addition, the suppressive capabilities of these components will be determined through their incubation with human skin melanoma cells. Whole elderberry extract maintains proliferative effects on spleen cells as well as suppressive capabilities on tumor cells. Therefore, it was hypothesized that there would be specific fractions of the extract that would enhance spleen cell proliferation, and specific fractions of the extract that would specifically suppress tumor cells. Any samples that exhibit activity will be further analyzed using nuclear magnetic resonance in order to identify the composition of the sample.

MATERIALS AND METHODS

Sambucus Nigra Extract Characteristics

The sample used for these studies was a 13% standardized elderberry powder prepared by Artemis, International, Inc. Pure elderberry was concentrated without any solvent through physical means and spray dried onto maltodextrin (Artemis International, Inc. 2005). The most significant components of this standardized elderberry extract includes anthocyanins and polyphenols. For complete composition of Sambucus Nigra, see Table A1.

Resolution of Elderberry Extract

The components of the Sambucus Nigra extract were separated utilizing column chromatography. The different chemical components elute off of the column based on their polarity and that of the eluent, and enable further analysis of the individual chemicals found in elderberry. Separation of the components proved to be infeasible by column chromatography using silica gel as the adsorbent and ethyl acetate/formic acid/acetic acid/water as the eluent, see Appendix C.

A second protocol was adapted from Strack et al., with poly(vinylpolypyrrolidone) (PVP) serving as the stationary phase. This polymer has been widely used in the separatory techniques of anthocyanins, so a finer resolution would be

expected. 2 g of the 13% standardized elderberry extract was dissolved in 4 mL of 0.01N hydrochloric acid (HCl). A 250-mL column (d ≈ 2.5 cm) was filled with 25 g of dry PVP (height ≈ 18.4 cm), and the prepared elderberry extract was added. The mobile phase was a gradient containing an increasing ratio of methanol to deionized water, and an equal amount of 0.01N HCl, see Appendix C. The solutions were added sequentially in order of descending polarity.

Concentration of Resolved Samples

16.5-mL fractions were collected from the column (Figure B1) and stored at 4^{o}C to maintain the integrity of the contents. For the collection times, see Table A2. Once the fractions were collected, the bulk of the solvent was evaporated off using a rotary evaporator (Figure B2a) to achieve more concentrated samples for the experimental analysis. The samples were concentrated down to approximately 2 mL, to ensure a sufficient amount for both experimental use and identification purposes. Depending on the percentage of methanol in the sample, the evaporation rates varied for each of the fractions (Table A3).

Preliminary Identification through Thin Layer Chromatography

To obtain a finer resolution of the compounds collected through the column chromatography, the fractions were separated further through thin layer chromatography. As the stationary phase, mylar backed silica gel (JT Baker) plates were used. The plates were prepared by creating a base line 0.5 cm from one end of the plate as well as a solvent front line 0.5 cm from the other side of the plate. Each fraction was spotted

(diameter of the spots = 2mm) on the midpoint of the baseline of the plate using microcapillary tubes. A mixture of butanol, acetic acid, and deionized water (4:1:5) was used as the mobile phase (Hurst 2002). In a 100-mL beaker, which was used as the developing chamber, the solvent was filled 0.5 cm from the bottom, or 8.3 mL. A strip of filter paper was placed in the beaker, and the plate was placed in the beaker and covered with a watch glass to develop. Each plate took approximately 1 hour and 20 minutes to run to completion. See Table A4 for calculated retention factor values.

Cells

Spleen cells obtained from either the C57 mouse strain or Balb/c mouse strain were used for the assessment of the proliferative effects of the elderberry extract samples on spleen cells. Initial experiments were conducted with C57 mice, but due to the low counts obtained, the spleen cells were replaced with those of Balb/c mice. A human skin melanoma cell line (Mewo) was obtained from Dr. Visalli's laboratory and was used to assess the suppressive capabilities of the elderberry extracts on tumor cell propagation. The Mewo cells were maintained in Eagle MEM X media supplemented with 10% fetal bovine serum (FBS), and sub-cultured every 4-5 days.

Spleen Cell Preparation

Each mouse was sacrificed through cervical dislocation. The spleen was removed from the mouse by first thoroughly wetting the right side of the mouse with ethanol to eliminate interference. Using a pair of heat-sterilized scissors, a left paramedial dorsal incision was made to expose the abdominal wall. Another incision was made on the

abdominal wall to provide access to the spleen. The spleen was lifted out of the cavity using heat-sterilized forceps, and any fatty tissue was cut away. The spleen was placed in a sterile petri dish, containing 1 mL of RPMI-1640 nutrient media supplemented with 10% FBS, on top of a heat-sterilized mesh screen. Using the plunger of a syringe, the spleen was pushed through the mesh screen in order to create a single cell suspension. Using a syringe and a 25 gauge needle, the cell suspension was collected and placed in a sterile vial. In a separate tube, 295 µL of isotonic buffer, 100 µL of 0.4% Trypan Blue in PBS, 100 µL of Lyse buffer, and 5 µL of the spleen cells were added. 10 µL of this mixture was pipetted onto a hemocytometer and the cells were counted. The 1 mL of cells was diluted with enough media to create a 1×10^6 cells/well concentration.

Spleen Cell Proliferation Assay with Resolved Components

To assess the mitogenic effects of the elderberry extract fractions collected from the column, a spleen-cell proliferation assay was conducted. For this assay, two C57 mice were sacrificed; one young (4 months) and one old (1 ½ year old). 100 µL of RPMI-1640 nutrient media containing 10% FBS were added to each experimental well of a 96-well cell culture plate, followed by 100 µL of spleen cells were added to the wells. 10 µL of each sample resolved from the column were applied to the cells, after which the cells were incubated in 5% CO_2 and 37^oC. After 48 hours, the plates were removed from the incubator, and 1 mCi/mL of tritiated thymidine was added to each experimental well. The plates were placed back into the incubator for an additional 24 hours, and then harvested.

Spleen Cell Proliferation Assay with 1:2 Methanol Dilution

Previously conducted experiments indicate the ability of methanol to function as a mitogen for murine spleen cells (Zandonai 2010). To eliminate the speculation of any interference of methanol on spleen cell propagation in these trials, a proliferation assay was performed with methanol. Due to the extremely low counts of the previous assays, the C57 mice were not used. Instead, a young Balb/c mouse (4 months old) was sacrificed for this experiment. The 100 µL of RPMI-1640 media containing 10% FBS and 100 µL of cells were added to each experimental well of a 96-well cell culture plate. A 1:2 serial dilution was conducted for methanol with deionized water, and 10 µL of each methanol dilution was applied to the spleen cells, followed by 25µg/mL of concanavalin A. The cells were incubated in 5% CO_2 and 37^{o}C. After 48 hours, the plates were removed from the incubator, and 1 mCi/mL of tritiated thymidine was added to each experimental well. The plates were placed back into the incubator for an additional 24 hours, and then harvested.

Spleen Cell Proliferation Assay with Column Eluent

A spleen cell proliferation assay with Balb/c spleen cells was conducted using the eleven solutions prepared as the mobile phase (Appendix C) during the column chromatography without running them through the column.16.5 mL of each individual solution was evaporated down to 2 mL samples using the rotary evaporator as had been done to the fractions resolved from the column. 100 µL of RPMI-1640 containing 10% FBS and 100 µL of cells were added to each experimental well of a 96-well cell culture plate. 10 µL of the 2-mL mobile phase samples were incubated with the cells and 25

µg/mL of concanavalin A. The cells were incubated in 37^{o}C and 5% CO_2. After 48 hours, the cells were tagged with 1 mCi/mL of tritiated thymidine. The plates were placed back into the incubator for an additional 24 hours, and then harvested.

Pooling and Drying Samples

According to Figure B9 produced from the results derived from the first assay, the 94 samples were pooled according to their levels of activity (Table A8).

Due to the relatively high counts produced during the spleen-cell assays conducted with methanol, two alternatives routes were proposed to rid the samples of their methanol content. One route was to dialyze the samples with sterile phosphate buffered saline (PBS). 500 µL of pooled sample numbers 14, 16, 22, 24, 25, 29, and 32 were dialyzed. 0.5-3-mL capacity Slide-A-Lyzer dialysis cassettes were obtained and prepared by removing them from their protective pouches and immersing them in PBS (the chosen dialysis buffer) for 2 minutes. Next each sample was added to a separate cassette by filling a syringe with the sample and inserting the tip of the needle through one of the syringe ports on the corner of the cassette. The sample was injected slowly and the air was removed by pulling up the syringe piston before removing the needle. The dialysis cassettes were attached to buoys to allow for floatation and placed in beakers contained 250 mL of PBS. The PBS was replaced with a fresh amount every 2 hours for 4 hours, and finally the samples were left overnight in the dialysis buffer. The dialyzed samples were removed from the cassettes by inserting the tip of the needle of a syringe into a portal other than that of the one used to insert the sample. The samples were stored in sterile micro-centrifuge tubes. Another drying technique involved the complete

evaporation off the solvent from each sample using the rotary evaporator, followed by dissolving the dry sample in PBS. Another 500 µL portion of the same pooled samples was evaporated using the rotary evaporator until just a film coated the round bottom flask (Figure B2a). Then, 500 µL of PBS was added to the round bottom flask and the film of sample was dissolved into the PBS and stored in sterile tubes.

To eliminate any doubt of the presence of methanol in the samples, a final drying technique was instated. A centrifuge connected to a vacuum pump (Figure B2b) was used to dry the pooled samples completely. 0.5 mL of sample each sample was placed in an autoclaved microcentrifuge tube, and the opening of the tubes was covered with parafilm. Using a 22 gauge needle, a series of holes were poked into the parafilm, which would ensure the removal of the liquid from the tube and maintenance of the desired compounds in the tube. The samples were placed into the centrifuge chamber and the centrifuge was allowed to run on medium heat (36^{o}C), until the samples were dry. The samples were dissolved in sterile PBS by adding 0.5 mL of PBS to the microcentrifuge tube and vortexing until homogenous.

Spleen Cell Proliferation Assay with Dialyzed and Evaporated Samples

A spleen cell proliferation assay of the dialyzed and evaporated samples was conducted using the spleen of a young Balb/c mouse. 100 µL of RPMI-1640 containing 10% FBS and 100 µL of cells were added to each experimental well. 10 µL and 20 µL of each samples were incubated with the cells and 25 µg/µL of concanavalin A. The cells were incubated in 37^{o}C and 5% CO_2. After 48 hours, the cells were tagged with 1

mCi/mL tritiated thymidine. The plates were placed back into the incubator for an additional 24 hours, and then harvested.

Spleen Cell Proliferation Assay with Dried Samples

A spleen cell proliferation assay was initiated using the spleen of a young Balb/c mouse. A 96-well cell culture plate was obtained and 100 µL of RPMI-1640 containing 10% FBS and 100 µL of cells were added to each experimental well. 10 µL and 20 µL of each sample were incubated with the cells and 2.5 µL of concanavalin A. The cells were incubated in 37^{o}C and 5% CO_2. After 48 hours, the cells were tagged with 1 mCi/mL of tritiated thymidine. The plates were placed back into the incubator for an additional 24 hours, and then harvested. This assay was repeated to confirm the results obtained.

Cell Harvesting

The cells were washed with PBS. The DNA of the cells was precipitated onto filter paper using 10%TCA and the discs were placed in vials and into a scintillation counter to assess the counts per minute of cellular division (Figure B3a,b).

Tumor Cell Proliferation Assay with Dried Samples

To assess the inhibitory effects of the elderberry extract samples on tumor cell proliferation, an assay was set up to incubate the cells with each pooled sample. 100 µL of Eagle MEM X media supplemented with 10% FBS was added to each experimental well of a 96-well cell culture plate, followed by 5 µL of cells (50,000 cells). The cells were seeded 24 hours before adding sample. 10 µL and 20 µL of each sample were each

added to an experimental well. The cells were incubated in 5% CO_2 and 37^{o}C for 48 hours. The cells were tagged with 1 mCi/mL of tritiated thymidine. The plates were placed back into the incubator for an additional 24 hours, and then harvested. This assay was repeated to validate the results achieved.

Apoptosis

To attribute the tumor cell death to the samples resolved from the elderberry extract, the apoptotic activity of the cells following exposure to the samples was evaluated. The colorimetric assay used assessed caspase 8 activity through the hydrolysis of the peptide Acetyl-Ile-Glu-Thr-Asp p-nitroaniline, which results in the release of a p-Nitroaniline (pNA) moiety.

In order to do this, a 96-well was obtained and 5 μL of Mewo cells (50,000 cells) were seeded into each well, followed by 100 μL of Eagle MEM X, containing 10% FBS and left overnight. 10 μL of each active berry (Figure B15) was added to a well of cells and allowed to incubate for either an additional 24 or 48 hours. The cells were lysed to reveal their contents.

RESULTS

Resolution of Elderberry Extract

The resolution of the standardized elderberry extract resulted in the acquisition of 94 separate 16.5-mL increment samples. The polarity of the mobile phase altered the colors of the fractions that were pulled through the column; as the polarity decreased, darker pigmented liquid eluted through the column. The colors of the samples collected varied from a pale orange color to a deep purple color. The time at which it took to collect each fraction also varied according to the polarity of the eluent added to the column. The initial collection time of the fractions containing 100% water and 50 mL 0.01N HCl was 0.4 mL/min while the final collection time of the fractions containing 100% methanol and 50 mL 0.01N HCl was 0.15 mL/min. For complete collection times, see Table A2.

Preliminary Identification through Thin Layer Chromatography

Thin layer chromatography resulted in obtaining several R_f values for the 94 samples from the column. Without a standard, one cannot compare the R_f values and therefore cannot identify the composition of the samples.

Spleen Cell Proliferation Assay with Resolved Components

The spleen cell proliferation assay resulted in the varying induction of spleen cell proliferation from the different resolved components of the column (Figure B9). The samples were pooled based on similar proliferative characteristics. Based on the published data reflecting the immune-enhancing capability of elderberry as a whole, it was hypothesized that the elderberry fractions would induce a significantly positive effect upon spleen cell proliferation. The counts were still relatively low as compared to the previous counts obtained from spleen cell incubation with concanavalin A, the positive control for these proliferative assays. Concanavalin A induces spleen cell proliferation, obtaining counts over 10,000 cpm. The highest count obtained from incubation of the spleen cells with the fractions resolved from the column was 76% less than that of con A. Although the counts obtained were relatively low, peaks of activity were noted, indicating a varying range of composition between the fractions obtained from the column.

Spleen Cell Proliferation Assay with 1:2 Methanol Dilution

As shown in previous experiments, methanol proved to induce significant proliferation in murine spleen cells (Figure B10) and therefore had to be completely eliminated from the samples in order to obtain a more accurate assessment of the berry extract samples' induction of spleen cell propagation. As compared to the cells incubated with con A alone (shown in light pink), the 1:2, 1:16, and 1:32 dilutions of methanol induced significantly higher counts. Due to the fact that even trace amounts of methanol (1:32 dilution) resulted in 4% higher counts than those of the cells incubated with con A,

proved that methanol must be eliminated from the samples in order to obtain an accurate assessment of the elderberry extract samples' ability to induce spleen cell proliferation.

Spleen Cell Proliferation Assay with Column Eluent

The mobile phase used in running the column contained varying amounts of methanol and therefore could be the sole inducer of spleen cell proliferation in the previous experiments. Each of the solutions of the mobile phase (10% MeOH thru 80% MeOH) produced significantly high counts comparable to those of the spleen cells incubated with just con A. The samples containing 90% and 100% MeOH, did not induce significant proliferation in the spleen cells. The results from this assay confirm the presence of methanol in the evaporated samples and their stimulatory effects on spleen cell propagation (Figure B11). These results confirmed that another drying technique must be performed to rid the solvent from the berry extract samples.

Spleen Cell Proliferation Assay with Dialyzed and Evaporated Samples

This assay was performed on the samples that had been either dialyzed with PBS or evaporated off using a rotary evaporator. As compared to the control (shown in light pink), dialyzed samples 14, 16, 24, and 25 all induced higher proliferative counts (Figure B12). The rest of the dialyzed samples and all of the evaporated samples did not induce signification spleen cell proliferation.

Pooling and Drying Samples

The initial rotary evaporation of the solvents resulted in the removal of the bulk of the solvents in the elderberry fractions resolved from the column. Further evaporation using the rotary evaporator and dialysis also failed in the complete removal of the methanol from the samples. The final method of centrifugation using a vacuum pump was successful in eliminating any traces of methanol that would affect the results of the experiments. Through GCMS analysis, the samples were tested for the absence of methanol and proved successfully free of the solvent.

Spleen Cell Proliferation Assay with Dried Samples

Of the 41 total elderberry extract samples resolved from the column, several of the elderberry samples induced a greater amount of spleen cell proliferation as compared to samples just incubated with con A. Samples numbers 5, 6, 12, 13, 14, 15, 16, 17, 19, 20, 22, 24, 25, 29, and 32 all induced proliferation to a greater extent than the positive control, with at least a 4% greater proliferation than the positive control when 10 μL of each sample was added. Samples 14, 16, 17, 22, 24, and 32 all exhibited increased proliferative effects when 20 μL of sample were added, but to a lesser degree. When 20 μL of sample was added, samples 5, 6, 12, 13, 15, 19, 20, 25, and 29 had reduced proliferation; less than that of the positive control. The increased sample amount induced proliferation in sample #28, which did not induce a greater proliferation when 10 μL of sample was added. This assay was repeated to confirm the results and a similar pattern of results was obtained (Figures B13, B14).

Tumor Cell Proliferation Assay with Dried Samples

This proliferative assay revealed significant suppressive effects of the elderberry extract samples. This assay was repeated and similar results were obtained each time (Figures B15, B16). When 10 μL of sample were added, samples numbers 1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 13, 15, 19, 21, 23, 26, 27, 28, 30, 31, 39, 40, 41 achieved successful suppression over 60%. All but samples 14, 22, and 32 suppressed tumor proliferation by at least 60%.

Apoptosis

After 24 hours, significantly increased apoptotic activity was noted in tumor cells incubated with 10 μL of samples 1, 11, 26, 31, 36, and 39 (Table A16). Tumor cells incubated with sample for 48 hours, showed a couple of different activities. In the well containing sample #2, the apoptotic increased between the 24-hour interval and the 48-hour assessment. However, in the wells containing samples 11, 26, 27, 31, and 36, apoptotic activity actually decreased. This could be attributed to the fact that certain peptides are degraded in a timely manner and would therefore not show up after 48 hours.

DISCUSSION

Although thorough investigation of the therapeutic effects of elderberry must still be conducted, preliminary research suggests that elderberry fruit may be useful in treating various ailments. Initial trials were conducted applying the elderberry extract fractions with spleen cells from both C57 and Balb/c mice, and they resulted in very low counts of both the experimental groups and the controls. This possibly corresponded with the fact that the accuracy of tritiated thymidine diminshes with age. This was rectified through the purchase of a new batch of tritiated thymidine, and the rest of the trials were conducted with cells obtained from only Balb/c mice. The results from the spleen cell proliferation assay indicated a direct effect of the components of elderberry on the proliferative behavior of the immune cells vital in the recognition and targeting of tumor cells. As a whole, elderberry possesses several beneficial qualities including antiangiogenic,anti-carcinogenic, and immune-enhancing capabilities. All of which are essential in the combat against tumor cell propagation. Therefore, it was hypothesized that elderberry extract fractions would induce significant proliferative effects on murine spleen cells. However, the counts obtained were greater than expected and provide an optimistic outlook on the potential of the individual components of elderberry. The overall decrease in efficiency of most of the samples in their mitogenic effects on T cell lymphocytes, seen when 20 μL of each sample were added to the spleen cells, indicated the need to

determine an optimal concentration of the therapeutic agents. An excess amount of sample proved to reduce the beneficial properties of the extracts.

The tumor cell proliferation assays served to assess the inhibitory effects of the resolved fractions of elderberry, and resulted in surprisingly high levels of suppression. Not only did most of the samples repress the proliferative behavior of the tumor cells, the level of suppression achieved was exceedingly high.

The assessments of immune cell proliferation and tumor cell suppression were in vitro studies, designed to evaluate the direct effects of the different components of elderberry. The results validate the role of elderberry as a possible therapeutic agent in both augmenting the immune system's response to tumor cells, as well as an agent that will directly target and eliminate tumor cells.

The results from the assessment of apoptotic activity reveal an additional activity of the elderberry extract fractions. The samples evaluated, not only inhibit tumor cell proliferation as displayed in the previous proliferation assays, but they actually induce apoptosis in the tumor cells as displayed in this colorimetric assay.

Upon the successful completion of these in vitro studies, the separation techniques of elderberry may be applied to in vivo studies that may validate the use of the identified components in the elderberry extract as viable therapeutic agents both in immunotherapy as well as natural chemotherapy.

Proposed Method of Identification

As a means of identification of the actual stimulatory components and inhibitory components of the berry extract, there are several proposed techniques that can be

utilized. The composition of the samples will be tested through two-dimensional nuclear magnetic resonance techniques including Correlation Spectroscopy (COSY) and Distortionless Enhancement by Polarization Transfer (DEPT) to elucidate the structures of the compounds contained within the active samples. COSY will contribute to the identification of nuclei that are coupled to each other, and it is useful for interpreting the complicated spectra which will be produced. Diffusion Ordered Spectroscopy (DOSY) NMR will be utilized due to its ability to differentiate NMR signals based on the diffusion rates of component compounds in a solvent. Diffusion coefficient differences will be enhanced by polyvinylpyrrolidone.

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APPENDICES

Appendix A: Tables

Table A1. The individual components found in elderberry and their proposed benefits.

Category	Compounds	General known Therapeutic Effects
Phenolic acids	Malic acid Benzoic acid Cinnamic acid Cholorogenic acid Caffic acid Palmitic acid	Apoptotic effects on tumor cells Inhibitory effects on tumor cell proliferation
Flavonoids	Rutin Isoquercitrin Quercitrin Hyperoside Astagalin Nicotoflorin	Fight arterial and immune system aging Antioxidants
Tannins	Tannins	Immune stimulant Tumor suppressors
Epicatechin	Epicatechin	Reduced risk of cancer
Catechin	Catechin	Tumor suppressor
Anthocyanins	Cyanidin-3-glucoside Cyanidin-3,5-diglucoside Cyanidin-3 sambubioside-5-glycoside Cyanidin-3-rhamnoglucoside Cyanidin-3-xyloglucoside	Inhibitory effects on tumor cell proliferation Inhibitors of angiogenesis Initiators of apoptosis
Proanthocyanidins	Proanthocyanidins	Antioxidant
Vitamin A	Vitamin A	Immune enhancement Antioxidant
Vitamin C	Vitamin C	Immune enhancement Antioxidant Anti-histamine

Table A2. The collection times of the berry extract fractions resolved through column chromatography.

Fraction Numbers	mL/min
1-2	0.4
3-10	0.5
11-19	0.4
20-25	0.3
26-85	0.2
86-94	0.15

Table A3. The evaporation times of the berry extract fractions using a rotary evaporator.

Fraction Numbers	Methanol Content	Time (minutes)
1-6	0%	17.5
7-15	0-10%	16.25
16-22	10-20%	16
23-46	20-50%	14.5
47-53	50-60%	14.25
54-66	60-70%	13
67-78	70-80%	11.5
79-86	80-90%	10
87-93	90-100%	8.75

Table A4. R_f values for berry extract fractions calculated using the following equation: R_f = (distance traveled by sample) ÷ (distance traveled by solvent).

Sample Number	Distance Traveled By Sample	Distance Traveled By Solvent	Calculated R_f Value
8	4.70 cm	6.49 cm	0.724
9	4.55 cm	6.45 cm	0.705
10	5.05 cm	6.50 cm	0.777
11	4.70 cm	6.55 cm	0.718
14	3.65 cm	6.45 cm	0.566
17	3.40 cm	6.50 cm	0.523
18	4.35 cm	6.46 cm	0.673
19	4.65 cm	6.45 cm	0.721
20	3.65 cm	6.50 cm	0.562
21	3.70 cm	6.40 cm	0.578
22	3.90 cm	6.45 cm	0.605
24	3.10 cm	6.42 cm	0.483
26	4.10 cm	6.45 cm	0.636
27	3.79 cm	6.50 cm	0.583
29	4.08 cm	6.45 cm	0.633
31	4.40 cm	6.43 cm	0.684
32	3.75 cm	6.65 cm	0.564
34	4.25 cm	6.50 cm	0.654
35	4.50 cm	6.50 cm	0.692
38	4.50 cm	6.52 cm	0.690
39	4.45 cm	6.50 cm	0.685
40	4.12 cm	6.52 cm	0.632
41	3.90 cm	6.50 cm	0.600
42	4.59 cm	6.53 cm	0.703
44	4.80 cm	6.49 cm	0.740
45	5.35 cm	6.51 cm	0.822
47	4.75 cm	6.48 cm	0.733
48	5.85 cm	6.45 cm	0.907
50	4.30 cm	6.40 cm	0.672
51	4.91 cm	6.45 cm	0.761
52	5.10 cm	6.52 cm	0.782
53	5.25 cm	6.45 cm	0.814
54	5.72 cm	6.50 cm	0.880
55	5.62 cm	6.50 cm	0.865
57	4.68 cm	6.42 cm	0.729
60	4.75 cm	6.45 cm	0.736
61	4.70 cm	6.48 cm	0.725
62	4.82 cm	6.45 cm	0.747
63	4.80 cm	6.45 cm	0.744
64	5.00 cm	6.45 cm	0.775
65	4.75 cm	6.50 cm	0.731
66	4.95 cm	6.49 cm	0.763
68	4.15 cm	6.50 cm	0.638
71	4.86 cm	6.53 cm	0.744
73	5.03 cm	6.55 cm	0.768

Table A4, continued.

74	4.99 cm & 5.81 cm	6.58 cm	0.758 & 0.883
75	5.18 cm & 6.00 cm	6.47 cm	0.801 & 0.927
76	4.91 cm	6.45 cm	0.761
77	4.78 cm	6.51 cm	0.734
78	4.20 cm & 5.48 cm	6.45 cm	0.651 & 0.850
79	4.58 cm & 5.47 cm	6.45 cm	0.837 & 0.848

Table A5. Results for spleen cell proliferation assay with resolved berry components.

Well Contents	CPM	Well Contents	CPM
Young Resting	73.00	Old + 10 µL Sample #30	6,975.00
Young Resting	81.00	Old + 10 µL Sample #31	4,503.00
Young Resting	83.00	Old + 10 µL Sample #32	3,630.00
Young + 2.5 µL 1:10 conA	516.00	Old + 10 µL Sample #33	4,297.00
Young + 2.5 µL 1:10 conA	603.00	Old + 10 µL Sample #34	7,625.00
Young + 2.5 µL 1:10 conA	524.00	Old + 10 µL Sample #35	4,298.00
Young + 2.5 µL 1:20 conA	726.00	Old + 10 µL Sample #36	4,698.00
Young + 2.5 µL 1:20 conA	689.00	Old + 10 µL Sample #37	419.00
Young + 2.5 µL 1:20 conA	635.00	Old + 10 µL Sample #38	421.00
Young + 2.5 µL crude berry	104.00	Old + 10 µL Sample #39	2,071.00
Young + 2.5 µL crude berry	94.00	Old + 10 µL Sample #40	2,713.00
Young + 2.5 µL crude berry	98.00	Old + 10 µL Sample #41	2,383.00
Young + 5 µL crude berry	77.00	Old + 10 µL Sample #42	2,479.00
Young + 5 µL crude berry	95.00	Old + 10 µL Sample #43	2,794.00
Young + 5 µL crude berry	95.00	Old + 10 µL Sample #44	4,354.00
Young + 10 µL crude berry	175.00	Old + 10 µL Sample #45	2,523.00
Young + 10 µL crude berry	148.00	Old + 10 µL Sample #46	1,867.00
Young + 10 µL crude berry	142.00	Old + 10 µL Sample #47	429.00
Old Resting	327.00	Old + 10 µL Sample #48	2,270.00
Old Resting	423.00	Old + 10 µL Sample #49	1,025.00
Old Resting	450.00	Old + 10 µL Sample #50	1,010.00
Old + 2.5 µL 1:10 conA	404.00	Old + 10 µL Sample #51	834.00
Old + 2.5 µL 1:10 conA	419.00	Old + 10 µL Sample #52	3,335.00
Old + 2.5 µL 1:10 conA	338.00	Old + 10 µL Sample #53	4,379.00
Old + 2.5 µL 1:20 conA	98.00	Old + 10 µL Sample #54	3,850.00
Old + 2.5 µL 1:20 conA	99.00	Old + 10 µL Sample #55	4,291.00
Old + 2.5 µL 1:20 conA	118.00	Old + 10 µL Sample #56	3,844.00
Old + 2.5 µL crude berry	72.00	Old + 10 µL Sample #57	1,323.00
Old + 2.5 µL crude berry	92.00	Old + 10 µL Sample #58	3,498.00
Old + 2.5 µL crude berry	76.00	Old + 10 µL Sample #59	1,933.00
Old + 5 µL crude berry	121.00	Old + 10 µL Sample #60	364.00
Old + 5 µL crude berry	152.00	Old + 10 µL Sample #61	1,048.00
Old + 5 µL crude berry	202.00	Old + 10 µL Sample #62	2,037.00
Old + 10 µL crude berry	731.00	Old + 10 µL Sample #63	1,007.00
Old + 10 µL crude berry	780.00	Old + 10 µL Sample #64	2,039.00
Old + 10 µL crude berry	623.00	Old + 10 µL Sample #65	4,397.00

Table A5, continued.

Old + 10 µL Sample #1	180.00	Old + 10 µL Sample #66	4,066.00
Old + 10 µL Sample #2	62.00	Old + 10 µL Sample #67	3,721.00
Old + 10 µL Sample #3	78.00	Old + 10 µL Sample #68	3,680.00
Old + 10 µL Sample #4	50.00	Old + 10 µL Sample #69	1,386.00
Old + 10 µL Sample #5	103.00	Old + 10 µL Sample #70	2,335.00
Old + 10 µL Sample #6	318.00	Old + 10 µL Sample #71	3,906.00
Old + 10 µL Sample #7	1,794.00	Old + 10 µL Sample #72	3,508.00
Old + 10 µL Sample #8	207.00	Old + 10 µL Sample #73	2,628.00
Old + 10 µL Sample #9	315.00	Old + 10 µL Sample #74	1,822.00
Old + 10 µL Sample #10	392.00	Old + 10 µL Sample #75	2,466.00
Old + 10 µL Sample #11	316.00	Old + 10 µL Sample #76	3,092.00
Old + 10 µL Sample #12	550.00	Old + 10 µL Sample #77	5.00
Old + 10 µL Sample #13	929.00	Old + 10 µL Sample #78	4.00
Old + 10 µL Sample #14	214.00	Old + 10 µL Sample #79	13.00
Old + 10 µL Sample #15	319.00	Old + 10 µL Sample #80	3.00
Old + 10 µL Sample #16	1,845.00	Old + 10 µL Sample #81	369.00
Old + 10 µL Sample #17	929.00	Old + 10 µL Sample #82	1,659.00
Old + 10 µL Sample #18	1,368.00	Old + 10 µL Sample #83	1,850.00
Old + 10 µL Sample #19	380.00	Old + 10 µL Sample #84	2,971.00
Old + 10 µL Sample #20	1,259.00	Old + 10 µL Sample #85	611.00
Old + 10 µL Sample #21	2,069.00	Old + 10 µL Sample #86	1,039.00
Old + 10 µL Sample #22	724.00	Old + 10 µL Sample #87	1,328.00
Old + 10 µL Sample #23	2,896.00	Old + 10 µL Sample #88	1,844.00
Old + 10 µL Sample #24	6,012.00	Old + 10 µL Sample #89	230.00
Old + 10 µL Sample #25	3,888.00	Old + 10 µL Sample #90	961.00
Old + 10 µL Sample #26	4,136.00	Old + 10 µL Sample #91	671.00
Old + 10 µL Sample #27	4,147.00	Old + 10 µL Sample #92	168.00
Old + 10 µL Sample #28	3,961.00	Old + 10 µL Sample #93	285.00
Old + 10 µL Sample #29	3,041.00	Old + 10 µL Sample #94	207.00

Table A6. Results for spleen cell proliferation assay with 1:2 dilution of methanol.

Well Contents	CPM	Well Contents	CPM
Resting	217.00	MeOH 1:16	236.00
Resting	189.00	MeOH 1:16	170.00
Resting	326.00	MeOH 1:16	166.00
2.5 µL 1:10 conA	14,042.00	MeOH 1:32	151.00
2.5 µL 1:10 conA	11,816.00	MeOH 1:32	269.00
2.5 µL 1:10 conA	10,865.00	MeOH 1:32	237.00
5 µL 1:10 conA	6,029.00	MeOH + conA	10,077.00
5 µL 1:10 conA	6,079.00	MeOH + conA	13,314.00
5 µL 1:10 conA	8,922.00	MeOH + conA	27,005.00
10 µL 1:10 conA	4,413.00	MeOH 1:2 + conA	29,545.00
10 µL 1:10 conA	4,115.00	MeOH 1:2 + conA	22,392.00
10 µL 1:10 conA	166.00	MeOH 1:2 + conA	20,792.00
MeOH	103.00	MeOH 1:4 + conA	13,949.00
MeOH	158.00	MeOH 1:4 + conA	12,542.00
MeOH	120.00	MeOH 1:4 + conA	13,668.00
MeOH 1:2	169.00	MeOH 1:8 + conA	14,928.00
MeOH 1:2	137.00	MeOH 1:8 + conA	15,867.00
MeOH 1:2	164.00	MeOH 1:8 + conA	19,069.00
MeOH 1:4	121.00	MeOH 1:16 + conA	29,783.00
MeOH 1:4	128.00	MeOH 1:16 + conA	30,328.00
MeOH 1:4	188.00	MeOH 1:16 + conA	26,508.00
MeOH 1:8	318.00	MeOH 1:32 + conA	19,524.00
MeOH 1:8	129.00	MeOH 1:32 + conA	16,779.00
MeOH 1:8	391.00	MeOH 1:32 + conA	16,530.00

Table A7. Results for spleen cell proliferation assay with mobile phase.

Well Contents	CPM	Well Contents	CPM
Resting	187.00	50% MeOH + conA	47.00
Resting	272.00	50% MeOH + conA	7,477.00
Resting	381.00	50% MeOH + conA	6,892.00
2.5 µL 1:10 conA	5,555.00	60% MeOH	237.00
2.5 µL 1:10 conA	14,912.00	60% MeOH	187.00
2.5 µL 1:10 conA	17,658.00	60% MeOH	19.00
10% MeOH	215.00	60% MeOH + conA	6,838.00
10% MeOH	160.00	60% MeOH + conA	5,502.00
10% MeOH	86.00	60% MeOH + conA	8,253.00
10% MeOH + conA	9,341.00	70% MeOH	108.00
10% MeOH + conA	6,383.00	70% MeOH	110.00
10% MeOH + conA	7,313.00	70% MeOH	139.00
20% MeOH	24.00	70% MeOH + conA	8,629.00
20% MeOH	98.00	70% MeOH + conA	17.00
20% MeOH	491.00	70% MeOH + conA	6,250.00
20% MeOH + conA	14,030.00	80% MeOH	158.00
20% MeOH + conA	13,016.00	80% MeOH	171.00
20% MeOH + conA	12,082.00	80% MeOH	363.00
30% MeOH	17.00	80% MeOH + conA	2,515.00
30% MeOH	75.00	80% MeOH + conA	4,966.00
30% MeOH	218.00	80% MeOH + conA	4,563.00
30% MeOH + conA	11,403.00	90% MeOH	391.00
30% MeOH + conA	12,213.00	90% MeOH	117.00
30% MeOH + conA	8,394.00	90% MeOH	87.00
40% MeOH	22.00	90% MeOH + conA	142.00
40% MeOH	254.00	90% MeOH + conA	87.00
40% MeOH	111.00	90% MeOH + conA	186.00
40% MeOH + conA	11,241.00	100% MeOH	135.00
40% MeOH + conA	7,037.00	100% MeOH	108.00
40% MeOH + conA	2,071.00	100% MeOH	522.00
50% MeOH	138.00	100% MeOH + conA	303.00
50% MeOH	122.00	100% MeOH + conA	551.00
50% MeOH	119.00	100% MeOH + conA	711.00

Table A8. Pooled samples based on the results in Figure B9.

Sample Number	Pooled Sample #
1	1
2	
3	2
4	
5	
6	3
7	
8	4
9	
10	5
11	
12	6
13	
14	7
15	
16	8
17	
18	
19	9
20	
21	10
22	
23	
24	11
25	12
26	
27	
28	13
29	
30	14
31	
32	15
33	
34	16
35	
36	
37	17
38	
39	18
40	
41	19
42	
43	20
44	
45	21
46	
47	22
48	

Sample Number	Pooled Sample #
49	23
50	
51	
52	24
53	
54	25
55	
56	
57	26
58	
59	27
60	
61	
62	28
63	
64	
65	29
66	
67	30
68	
69	31
70	
71	32
72	
73	33
74	
75	34
76	
77	35
78	
79	
80	36
81	
82	37
83	
84	
85	38
86	
87	39
88	
89	40
90	
91	
92	41
93	
94	

Table A9. Results for spleen cell proliferation assay with select dialyzed or evaporated samples.

Well Contents	CPM	Well Contents	CPM
Resting	242.00	Evap PS #25	554.00
Resting	274.00	Evap PS #29	589.00
Resting	472.00	Evap PS #32	505.00
2.5 µL 1:10 conA	2,915.00	Dialyzed PS #14 + conA	4,538.00
2.5 µL 1:10 conA	2,798.00	Dialyzed PS #16 + conA	4,541.00
2.5 µL 1:10 conA	2,779.00	Dialyzed PS #22 + conA	2,122.00
Dialyzed PS #14	511.00	Dialyzed PS #24 + conA	3,202.00
Dialyzed PS #16	526.00	Dialyzed PS #25 + conA	2,989.00
Dialyzed PS #22	575.00	Dialyzed PS #29 + conA	2,567.00
Dialyzed PS #24	595.00	Dialyzed PS #32 + conA	2,824.00
Dialyzed PS #25	492.00	Evap PS #14 + conA	2,501.00
Dialyzed PS #29	483.00	Evap PS #16 + conA	2,614.00
Dialyzed PS #32	538.00	Evap PS #22 + conA	2,822.00
Evap PS #14	515.00	Evap PS #24 + conA	2,852.00
Evap PS #16	513.00	Evap PS #25 + conA	2,667.00
Evap PS #22	490.00	Evap PS #29 + conA	543.00
Evap PS #24	469.00	Evap PS #32 + conA	1,885.00

Table A10. Results for spleen cell proliferation assay with pooled samples.

Well Contents	CPM
Resting	191.00
Resting	212.00
Resting	249.00
2.5 µL 1:10 conA	200.00
2.5 µL 1:10 conA	232.00
2.5 µL 1:10 conA	238.00
Pooled Sample #1	143.00
Pooled Sample #2	157.00
Pooled Sample #3	278.00
Pooled Sample #4	447.00
Pooled Sample #5	523.00
Pooled Sample #6	472.00
Pooled Sample #7	551.00
Pooled Sample #8	443.00
Pooled Sample #9	439.00
Pooled Sample #10	427.00
Pooled Sample #11	367.00
Pooled Sample #12	513.00
Pooled Sample #13	432.00
Pooled Sample #14	365.00
Pooled Sample #15	471.00
Pooled Sample #16	395.00
Pooled Sample #17	491.00
Pooled Sample #18	421.00
Pooled Sample #19	482.00
Pooled Sample #20	500.00
Pooled Sample #21	466.00
Pooled Sample #22	436.00
Pooled Sample #23	496.00
Pooled Sample #24	423.00
Pooled Sample #25	433.00
Pooled Sample #26	434.00
Pooled Sample #27	459.00
Pooled Sample #28	491.00
Pooled Sample #29	380.00
Pooled Sample #30	567.00

Well Contents	CPM
Pooled Sample #39	489.00
Pooled Sample #40	438.00
Pooled Sample #41	450.00
PS #1 + conA	1,594.00
PS #2 + conA	146.00
PS #3 + conA	265.00
PS #4 + conA	500.00
PS #5 + conA	476.00
PS #6 + conA	488.00
PS #7 + conA	397.00
PS #8 + conA	418.00
PS #9 + conA	348.00
PS #10 + conA	381.00
PS #11 + conA	458.00
PS #12 + conA	506.00
PS #13 + conA	547.00
PS #14 + conA	386.00
PS #15 + conA	465.00
PS #16 + conA	417.00
PS #17 + conA	481.00
PS #18 + conA	449.00
PS #19 + conA	440.00
PS #20 + conA	428.00
PS #21 + conA	411.00
PS #22 + conA	397.00
PS #23 + conA	396.00
PS #24 + conA	390.00
PS #25 + conA	487.00
PS #26 + conA	403.00
PS #27 + conA	393.00
PS #28 + conA	576.00
PS #29 + conA	307.00
PS #30 + conA	452.00
PS #31 + conA	422.00
PS #32 + conA	247.00
PS #33 + conA	502.00

Table A10, continued.

Pooled Sample #31	534.00
Pooled Sample #32	292.00
Pooled Sample #33	332.00
Pooled Sample #34	495.00
Pooled Sample #35	371.00
Pooled Sample #36	455.00
Pooled Sample #37	346.00
Pooled Sample #38	487.00

PS #34 + conA	426.00
PS #35 + conA	393.00
PS #36 + conA	472.00
PS #37 + conA	395.00
PS #38 + conA	465.00
PS #39 + conA	415.00
PS #40 + conA	440.00
PS #41 + conA	425.00

Table A11. Results for spleen cell proliferation assay with either 10 µL or 20 µL of sample.

	10 µL Sample	20 µL Sample
Well Contents	**CPM**	**CPM**
Resting	2,059.00	2,792.00
Resting	2,963.00	3,062.00
Resting	2,412.00	2,823.00
2.5 µL 1:10 conA	8,689.00	9,692.00
2.5 µL 1:10 conA	10,734.00	14,789.00
2.5 µL 1:10 conA	10,143.00	13,888.00
Pooled Sample #1	91.00	50.00
Pooled Sample #2	283.00	82.00
Pooled Sample #3	3,666.00	4,572.00
Pooled Sample #4	2,395.00	422.00
Pooled Sample #5	3,431.00	3,448.00
Pooled Sample #6	4,052.00	347.00
Pooled Sample #7	2,470.00	1,638.00
Pooled Sample #8	2,493.00	1,252.00
Pooled Sample #9	2,798.00	455.00
Pooled Sample #10	4,031.00	534.00
Pooled Sample #11	3,538.00	2,207.00
Pooled Sample #12	3,315.00	4,255.00
Pooled Sample #13	4,332.00	3,286.00
Pooled Sample #14	6,650.00	9,170.00
Pooled Sample #15	4,408.00	5,388.00
Pooled Sample #16	8,505.00	13,715.00
Pooled Sample #17	6,197.00	7,148.00
Pooled Sample #18	3,253.00	3,647.00
Pooled Sample #19	3,071.00	2,104.00
Pooled Sample #20	3,552.00	2,905.00
Pooled Sample #21	2,426.00	1,114.00
Pooled Sample #22	6,631.00	11,355.00
Pooled Sample #23	1,289.00	198.00
Pooled Sample #24	5,760.00	10,049.00
Pooled Sample #25	7,357.00	9,789.00
Pooled Sample #26	2,921.00	1,510.00
Pooled Sample #27	539.00	95.00
Pooled Sample #28	74.00	78.00

Table A11, continued.

Pooled Sample #29	6,843.00	7,958.00
Pooled Sample #30	154.00	307.00
Pooled Sample #31	192.00	79.00
Pooled Sample #32	4,549.00	4,535.00
Pooled Sample #33	3,155.00	2,281.00
Pooled Sample #34	3,213.00	1,318.00
Pooled Sample #35	2,634.00	514.00
Pooled Sample #36	1,763.00	926.00
Pooled Sample #37	2,467.00	1,467.00
Pooled Sample #38	3,798.00	3,039.00
Pooled Sample #39	448.00	117.00
Pooled Sample #40	1,210.00	85.00
Pooled Sample #41	3,028.00	390.00
PS #1 + conA	43.00	78.00
PS #2 + conA	51.00	33.00
PS #3 + conA	7,002.00	2,792.00
PS #4 + conA	6,970.00	757.00
PS #5 + conA	9,259.00	7,727.00
PS #6 + conA	7,801.00	5,020.00
PS #7 + conA	6,819.00	1,401.00
PS #8 + conA	6,414.00	1,665.00
PS #9 + conA	5,178.00	163.00
PS #10 + conA	7,418.00	999.00
PS #11 + conA	4,060.00	2,270.00
PS #12 + conA	6,428.00	5,431.00
PS #13 + conA	9,406.00	3,800.00
PS #14 + conA	15,394.00	17,635.00
PS #15 + conA	10,676.00	9,066.00
PS #16 + conA	16,108.00	23,236.00
PS #17 + conA	12,333.00	14,933.00
PS #18 + conA	6,971.00	6,942.00
PS #19 + conA	8,961.00	7,677.00
PS #20 + conA	10,478.00	6,722.00
PS #21 + conA	4,763.00	1,314.00
PS #22 + conA	12,579.00	18,312.00
PS #23 + conA	2,274.00	272.00
PS #24 + conA	13,684.00	30,985.00

Table A11, continued.

PS #25 + conA	16,650.00	1,833.00
PS #26 + conA	2,794.00	70.00
PS #27 + conA	649.00	66.00
PS #28 + conA	247.00	16,463.00
PS #29 + conA	15,226.00	63.00
PS #30 + conA	559.00	62.00
PS #31 + conA	232.00	17,927.00
PS #32 + conA	15,464.00	7,090.00
PS #33 + conA	7,698.00	4,303.00
PS #34 + conA	8,434.00	3,408.00
PS #35 + conA	5,771.00	1,489.00
PS #36 + conA	7,172.00	641.00
PS #37 + conA	7,178.00	2,050.00
PS #38 + conA	8,036.00	61.00
PS #39 + conA	352.00	89.00
PS #40 + conA	2,169.00	872.00
PS #41 + conA	5,937.00	870.00

Table A12. Results for spleen cell proliferation assay with either 10 µL or 20 µL of sample.

	10 µL Sample	20 µL Sample
Well Contents	**CPM**	**CPM**
Resting	691.00	1,463.00
Resting	1,124.00	2,218.00
Resting	1,152.00	1,288.00
2.5 µL 1:10 conA	8,490.00	16,280.00
2.5 µL 1:10 conA	13,798.00	16,853.00
2.5 µL 1:10 conA	16,358.00	17,172.00
Pooled Sample #1	171.00	54.00
Pooled Sample #2	140.00	58.00
Pooled Sample #3	980.00	263.00
Pooled Sample #4	943.00	108.00
Pooled Sample #5	2,344.00	1,055.00
Pooled Sample #6	1,303.00	881.00
Pooled Sample #7	941.00	186.00
Pooled Sample #8	1,457.00	391.00
Pooled Sample #9	906.00	142.00
Pooled Sample #10	1,275.00	246.00
Pooled Sample #11	1,241.00	481.00
Pooled Sample #12	2,558.00	1,381.00
Pooled Sample #13	2,460.00	1,268.00
Pooled Sample #14	3,221.00	3,836.00
Pooled Sample #15	4,458.00	1,421.00
Pooled Sample #16	3,849.00	2,256.00
Pooled Sample #17	3,739.00	2,141.00
Pooled Sample #18	2,625.00	1,595.00
Pooled Sample #19	3,876.00	687.00
Pooled Sample #20	3,387.00	923.00
Pooled Sample #21	2,656.00	310.00
Pooled Sample #22	4,003.00	3,135.00
Pooled Sample #23	1,161.00	223.00
Pooled Sample #24	3,147.00	3,235.00
Pooled Sample #25	2,320.00	2,609.00
Pooled Sample #26	1,632.00	521.00
Pooled Sample #27	553.00	130.00
Pooled Sample #28	323.00	255.00

Table A12, continued.

Pooled Sample #29	3,828.00	2,150.00
Pooled Sample #30	231.00	69.00
Pooled Sample #31	972.00	121.00
Pooled Sample #32	3,371.00	4,031.00
Pooled Sample #33	2,693.00	1,424.00
Pooled Sample #34	5,839.00	788.00
Pooled Sample #35	1,415.00	311.00
Pooled Sample #36	1,331.00	303.00
Pooled Sample #37	2,601.00	589.00
Pooled Sample #38	3,097.00	596.00
Pooled Sample #39	232.00	66.00
Pooled Sample #40	722.00	115.00
Pooled Sample #41	1,289.00	161.00
PS #1 + conA	357.00	1,202.00
PS #2 + conA	246.00	75.00
PS #3 + conA	10,536.00	1,577.00
PS #4 + conA	8,740.00	268.00
PS #5 + conA	19,597.00	12,351.00
PS #6 + conA	13,685.00	5,349.00
PS #7 + conA	6,509.00	1,127.00
PS #8 + conA	7,756.00	2,345.00
PS #9 + conA	8,110.00	128.00
PS #10 + conA	10,876.00	1,366.00
PS #11 + conA	11,337.00	3,797.00
PS #12 + conA	16,719.00	6,404.00
PS #13 + conA	19,613.00	9,202.00
PS #14 + conA	17,700.00	16,230.00
PS #15 + conA	14,901.00	11,444.00
PS #16 + conA	31,467.00	18,999.00
PS #17 + conA	16,843.00	15,853.00
PS #18 + conA	11,143.00	6,729.00
PS #19 + conA	22,316.00	7,133.00
PS #20 + conA	15,326.00	6,758.00
PS #21 + conA	13,190.00	1,099.00
PS #22 + conA	29,887.00	14,137.00
PS #23 + conA	5,760.00	324.00
PS #24 + conA	23,123.00	12,561.00

Table A12, continued.

PS #25 + conA	23,407.00	13,286.00
PS #26 + conA	9,460.00	2,011.00
PS #27 + conA	1,802.00	109.00
PS #28 + conA	176.00	72.00
PS #29 + conA	19,646.00	15,133.00
PS #30 + conA	814.00	91.00
PS #31 + conA	1,150.00	65.00
PS #32 + conA	22,482.00	18,525.00
PS #33 + conA	12,880.00	7,612.00
PS #34 + conA	15,019.00	5,309.00
PS #35 + conA	11,120.00	1,454.00
PS #36 + conA	6,757.00	741.00
PS #37 + conA	11,383.00	4,318.00
PS #38 + conA	11,083.00	6,437.00
PS #39 + conA	1,174.00	117.00
PS #40 + conA	4,208.00	129.00
PS #41 + conA	6,817.00	335.00

Table A13. Results for tumor cell proliferation assay with either 10 µL or 20 µL of sample.

	10 µL sample	20 µL sample
Well Contents	**CPM**	**CPM**
5 µL cells	133,865	
5 µL cells + Sample #1	7,772	98
5 µL cells + Sample #2	942	113
5 µL cells + Sample #3	99,781	9,149
5 µL cells + Sample #4	76,550	6,927
5 µL cells + Sample #5	92,439	61,373
5 µL cells + Sample #6	68,532	40,153
5 µL cells + Sample #7	49,940	14,029
5 µL cells + Sample #8	47,981	28,220
5 µL cells + Sample #9	37,102	566
5 µL cells + Sample #10	51,538	3,896
5 µL cells + Sample #11	50,241	10,673
5 µL cells + Sample #12	89,222	30,584
5 µL cells + Sample #13	78,424	24,265
5 µL cells + Sample #14	104,938	56,777
5 µL cells + Sample #15	98,336	37,278
5 µL cells + Sample #16	128,217	55,428
5 µL cells + Sample #17	106,168	52,356
5 µL cells + Sample #18	83,032	36,893
5 µL cells + Sample #19	78,356	26,885
5 µL cells + Sample #20	71,534	28,370
5 µL cells + Sample #21	43,152	508
5 µL cells + Sample #22	124,830	96,028
5 µL cells + Sample #23	18,586	4937
5 µL cells + Sample #24	75,868	44364
5 µL cells + Sample #25	116,613	67,067
5 µL cells + Sample #26	41,864	1,272
5 µL cells + Sample #27	13,132	2,780
5 µL cells + Sample #28	8,934	697
5 µL cells + Sample #29	83,238	59,730
5 µL cells + Sample #30	19,231	7,166
5 µL cells + Sample #31	17,839	3,284
5 µL cells + Sample #32	123,049	100,528
5 µL cells + Sample #33	81,110	70,606

Table A13, continued.

5 µL cells + Sample #34	26,647	65,098
5 µL cells + Sample #35	73,889	10,960
5 µL cells + Sample #36	54,560	553
5 µL cells + Sample #37	97,206	58,584
5 µL cells + Sample #38	69,957	5,181
5 µL cells + Sample #39	10,163	2,409
5 µL cells + Sample #40	24,757	4,676
5 µL cells + Sample #41	31,644	12,125

Table A14. Results for tumor cell proliferation assay with either 10 µL or 20 µL of sample.

	10 µL sample	20 µL sample
Well Contents	**CPM**	**CPM**
5 µL cells	46,485	
5 µL cells + PBS	35,452	
5 µL cells + Sample #1	152	80
5 µL cells + Sample #2	371	69
5 µL cells + Sample #3	18,187	685
5 µL cells + Sample #4	23,636	262
5 µL cells + Sample #5	27,957	30,214
5 µL cells + Sample #6	23,192	1,897
5 µL cells + Sample #7	14,752	2,781
5 µL cells + Sample #8	14,274	7,377
5 µL cells + Sample #9	6,457	193
5 µL cells + Sample #10	20,695	5,360
5 µL cells + Sample #11	14,607	1,131
5 µL cells + Sample #12	22,893	4,592
5 µL cells + Sample #13	13,560	10,303
5 µL cells + Sample #14	33,748	8,689
5 µL cells + Sample #15	23,337	10,062
5 µL cells + Sample #16	51,454	11,901
5 µL cells + Sample #17	37,253	376
5 µL cells + Sample #18	39,032	143
5 µL cells + Sample #19	17,538	8,579
5 µL cells + Sample #20	45,723	5,116
5 µL cells + Sample #21	5,442	129
5 µL cells + Sample #22	53,690	1,930
5 µL cells + Sample #23	7,052	955
5 µL cells + Sample #24	44,288	5,255
5 µL cells + Sample #25	69,288	13,180
5 µL cells + Sample #26	8,620	110
5 µL cells + Sample #27	7,140	164
5 µL cells + Sample #28	670	179
5 µL cells + Sample #29	74,205	147
5 µL cells + Sample #30	3,936	154
5 µL cells + Sample #31	7,177	119
5 µL cells + Sample #32	69,740	130

Table A14, continued.

5 µL cells + Sample #33	44,165	736
5 µL cells + Sample #34	78,044	14,622
5 µL cells + Sample #35	39,939	30,921
5 µL cells + Sample #36	52,412	124
5 µL cells + Sample #37	67,633	193
5 µL cells + Sample #38	31,563	159
5 µL cells + Sample #39	11,014	143
5 µL cells + Sample #40	26,231	7,322
5 µL cells + Sample #41	22,466	168

Table A15. Results for tumor cell proliferation assay with either 10 µL or 20 µL of sample.

	10 µL Sample	20 µL Sample
Well Contents	**CPM**	**CPM**
5 µL cells	6,301	
5 µL cells + PBS	7,198	
5 µL cells + Sample #1	89.00	160.00
5 µL cells + Sample #2	90.00	127.00
5 µL cells + Sample #3	192.00	66.00
5 µL cells + Sample #4	67.00	93.00
5 µL cells + Sample #5	684.00	134.00
5 µL cells + Sample #6	382.00	192.00
5 µL cells + Sample #7	122.00	189.00
5 µL cells + Sample #8	197.00	285.00
5 µL cells + Sample #9	116.00	173.00
5 µL cells + Sample #10	450.00	103.00
5 µL cells + Sample #11	281.00	298.00
5 µL cells + Sample #12	504.00	233.00
5 µL cells + Sample #13	793.00	277.00
5 µL cells + Sample #14	7,734.00	4,746.00
5 µL cells + Sample #15	3,151.00	516.00
5 µL cells + Sample #16	8,089.00	881.00
5 µL cells + Sample #17	3,697.00	894.00
5 µL cells + Sample #18	518.00	276.00
5 µL cells + Sample #19	510.00	172.00
5 µL cells + Sample #20	4,103.00	158.00
5 µL cells + Sample #21	761.00	76.00
5 µL cells + Sample #22	6,402.00	4,154.00
5 µL cells + Sample #23	179.00	85.00
5 µL cells + Sample #24	5,270.00	3230.00
5 µL cells + Sample #25	6,454.00	2,737.00
5 µL cells + Sample #26	215.00	93.00
5 µL cells + Sample #27	159.00	110.00
5 µL cells + Sample #28	488.00	155.00
5 µL cells + Sample #29	7,258.00	3,733.00
5 µL cells + Sample #30	807.00	136.00
5 µL cells + Sample #31	1,063.00	149.00
5 µL cells + Sample #32	7,308.00	4,891.00

Table A15, continued.

5 µL cells + Sample #33	1,281.00	214.00
5 µL cells + Sample #34	540.00	188.00
5 µL cells + Sample #35	1,534.00	127.00
5 µL cells + Sample #36	479.00	157.00
5 µL cells + Sample #37	657.00	206.00
5 µL cells + Sample #38	405.00	157.00
5 µL cells + Sample #39	132.00	168.00
5 µL cells + Sample #40	354.00	182.00
5 µL cells + Sample #41	124.00	138.00

Table A16. Results for caspase 8 assay with tumor cells and select samples.

Well Contents	pNA (nmol/min/mL) after 24 hours	pNA (nmol/min/mL) after 48 hours
Reagent Blank	0.090	
+ Control	9.525	
5 µL cells	0.023	0.791
5 µL cells + Sample #1	1.808	-2.667
5 µL cells + Sample #2	0.226	1.379
5 µL cells + Sample #7	0.249	0.090
5 µL cells + Sample #8	0.090	0.000
5 µL cells + Sample #9	0.407	-0.068
5 µL cells + Sample #10	-0.068	1.469
5 µL cells + Sample #11	1.175	0.158
5 µL cells + Sample #21	0.000	-0.927
5 µL cells + Sample #23	0.158	3.232
5 µL cells + Sample #26	4.497	1.469
5 µL cells + Sample #27	0.973	0.475
5 µL cells + Sample #28	0.723	3.028
5 µL cells + Sample #30	-0.520	0.746
5 µL cells + Sample #31	5.153	1.107
5 µL cells + Sample #34	0.610	1.266
5 µL cells + Sample #36	2.780	1.266
5 µL cells + Sample #39	3.503	-0.023
5 µL cells + Sample #40	-0.023	0.814
5 µL cells + Sample #41	-0.158	12.972

Appendix B: Figures

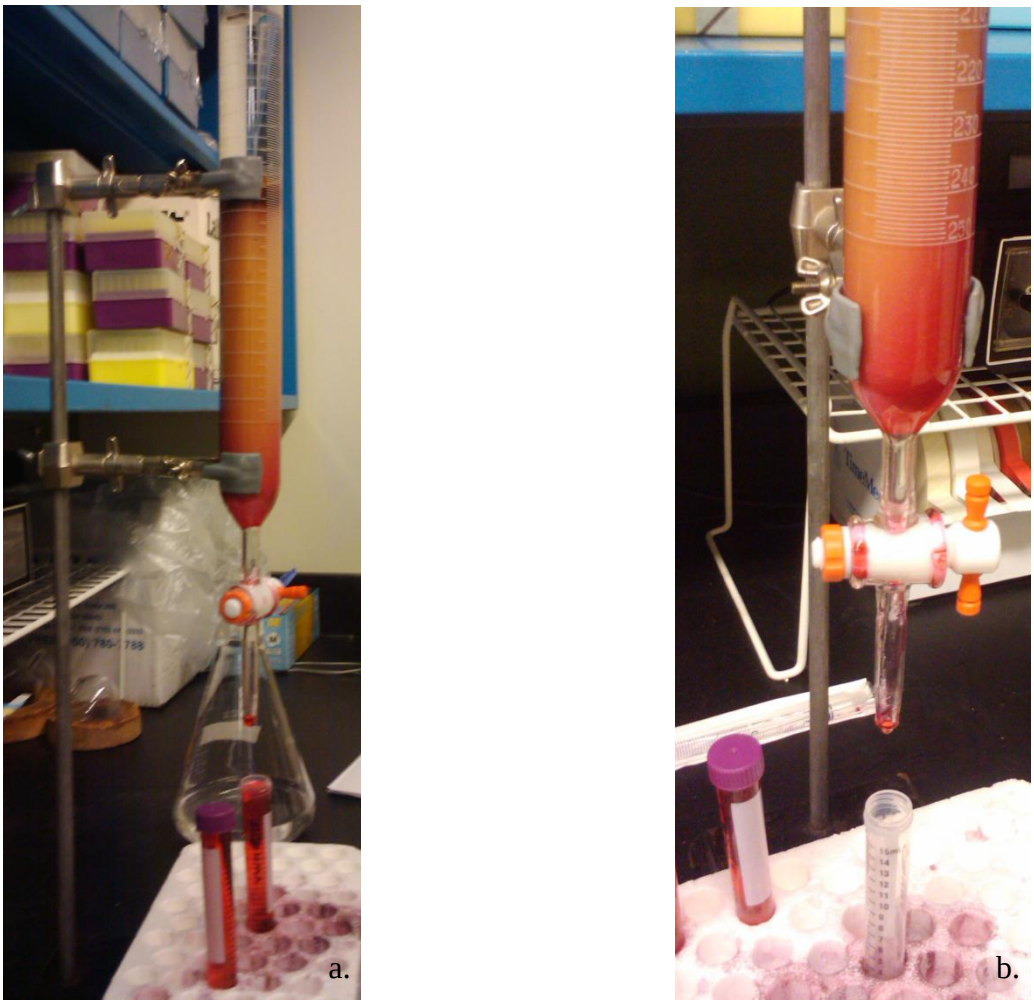


Figure B1. Collection of samples resolved through column chromatography (a,b).

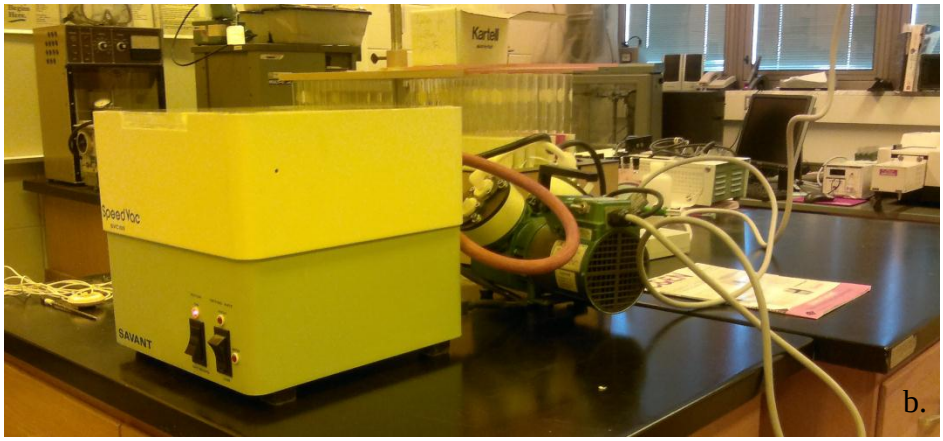
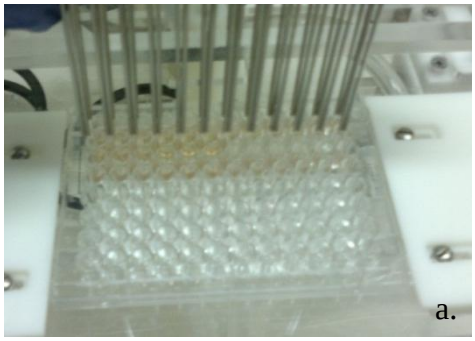


Figure B2. Evaporation techniques used to remove solvent (methanol). (a) Rotary evaporation and (b) Centrifugation with a vacuum pump.



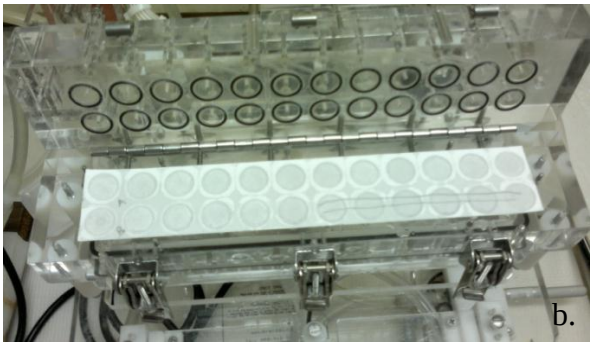


Figure B3. Cell harvesting. (a) Washing of the wells with PBS and (b) Removal of the filter discs.

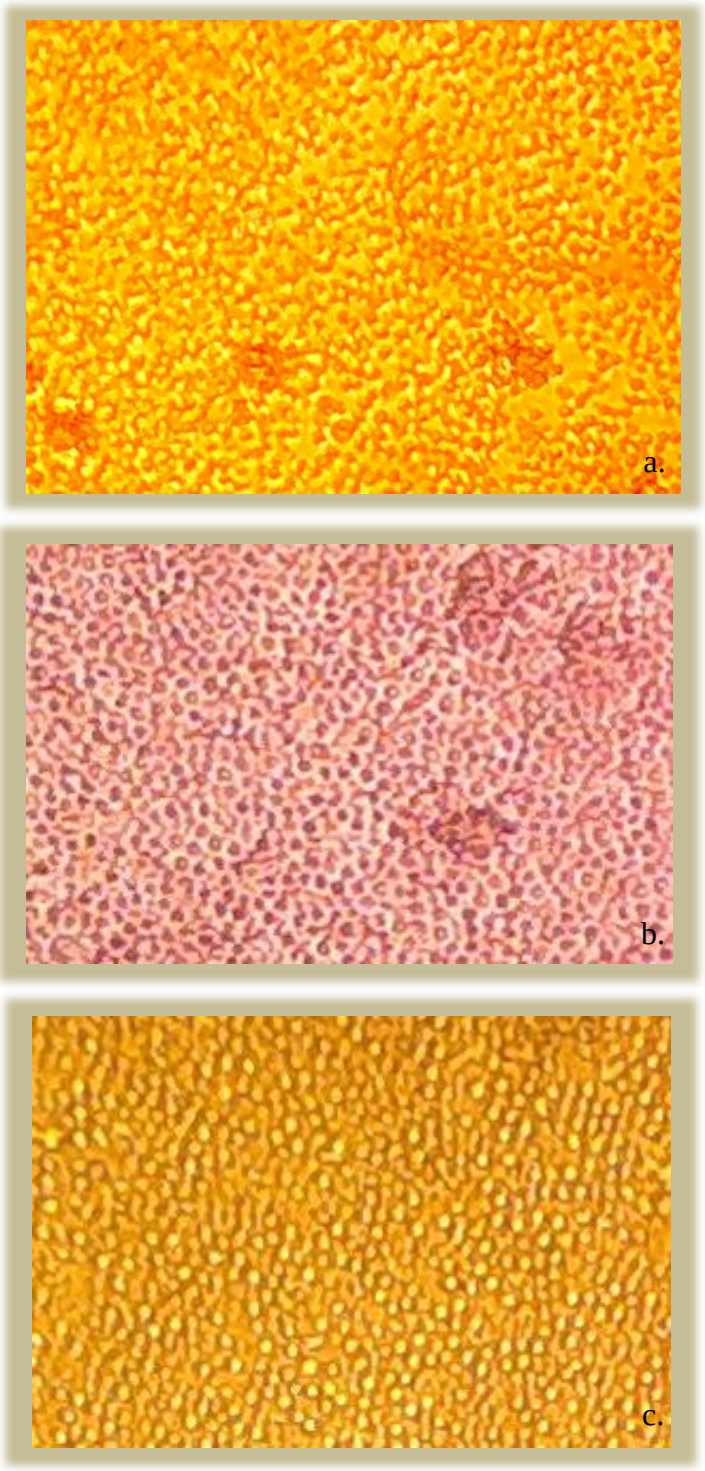


Figure B4. Spleen cell proliferation with 10 µL sample. (a) 2.5 µL 1:10 concanavalin A (b) 2.5 µL 1:10 concanavalin A + sample #19 (c) 2.5 µL 1:10 concanavalin A + sample #32.

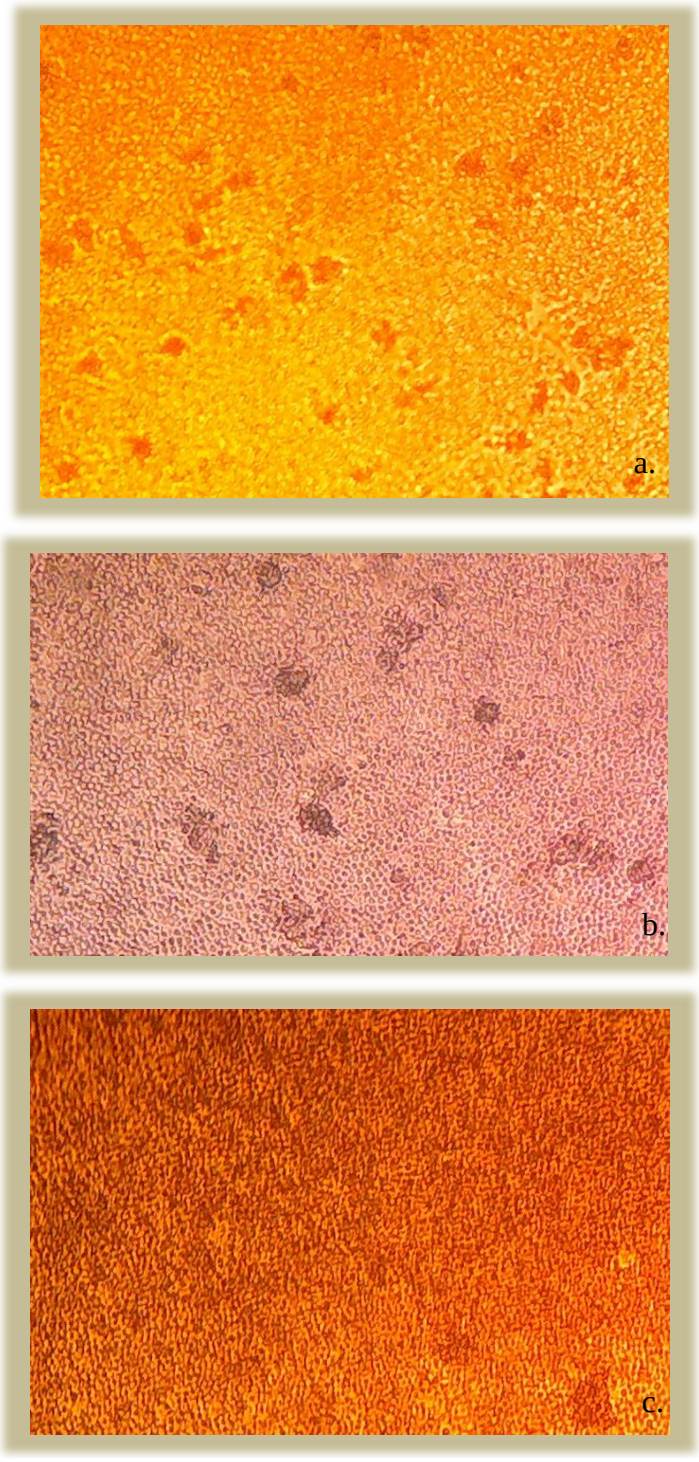


Figure B5. Spleen cell proliferation with 20 µL sample. (a) 2.5 µL 1:10 concanavalin A (b) 2.5 µL 1:10 concanavalin A + sample #17 (c) 2.5 µL 1:10 concanavalin A + sample #31.

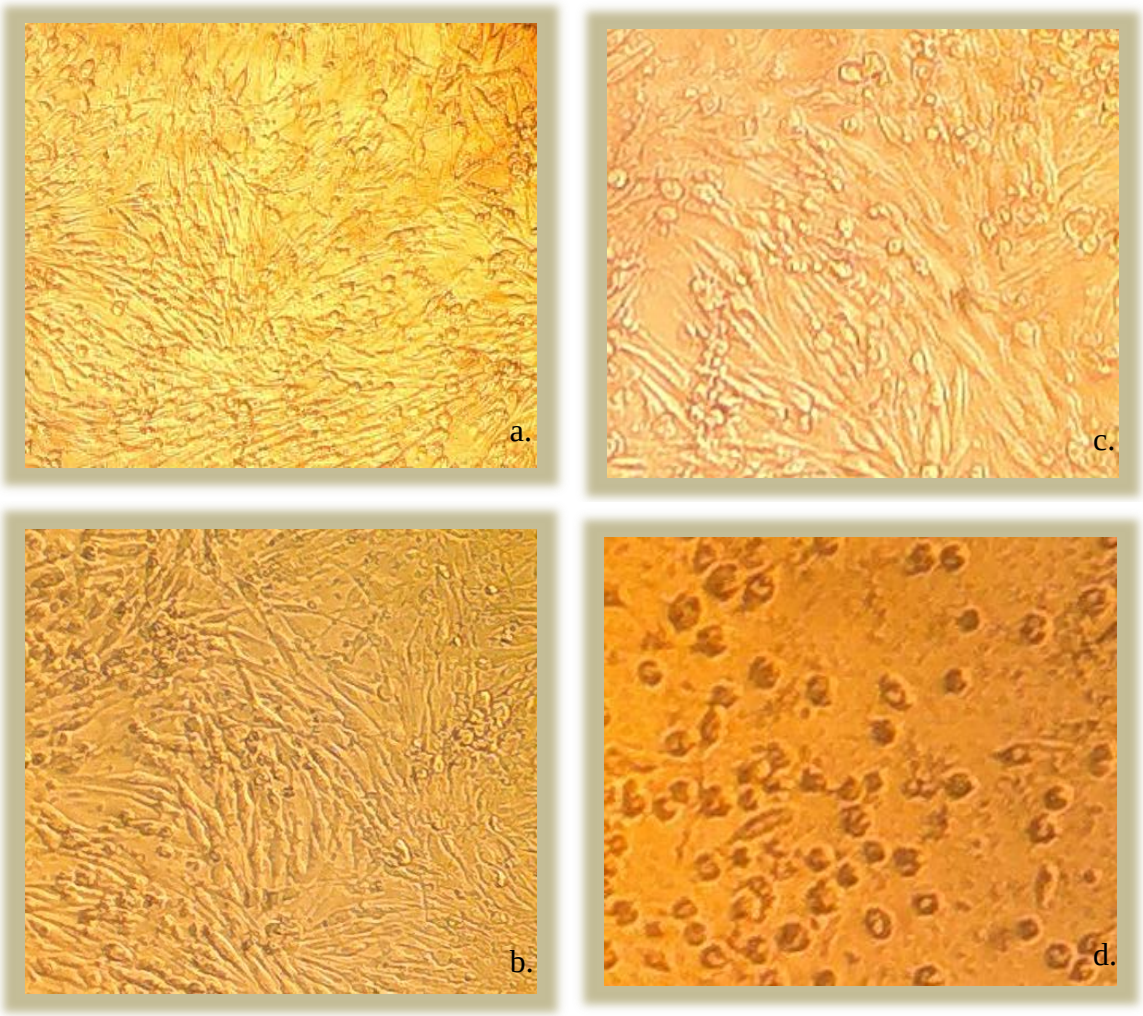


Figure B6. Tumor cell proliferation with 10 μL sample. (a) Control (b) + PBS (c) + sample #16 (d) + sample #27.

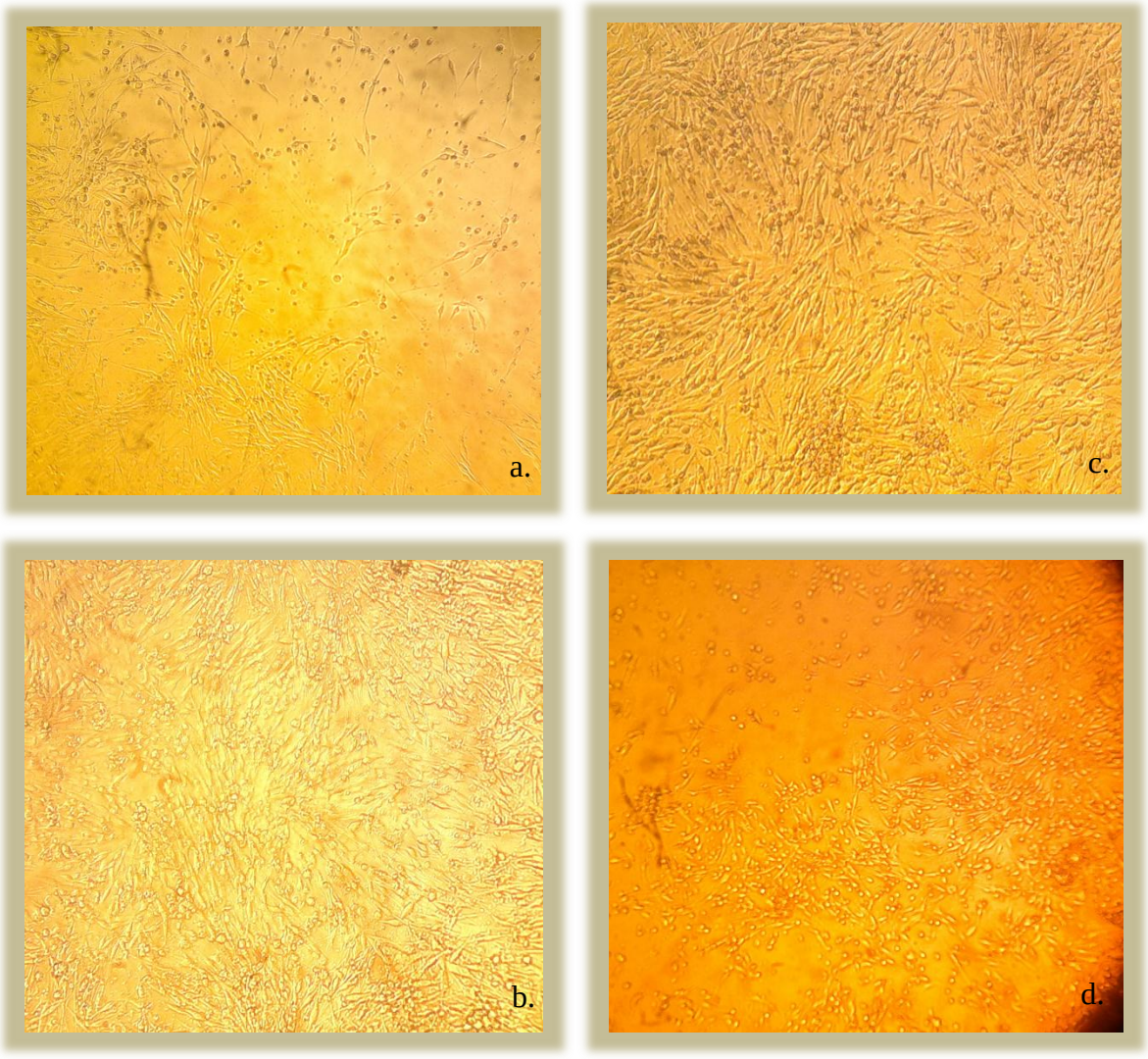


Figure B7. Tumor cell proliferation with 20 µL sample. (a) Control (b) + PBS (c) + sample #7 (d) + sample #11.

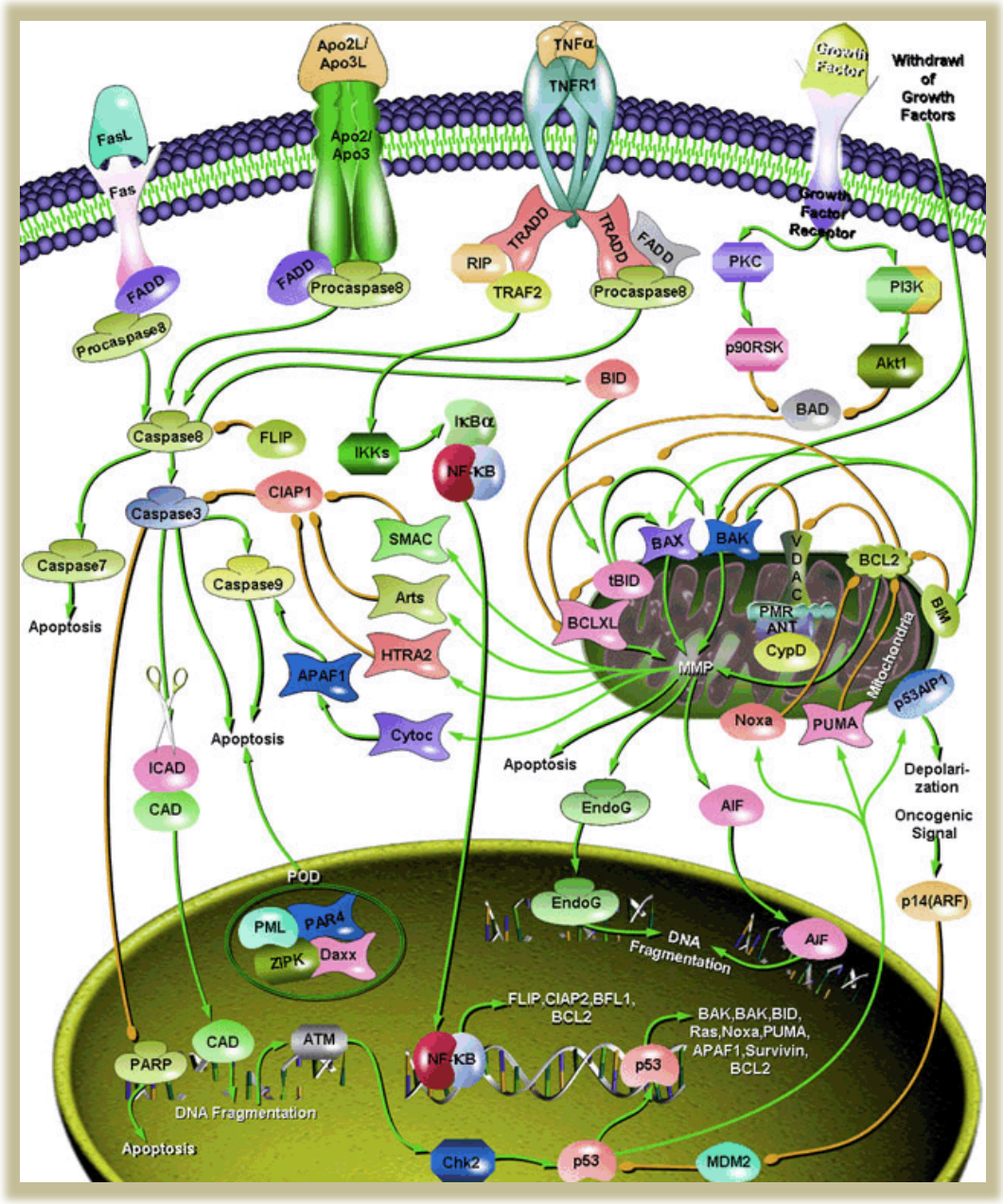


Figure B8. Apoptosis pathway.

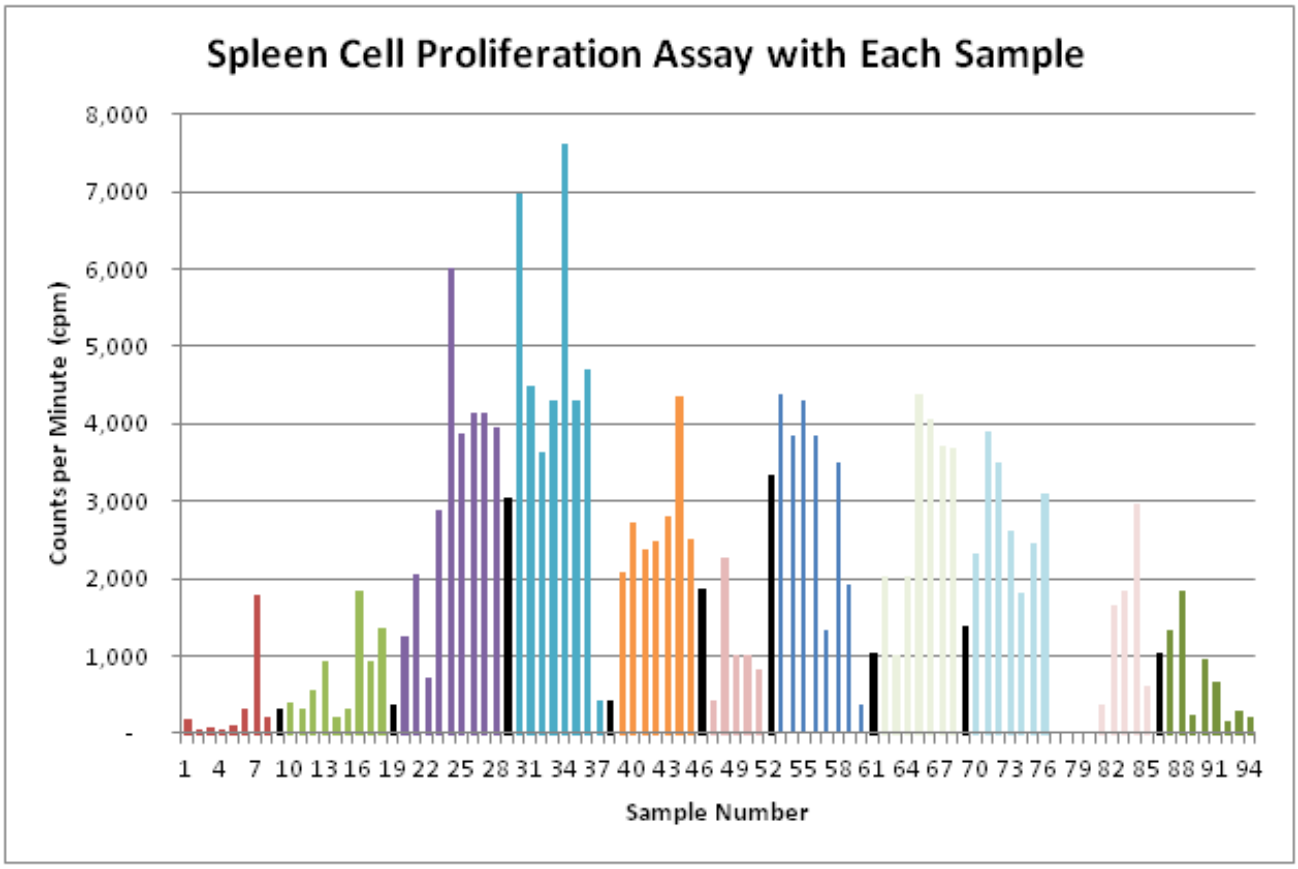


Figure B9. The results of the spleen cell proliferation assay conducted with all resolved samples through column chromatography.

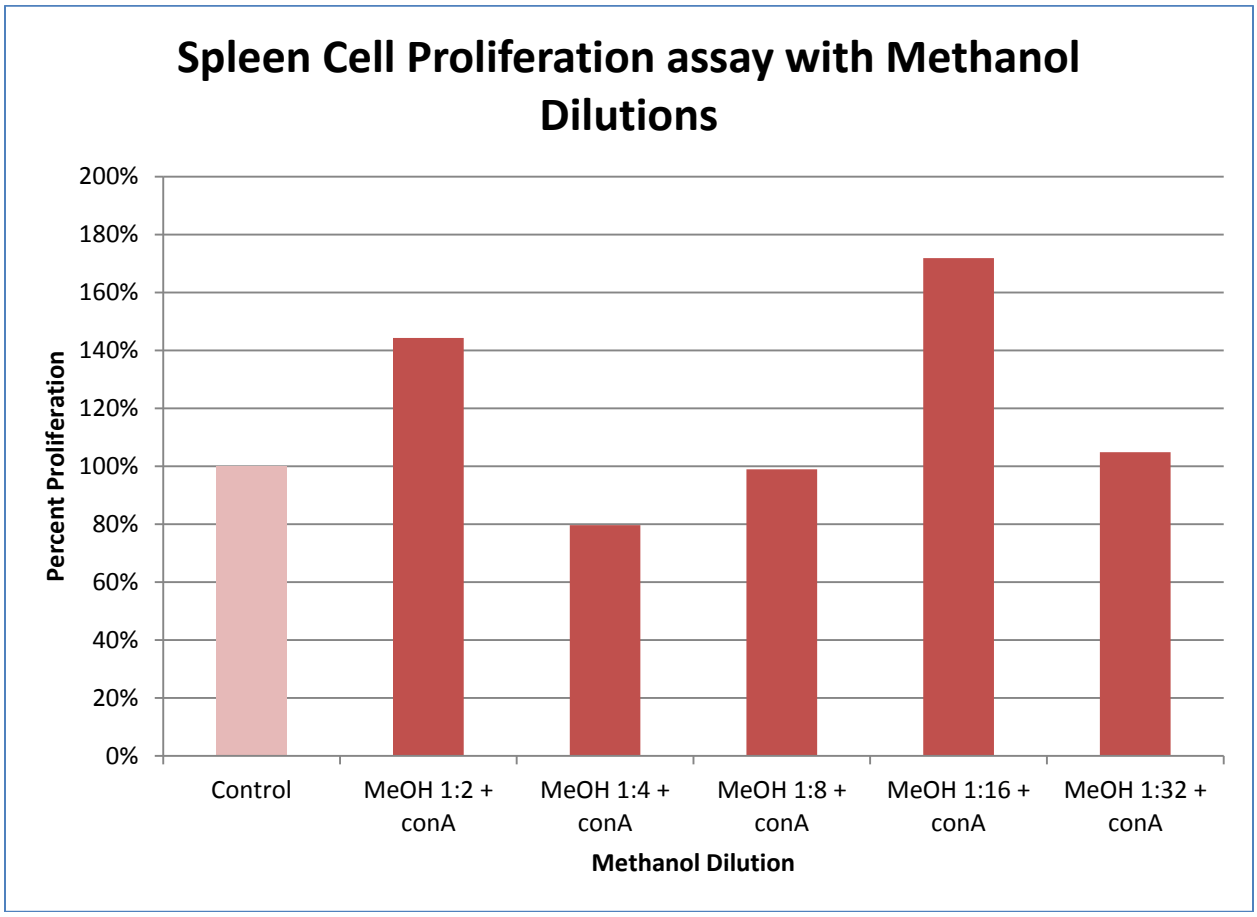


Figure B10. The results of a spleen cell proliferation assay conducted with 1:2 dilution of methanol.

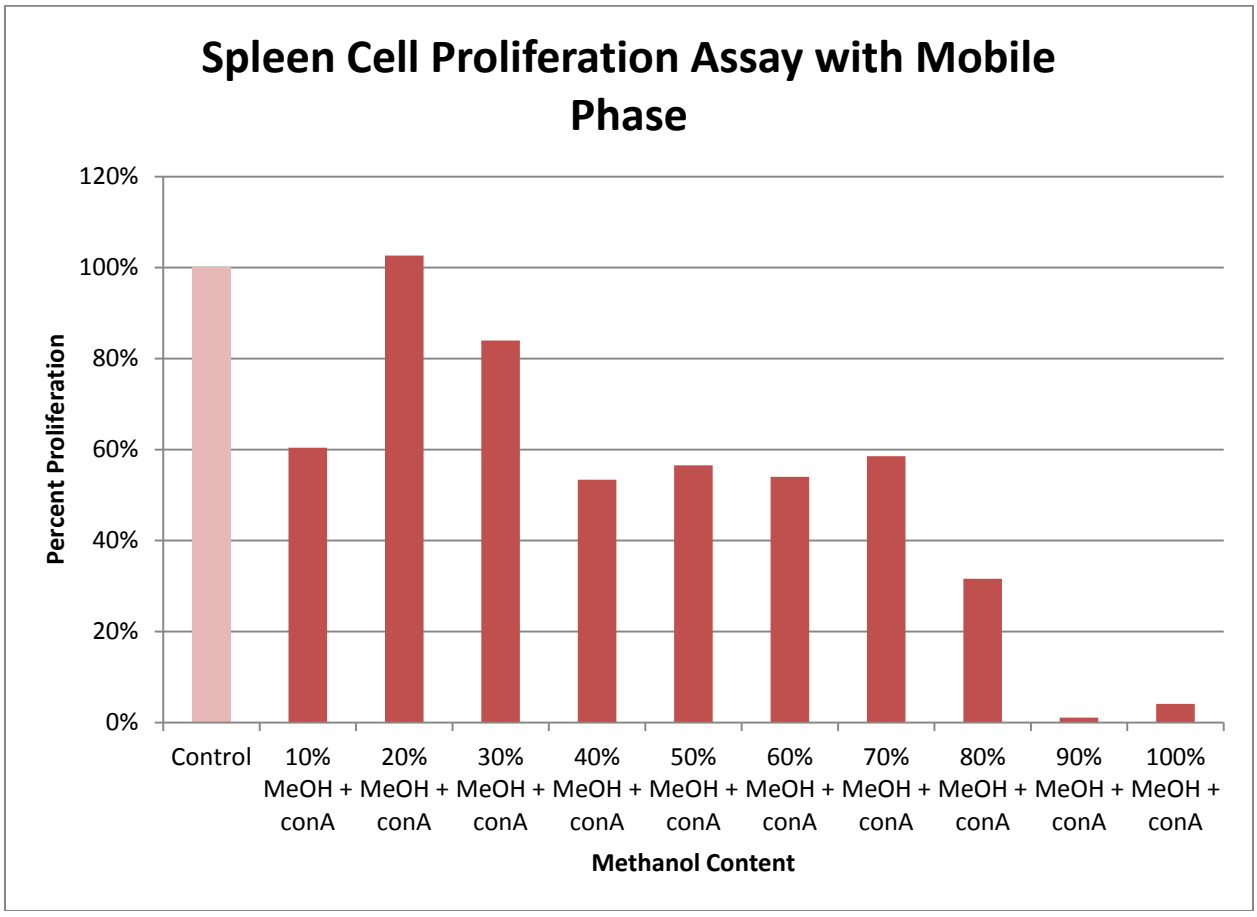


Figure B11. The results of a spleen cell proliferation assay conducted with the mobile phase used for column chromatography.

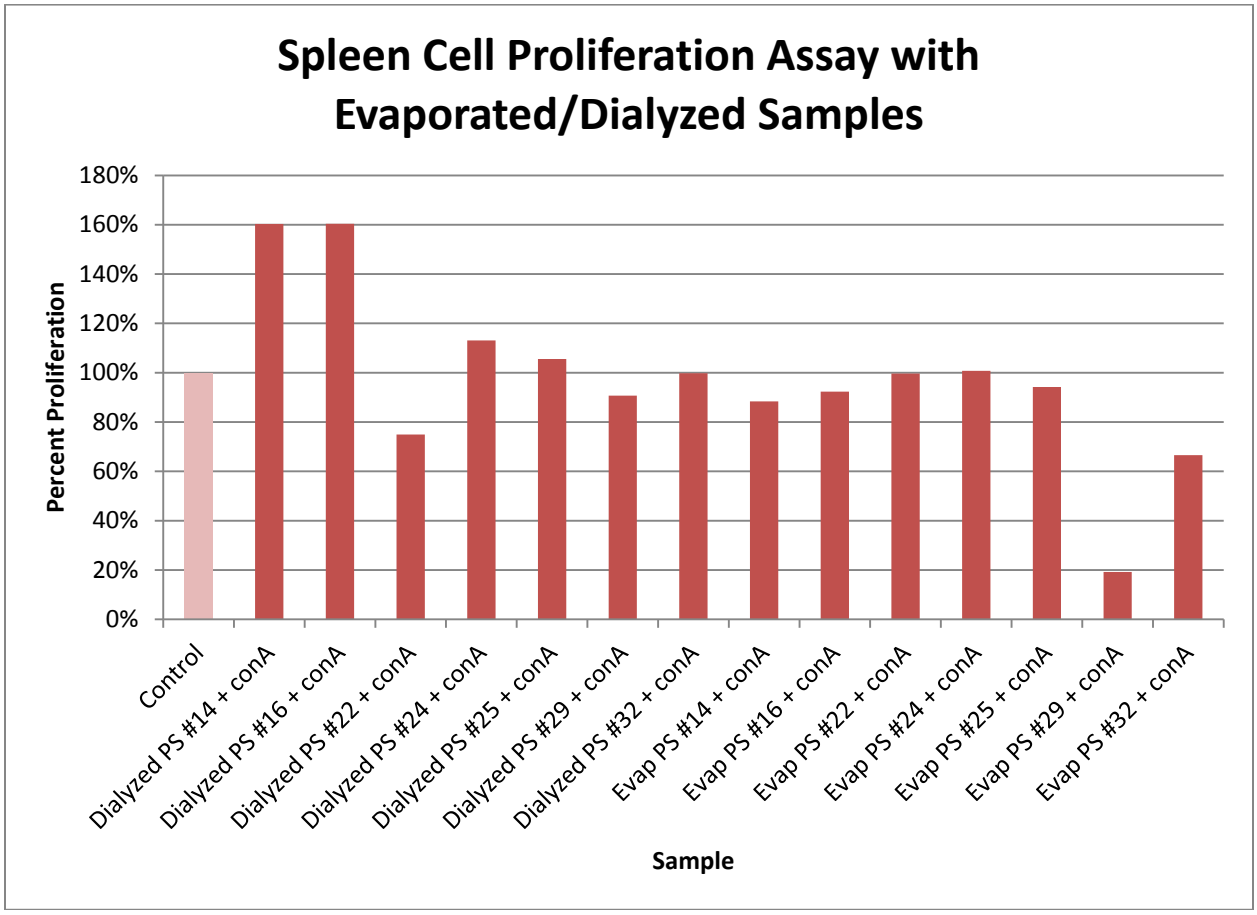


Figure B12. The results of a spleen cell proliferation assay conducted with dialyzed and fully evaporated samples.

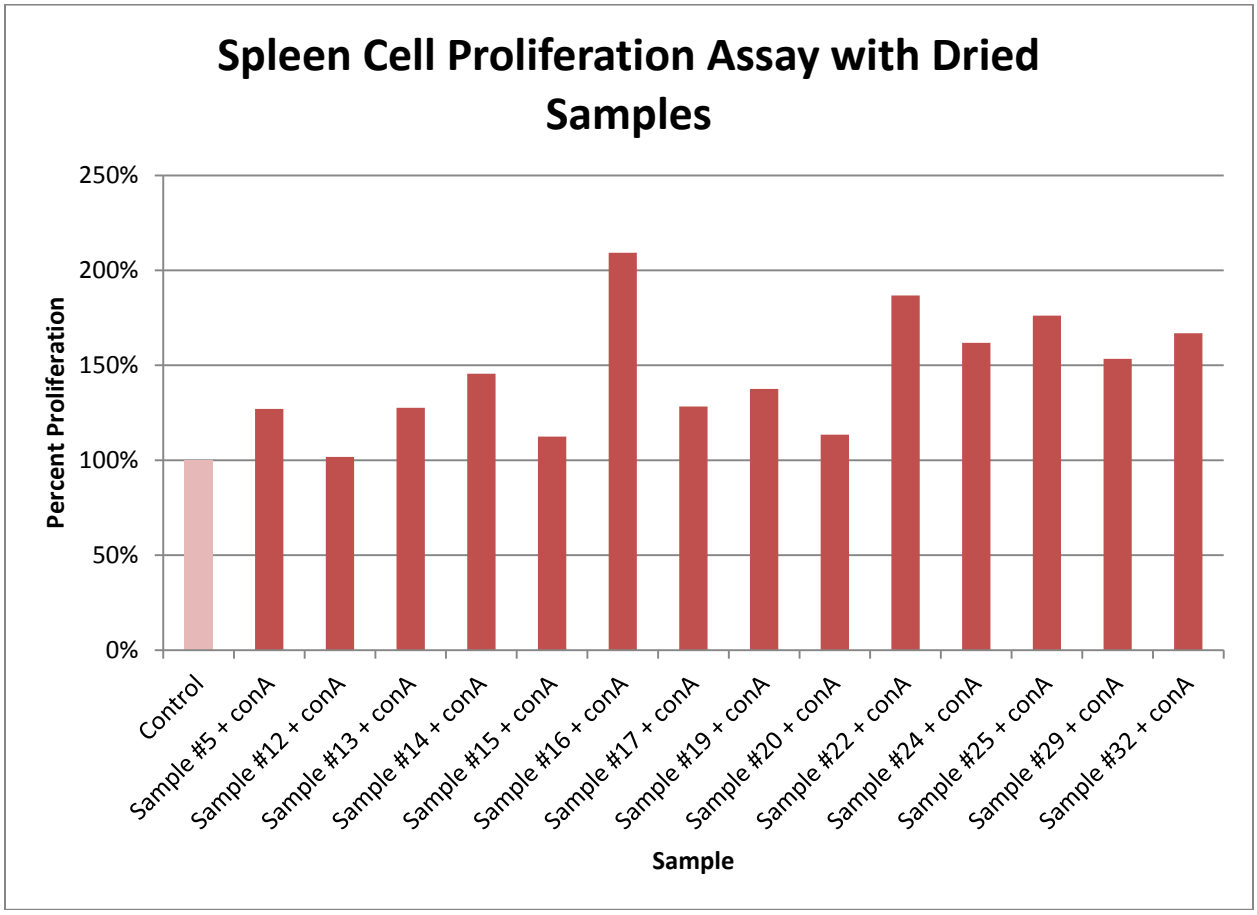


Figure B13. The combined results of spleen cell proliferation assays conducted with 10 µL of the samples dried through centrifugation with a vacuum pump.

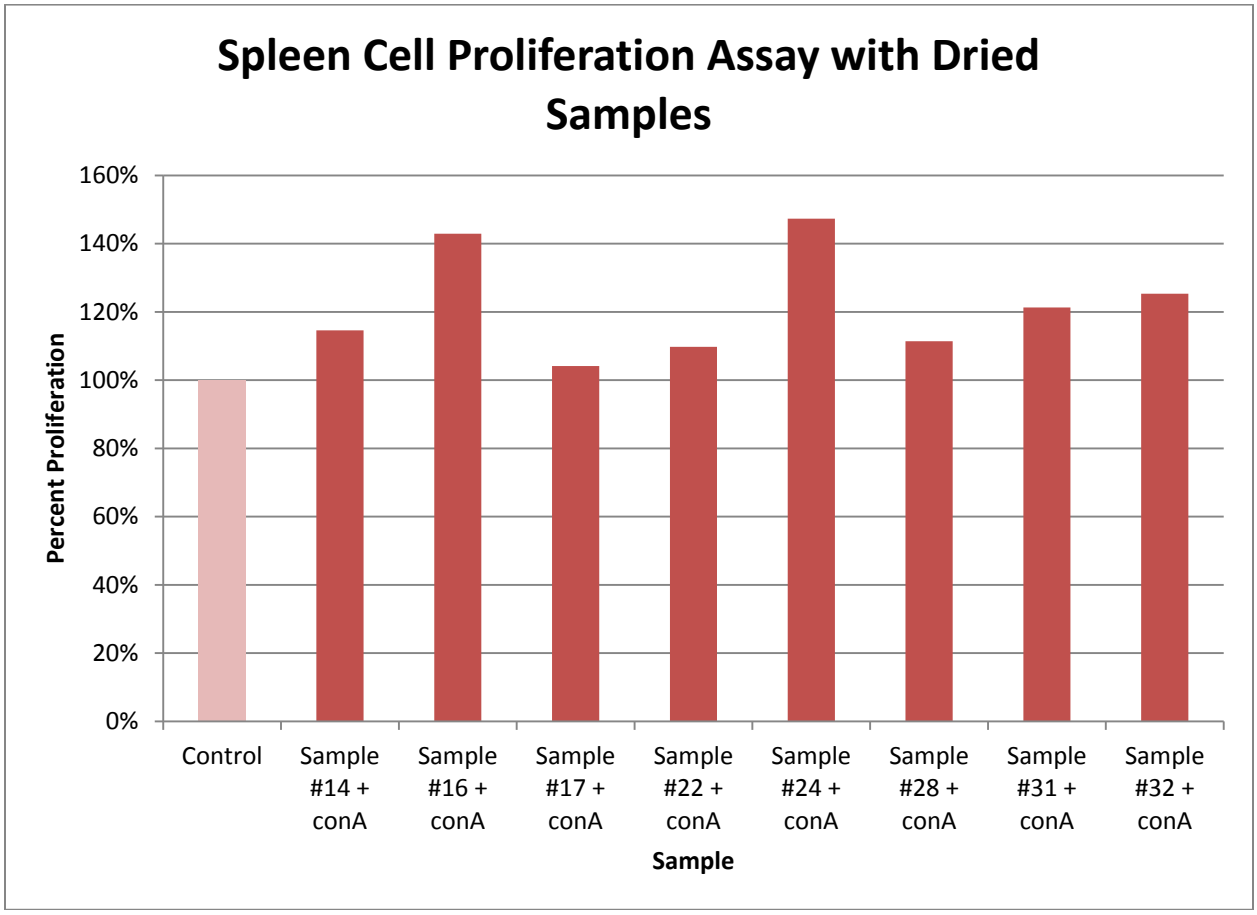


Figure B14. The combined results of spleen cell proliferation assays conducted with 20 µL of the samples dried through centrifugation with a vacuum pump.

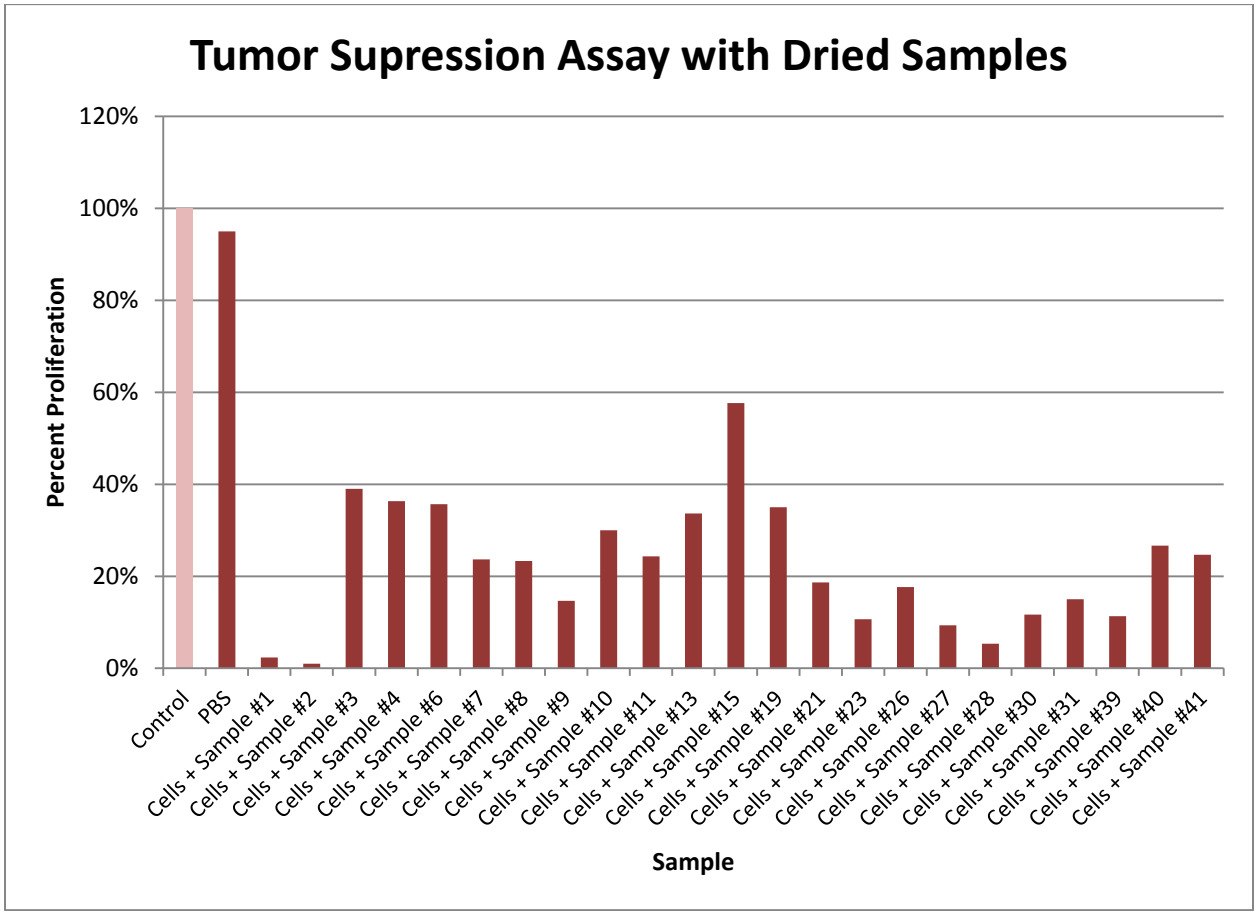


Figure B15. The combined results of tumor cell proliferation assays conducted with 10 µL of the samples dried through centrifugation with a vacuum pump.

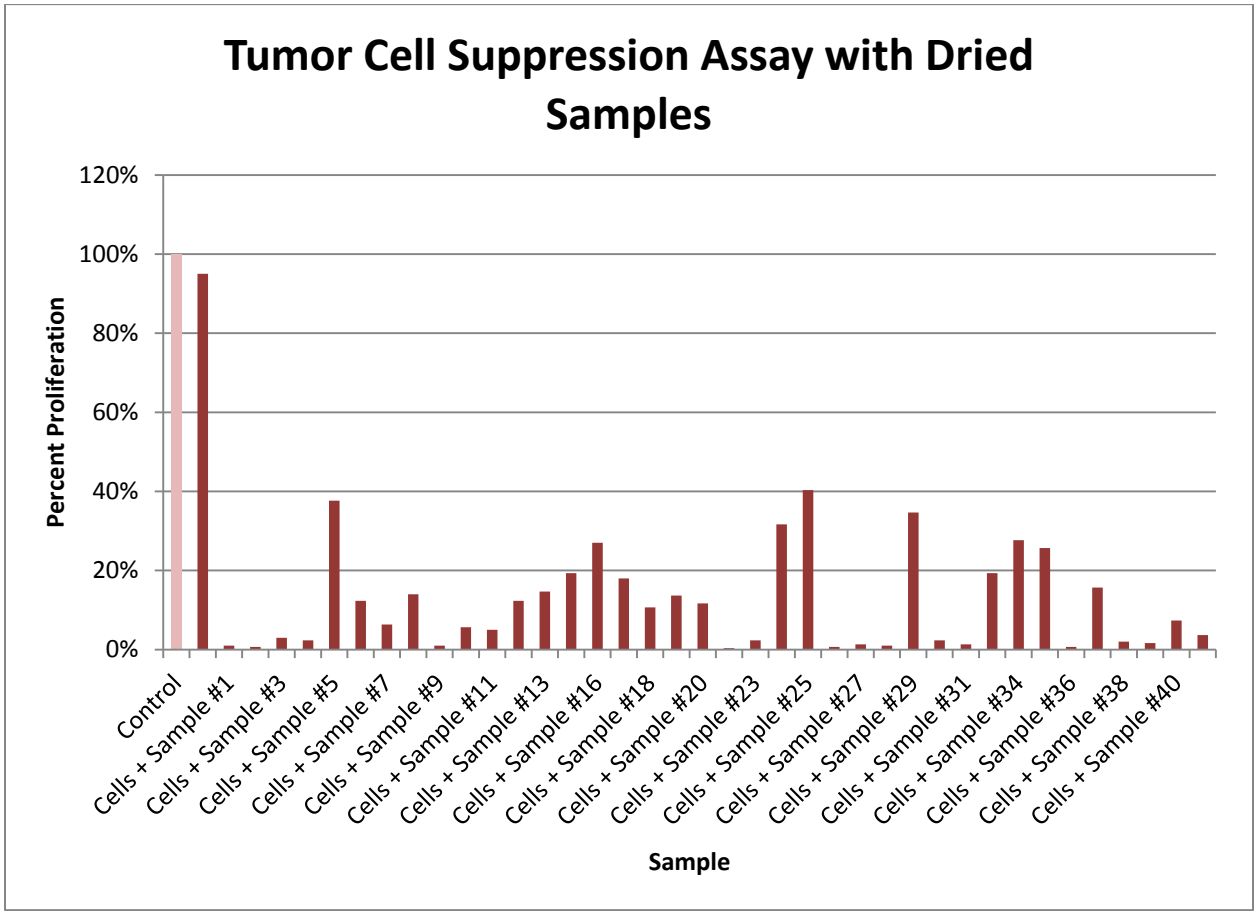


Figure B16. The combined results of tumor cell proliferation assays conducted with 20 µL of the samples dried through centrifugation with a vacuum pump.

Appendix C: Solution Recipes

Column Chromatography Mobile Phase (Silica Gel as the Stationary Phase)

The designated mobile phase for this procedure was a mixture of 200mL ethyl acetate, 22mL formic acid, 22 mL acetic acid, and 52 mL deionized water. The mixture was shaken rigorously due to the immiscible nature of the chemicals and poured through the column.

Column Chromatography Mobile Phase (nPVP as the Stationary Phase)

The mobile phase was prepared through the combination of varying mixtures of methanol and water with a consistent amount of 0.01N HCl. 150 mL of each solution was poured through the column.

1: 0% MeOH: 500-mL H_2O + 50-mL 0.01N HCl

2: 10% MeOH: 50-mL MeOH + 450-mL H_2O + 50-mL 0.01N HCl

3: 20% MeOH: 100-mL MeOH + 400-mL H_2O + 50-mL 0.01N HCl

4: 30% MeOH: 150-mL MeOH + 350-mL H_2O + 50-mL 0.01N HCl

5: 40% MeOH: 200-mL MeOH + 300-mL H_2O + 50-mL 0.01N HCl

6: 50% MeOH: 250-mL MeOH + 250-mL H_2O + 50-mL 0.01N HCl

7: 60% MeOH: 300-mL MeOH + 200-mL H_2O + 50-mL 0.01N HCl

8: 70% MeOH: 350-mL MeOH + 150-mL H_2O + 50-mL 0.01N HCl

9: 80% MeOH: 400-mL MeOH + 100-mL H_2O + 50-mL 0.01N HCl

10: 90% MeOH: 450-mL MeOH + 50-mL H_2O + 50-mL 0.01N HCl

11: 100% MeOH: 500-mL MeOH + 50-mL 0.01N HCl

RPMI-1640 Nutrient Media (Used for Spleen Cell Culture)

Add one jar of RMPI-1640 to 1 liter of deionized water. To this, add 2.0g sodium bicarbonate, and 10 mL of penicillin-streptomycin. Filter-sterilize the media using a vacuum filtration technique.

Eagle MEM X Nutrient Media (Used for the Mewo Cell Culture)

10 mL PSG (antibiotic + L-glutamine), 5 mL NEAA (non-essential amino acid solution), and 0.5 mL Ampho-Beta antifungal were added to one bottle of Eagle MEM 1X media (500-mL), The reagents were mixed by gently inverting the bottle.