ABSTRACT

Title of Document: SUPPRESSION OF THE HEPATITIS C VIRAL LOAD BY *PHYLLANTHUS NIRURI* EXTRACTS

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Hepatitis C virus (HCV) is a public health crisis, affecting over 170 million people by 2011. Conventional treatment, interferon alpha with ribavirin, can often be ineffective and may lead to severe side effects, and this lack of effective treatment poses significant challenges to patients, healthcare providers, and public health officials. As

a result, our team investigated the therapeutic potential of medicinal plant extracts from *Phyllanthus niruri* as an alternative treatment for HCV. Molecular barcoding of chloroplast genes rbcl and matk was used to document the genetic identity of our plants. Phytochemicals were then extracted from dried plant material to isolate potential active compounds. We applied the extraction products to HCV-infected cell lines, specifically Huh7.5 liver cancer cells with J6JFH virus, to determine their effects on viral load and cell toxicity. Our extracts significantly decrease the number of viral copies per cell with no significant toxicity. Viral load suppression was strongest 24 hours after treatment. This effect either declined (extracts 3 and 4) or was sustained (extracts 1 and 2) over time. Extract 4 at 1 μg/ml at 24 hours and 5 at 10 μg/ml at 96 hours reached near 100% suppression, demonstrating significant potent effect comparable to interferon-alpha, but the effect of Extract 4 is not sustained. It also demonstrates different extracts may exhibit different kinetics, suggesting different mechanisms of action. Overall, our study serves as a building block to develop novel treatments and contributes to the discovery of alternative drug options for HCV patients.

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Table of Contents

This Table of Contents is automatically generated by MS Word, linked to the Heading formats used within the Chapter text.

List of Tables

List of Figures

Chapter 1: Introduction

Section 1: 1.1.0 Team ANTIDOTE

The Gemstone Program at the University of Maryland, College Park is a living-learning program that enables teams of undergraduate students to engage in interdisciplinary research under the guidance of faculty mentors. Team ANTIDOTE comprises of fourteen undergraduate students in the Gemstone program that conducted research under the guidance of Dr. Andrea Ottesen of the College of Agriculture & Natural Resources at the University of Maryland, College Park, and the Food and Drug Administration (FDA), and Dr. Shyam Kottilil, of the National Institutes of Allergies and Infectious Diseases (NIAID) at the National Institutes of Health (NIH).

The use of natural products to treat ailments has been a common practice throughout history in many regions of the world. While herbal remedies are often trusted because they are considered to be time-tested, their potential has also captured the interest of scientists involved in drug discovery and development. Because natural products are so diverse in nature, research in this area permits "the identification of lead molecules of greater interest for the development of new therapeutic agents, as well as biochemical and molecular tools needed to clarify complex cellular and molecular mechanisms of action involved in most physiological and pathological processes" (Calixto, Santos, Filho, & Yunes, 1998). One subset of herbal medicine research involves the study of "edible plants containing antioxidants and health-promoting phytochemicals" (Sabir & Rocha, 2008). Polyphenolic compounds found

in the vegetables, fruits, and teas of such plants have been correlated with the low risk of chronic diseases such as heart disease, stroke, arthritis, diabetes and cancer (Sabir & Rocha, 2008). The members of our team are amongst those who are greatly intrigued by this prospect, and it was with this curiosity and concern that we formed Team ANTIDOTE. With our team, we planned to respond to the need for the development of alternative medicinal treatments for the hepatitis C Virus (HCV).

The project idea began after one team member spent a summer at the National Institutes of Health, where she heard about patients with HIV/hepatitis C co-infection. When she later traveled abroad to Peru to study medicinal plants of the Amazon rainforest, she encountered *phyllanthus niruri*, a plant that seemed tremendously understudied but held great therapeutic potential in the liver and against HIV (Chirdchupunseree & Pramyothin, 2010) (Qian-Cutrone, 1996). Having also learned that an infectious model of the hepatitis C virus cell culture system had only been developed in 2005 (Duverlie & Wychowski, 2007), just three and a half years prior to start of the project, and finding that little research had been done specifically with this plant on hepatitis C, she thought that combining *phyllanthus* and HCV would be a great opportunity to contribute new knowledge. The project idea was proposed through the Gemstone Program and Team ANTIDOTE was formed in spring of 2009.

Section 2: 1.2.0 Status of HCV treatment

Hepatitis C, an infectious liver disease, affects 170 million people worldwide, with almost 4 million cases in the United States alone (World Health Organization, 2011). Most people with hepatitis C, caused by the hepatitis C virus (HCV), have no

symptoms, or develop symptoms that eventually get better. Those who do develop symptoms often suffer from fatigue, jaundice, abdominal pain, and vomiting (NIAID). However, most people, 75%-85%, will also develop chronic hepatitis C. Chronic hepatitis C is a long-term illness that can lead to serious liver complications, including cirrhosis and liver cancer (CDC, 2009). In fact, hepatitis C is the leading cause of cirrhosis and liver cancer in the United States and the most common reason for liver transplantation, with 8,000-10,000 people dying from hepatitis C complications each year (CDC, 2009).

Currently, there is no vaccine available for hepatitis C, and the most common medication, pegylated interferon alpha and ribavirin, is often ineffective and induce very serious side effects. For patients treated with both pegylated interferon alpha and ribavirin, only 40% are able to clear HCV from the blood (Brok, Gluud, & Gluud, 2010). Typically, pegylated interferon alpha and ribavirin are given simultaneously for 24-48 weeks (Dugdale, Longstreth & Zieve, 2010). Interferon, given as a shot, is a protein that the body produces to fight infections. Pegylated interferon injections are given once a week. Ribavirin is a capsule that has to be taken twice a day. However, ribavirin by itself is ineffective and has to be taken along with interferon injections (Dugdale, Longstreth & Zieve, 2010).

Recently, the FDA approved protease inhibitors that can be given along with interferon and ribavirin to help improve effectiveness (FDA, 2011). Two new protease inhibitors, boceprevir and telaprevir, have been clinically shown to improve the cure rate from 50% to 70-80% (McQueen, 2011). However, the approval of these drugs does not eliminate the use of interferon, and these drugs are now prescribed in

combination with interferon. This means that the side effects of interferon can still persist in conventional treatment (Canavan, 2012).

Section 3: 1.3.0 Problems with HCV treatment

The need for research in remedial alternatives against HCV stems primarily from the multitude of side effects associated with the current treatment of the virus. As aforementioned, pegylated interferon alpha with ribavirin is used to increase the body's ability to fight off infection and to specifically combat the virus ("Hepatitis C treatment:," 2011). However, the side effects of these drugs include fatigue, gastrointestinal disturbances, influenza-like symptoms, and hematologic abnormalities (Fried, 2002). While these are the more commonly experienced side effects, some patients have experienced more serious effects such as retinopathy, retinal hemorrhage, visual loss, tinnitus, hearing loss, cardiac arrhythmias, and congestive heart failure (Fried, 2002). These side effects have caused patients to resort to lower drug dosages, which compromises the activity of drug against the virus. It has even been the case that patients have had to terminate treatment altogether in order to prevent the damage caused by the more serious side effects (Fried, 2002). Some side effects have been deemed transient during the patient's therapy, while others last throughout the entirety of the patient's treatment ("Hepatitis C treatment:," 2011). The lack of an alternative treatment has left these potentially detrimental side effects and discomforts to persist in many HCV patients – and unfortunately, the side-effect profile of standard treatment has remained "largely unchanged" since its initial release in the late 1990s and early 2000s (Russo & Fried,

2003). The search for alternative therapy, therefore, such as the exploration of herbal remedies, is necessary in order to see if there is another effective method against HCV that does not have to involve such uncomfortable -- if not detrimental -- side effects.

Section 4: 1.4.0 Problems with herbal remedies

In recent decades, the recognition of alternative medicine has become increasing popular in North America. Since the adverse effects of conventional drug therapies are well documented, people have started to use natural remedies as potentially effective and less toxic alternatives to pharmaceutical agents (Elvin-Lewis, 2000). Herbal medicine can be separated into four major types: Indigenous herbalism, Asian medicinal systems, European herbalism, and Neo-Western herbalism (Elvin-Lewis, 2000), and with this wide range of sources, there are numerous regulatory challenges to alternative medicine. In the United States, regulation of herbal products only began around 1994 when the Dietary Supplement Health and Education Act (DSHEA) was enacted. Under this act, herbal remedies became exempt from strict FDA regulations as long as the labeling of these products did not contain medical claims (Elvin-Lewis, 2000). However, these broad guidelines lack proper evaluation of herbal remedies in terms of safety and efficacy. The lack of regulation means that a variety of factors can cause herb toxicity including misidentification of plants, contamination, incorrect handling of the plant, and improper storage (Whiting, Clouston, & Kerlin, 2002). In addition, the use of herbal medication in conjunction with pharmaceutical drugs can cause a variety of adverse side effects due to the antagonism of the drug by herbal compounds. For instance,

cinnamon with tetracycline can cause hallucinations and valerian with anti-histamines can cause sedative effects (Elvin-Lewis, 2000).

For chronic diseases without effective treatments such as hepatitis C, the use of alternative medicine is becoming more prevalent. In one particular study, up to 46 percent of patients attending liver disease clinics admitted to using some form of alternative medicine at least once in the month prior (White, Hirsch, Patel, Adams, & Peltekian, 2007). The high percentage of HCV patients who use alternative therapies is not surprising given that they are perceived to be safe and without side effects. However, this overconfidence in the safety of herbal therapies can be dangerous since the improper use, preparation, and quality of herbal therapies can have significant harmful effects. A universal regulation system with documented efficacy would be helpful in evaluating herbal remedies and preventing improper use.

Section 5: 1.5.0 Objectives

To address the need for additional research in the area of herbal medicine and alternative treatments for HCV, the primary goal of our research project was to examine the manner in which extracts of *P. niruri* affect the cell viability and viral load in cells infected with HCV. The research questions guiding this project were:

- Are there any biologically active fractions in *P. niruri* against hepatitis C virus and how do they affect viral load and cell viability?
- What is the effect of extracts from *P. niruri* when combined with and compared to medicines used in conventional treatment of HCV, specifically standard interferon alpha for HCV?

In order to organize and facilitate the answering of these overarching questions, we divided our primary research objective into three specific objectives:

1. To extract and identify the active compounds from *P. niruri* that were said to have therapeutic potential against HCV.
2. To genetically barcode *P. niruri* plant samples grown and used in chemical extractions to ensure that all plants used were from one species and to establish a clarified taxonomic identity for *P. niruri*.
3. To quantitatively and qualitatively determine the efficacy of active *P. niruri* extracts on HCV-infected cell lines via cell studies which focus on measuring cell viability and viral load.

Section 6: 1.6.0 Outline of Study

P. niruri seeds were purchased through online vendors and cultivated with assistance from U.S Botanical Garden and sustained by the University of Maryland Greenhouse.

Objective 1 was accomplished through a methanol extraction and partitioning of the different extract layers. These fractions were later analyzed through HPLC to compare the phytochemical content between fractions of different purity.

Objective 2 was accomplished by tracking the plants used in the chemical extractions to determine whether the samples used were genetically identical. DNA barcoding was done by amplifying and sequencing target regions of the *P. niruri* genome in order to construct phylogenetic trees of all plants used.

Objective 3 was accomplished by analyzing the efficacy of *P. niruri* extracts on Huh7.5 liver cancer cell line infected with a strain of the HCV virus. Cell viability and viral load was quantified after treating with the cell line with the various fractions of extract. This was done by employing the MTS assay and quantitative real-time PCR. The results of these experiments allowed for a correlation to be made between the use of *P. niruri* as a form of treatment and HCV.

Section: 1.7.0 Hypothesis

We hypothesize that individual *P. niruri* plants used during experimentation are genetically identical. Furthermore, we hypothesized that the extracted compounds from *P. niruri* will have antiviral effects on cell cultures for hepatitis C as seen through decreased viral load without significant cell toxicity.

Chapter 2: Literature Review

Section 1: 2.1.0 Phyllanthus

Phyllanthus (Euphorbiaceae) is a broad genus, comprising between 550-750 species and 10 to 11 subgenera, with roughly 200 species growing in tropical and temperate regions of the Americas and the Caribbean (Calixto, Santos, Filho, & Yunes, 1998). Species of *Phyllanthus* are found growing in many tropical regions such as India, Africa, the West Indies, and South America. It has a geographically wide spread distribution which makes it difficult to find its point of origin.

Plants of this genus were first recorded by Swedish botanist and taxonomic pioneer, Carl Linneaus, in 1737 (Linneaus, 1738). However, prior to its first taxonomic record, *phyllanthus* had a long tradition in local medicinal therapies (Webster, 1957). Specifically, members of this genus had already been used for thousands of years in the folkloric medicine of Brazil, India, and China. Known in Brazil as "quebra pedra," or "stone breaker," Phyllanthus was often used to treat a wide range of ailments, particularly those affecting the kidneys and urinary bladder (Calixto, Santos, Filho, & Yunes, 1998). "Quebra pedra" is a general name that encompasses a handful of different Phyllanthus species, especially, *P. niruri*, *P. uririnaria*, *P. amarus*, *P. tenellus*, and *P. caroliniensis* (Calixto, Santos, Filho, & Yunes, 1998).

This study focuses on one particular species in the *Phyllanthus* genus, *P. niruri*, and its potential for use in the treatment of hepatitis C. In Brazilian folk medicine, *P. niruri* is among the most commonly used plant that is classified as "quebra pedra" due to its widespread availability in Brazilian municipalities (Calixto,

Santos, Filho, & Yunes, 1998). In more recent years, however, *P. niruri* has garnered the attention of the international medical community due to its inhibitory effects against hepatitis B, and most recently, antiviral activity against the human immunodeficiency virus (HIV) and hepatitis C (Qian-Cutrone et al., 1996).

Section 2: 2.2.0 P. Niruri

P. niruri is a small, erect annual herb that grows 30 to 50 cm (1 to 2 feet) in height (Vieira, 1999, Wiart 2006a). It has green oblong, oblate, alternate leaves up to 9 millimeters in length and two to five millimeters wide (Wiart, 2006a). The name *Phyllanthus* means "leaf and flower," because the fruit and flower of *Phyllanthus* species seem to become one with the leaf (Patel, Tripathi, Sharm, Cauhan, & Dixit, 2011). The petiole is indistinct, and the base and the apex are around. The male flowers are very small, comprised of three stamens and often found in groups of two to three in the lowest axils. The female flowers are much larger, in higher axils with oval sepals. The fruits are tiny, smooth, three-lobed capsules. The seeds are very small and longitudinally ribbed (Wiart, 2006b). See Appendix M for a diagram.

P. niruri is thought to be mainly localized to the tropical regions in the Americas, however it is possible that the same species may be native to West Africa (Webster, 1957)

Subsection 1: 2.2.1 Taxonomy

The anatomical features of members of the *Phyllanthus* genus have been described by various sources. Related sister species are very similar in appearance and are thus easily mistaken for each other. Two common *Phyllanthus* species are described below.

P. amarus stands at roughly 1.5 feet tall and has smooth, reddish stems that are extensively branched. Their simple leaves have stipules and are light green in color, alternately attached to a long skinny stem. The petiole, which is the stalk that attaches the leaf to the stem, is not prominent in the *P. amarus* species. The plant is monoecious; having both male and female reproductive parts on the same specimen. Male flowers of this species have three stamens. They are small and clustered at the lower portion of the plant while the female flowers are large, non-clustered, and located in the upper portion of the plant. Fruits are spherical and smooth containing three lobes with very small seeds (Wiart, 2006a).

P. niruri Linn., stands roughly 2 feet tall. Its stem is smooth and does not have a bark layer. Leaves, similar to that of *P. amarus*, are small, alternating, and oblong in shape. Leaves contain ranunculaceous stomates, similar to that of the buttercup family. Some vascular tissue is visible under the palisade cell layer. Leaves are covered with a thin cuticle over the epidermis (Kapoor, 2001). This monoecious species has small flowers that grow in pairs parallel to the position of leaves on the stem. Fruits are capsulated, small, and ridged. Further anatomical features are gathered through a cross section of the stem. The outer epidermis layer is made up of box-shaped cells. The middle cortex layer is thick and the inner pith can be

distinctively identified. Vascular tissue, xylem and phloem, are arranged in rings. *P. niruri* has been deemed to be synonymous to species previously misclassified as *P. fraternus Web.* (Kapoor, 2001).

Subsection 2: 2.2.2 Taxonomic Confusion

P. niruri is taxonomically confused with other species classified as "quebra pedra," especially *P. amarus* and *P. urinaria*. This confusion stems from its original classification by Carl Linnaeus, who erroneously classified a number of closely related and taxonomically similar species as *P. niruri*, leading to further misidentification and misclassifications by subsequent scientists (Patel, Tripathi, Sharm, Chauhan, & Dixit, 2011). To complicate matters, the Brazilian name "quebra pedra" does not identify a single species, but instead refers to a handful of similar species. Likewise, in India, the common name of this class of herb applies for both *P. niruri* and *P. urinaria*, even though *P. urinaria* usually appears to be somewhat visually different (Patel, Tripathi, Sharm, Chauhan, & Dixit, 2011). The original specimens of *P. niruri* first identified by Linnaeus in 1737 were collected in Barbados, and it remains unclear whether true specimens matching these originals have been observed outside of the Americas (Webster, 1957). In fact, the so-called *P. niruri* described in India has been reported to be a mixture of three distinct species: *P. amarus, P. fraternus*, and *P. debilis* (Mitra & Jain, 1985).

The lack of consensus about the identity of *P. niruri* has led to some confusion and disagreement among researchers studying this species. This becomes especially problematic in the fabrication and regulation of herbal medicines, where the authenticity of the plants must be verified in order to ensure the quality and safety

of the herbal remedy in question (Martins, Pereira-Filho, & Cass, 2011). For instance, the Brazilian Pharmacopeia only recognizes two *Phyllanthus* species: *P. niruri* and *P. tenellus* as being therapeutically active and safe for human consumption (Martins, Pereira-Filho, & Cass, 2011). Due to the prevalence of co-habiting species of morphologically similar *Phyllanthus*, there is a high probability that local farmers or collectors create admixtures of several different species, especially since taxonomists are rarely consulted during the collection process (Srirama et al., 2010).

Since different species within the *Phyllanthus* genus have differing composition percentages of bioactive compounds, different plants can elicit different degrees of biological activity once they are consumed (Lee, Jagannath, Wang, & Sekaran, 2011). In some cases, the taxonomic similarity of *Phyllanthus* plants can cause the unintentional substitution of a plant that is not approved to be therapeutically active (Martins, Pereira-Filho, & Cass, 2011). In a comparative study by Khatoon et al. in 2006, it was shown that three commonly mixed species of *Phyllanthus* contained significantly different phytochemistries. *P. amarus*, *P. fraternus*, and *P. maderaspatensis* are all sold and used in India for the treatment of liver and kidney ailments, yet only one of them, *P. amarus,* contained the known hepatoprotective compounds, phyllanthin and hypophyllanthin. Such admixtures could lead to a decreased efficacy of treatment or even toxic effects (Khatoon, Rai, Raway & Mehrotra, 2006). Therefore, a major concern for phytomedicine is the creation of simple, cost-effective methods to assure the identity of raw plant material and the herbal medicines created from them.

Section 3: 2.3.0 Taxonomic identification and chromatographic fingerprinting

Until the recent past, most plant samples were taxonomically identified according to the physical appearance -- such as the size, shape, and color -- of features such as the leaves, stem, flowers, and seed. However, since this method relies heavily on the quality and age of the sample, it is often difficult to make these determinations based on physical appearances alone. ("What is dna," 2012) Chromatographic fingerprinting recently has emerged as a simple method of validating herbal samples and differentiating species. This method allows for validation and comparison between the complex phytochemical products of herbal extracts and plant-based medicine against a known or "standard" extract. In the context of herbal medicines, it allows comparison on the chemical level between herbal batches supposed to contain a single plant species (Martins, Periera-Filho, & Cass, 2011).

Brazilian scientists have attempted to combat these issues utilizing chromatographic fingerprinting as a technique for quick and accurate identification of *P. niruri*. In 2009, Colombo et al. published a standard high performance liquid chromatography (HPLC) method for evaluating *P. niruri* samples obtained from commercial vendors. This was done through the identification of three major compounds present in both the leaves of the herb and commercial extracts: corilagin, rutin, and ethyl 3,4,5-trihydroxybenzoate. The first of these compounds, corilagin has been proposed as a phytochemical marker for *P. niruri* and was used to develop rapid and efficient HPLC conditions to assay for the presence of this chemical in the plant samples. Theoretically, this could be expanded to test the reliability and authenticity

of samples commonly sold on the market. This method is beneficial because it is quick and simple, requires little plant matter to perform HPLC analysis, and does not require the whole plant for physical taxonomical identification (Colombo, 2009).

However, this method is not without limitations. There is often a degree of chemical variation within a plant species since factors such as growing conditions may cause the relative concentrations of compounds between plant specimens to vary (Martins, Periera-Filho, & Cass, 2011). Such growing conditions include sunlight, temperature, water, location, and the availability of nutrients. Thus, while it is possible to use chromatographic differences to discern between plant samples from different genera, it does not provide an ideal method for taxonomic speciation (Martins, Periera-Filho, & Cass, 2011).

Section 4: 2.4.0 DNA Barcoding

Due to the limitations of taxonomic and chromatographic methods of identification, biodiversity research has been trending towards DNA barcoding. DNA barcoding uses short DNA sequences between 400 and 800 base pairs as "barcoding regions" that can be used to characterize and identify species, utilizing the fact that there is a degree of genetic variation between different species within the same genus (Kress, Wurdack, Zimmer, Weigt, & Janzen, 2005). Barcoding regions must exhibit sufficient genetic divergence between species in order to be used as effective markers (Kress & Erickson, 2008b). By cataloguing the barcoding regions of as many species as possible, an organization called the Consortium for the Barcode of Life aims to create a virtual database of species identifiers that can be publicly accessed to match

unknown specimens to known species (Kress, Wurdack, Zimmer, Weigt, & Janzen, 2005).

However, there has been difficulty in finding regions that meet the criteria necessary for a DNA barcode. These requirements include high variation of DNA regions within species of the same genus, conserved barcode flanking regions that allow for the use of universal primers, and a relatively short length to facilitate DNA amplification (Kress & Erickson, 2008a). For animals, the most successful region has been a short sequence in the cytochrome c oxidase 1 (CO1) gene (Kress, Wurdack, Zimmer, Weigt, & Janzen, 2005). The CO1 region has already been used in a number of studies. For plants, however, the identification of a region has been more challenging due to the fact that the CO1 region evolves much more slowly in plants than in animals (Kress, Wurdack, Zimmer, Weigt, & Janzen, 2005).

Despite these difficulties there has been some success in finding regions that show promise as possible DNA barcode loci for land plants. Kress and colleagues in 2005 and Kress and Erickson in 2007 both recommended the psbA-trnH spacer region and the rbcL gene as potential barcode loci. A more recent paper by Hollingsworth et al. (2009) recommended a combination of rbcL and matK, two gene regions in the chloroplast, as the most effective DNA barcode for plants. The matK chloroplast coding region has the advantage of having a rapid substitution rate compared to other regions. When used in conjunction with another region such as psbA-trnH (a chloroplast spacer region), it was found that matK could distinguish between 90% of species tested (Hollingsworth, 2008).

Recently, there has been some success in species-level discrimination of *Phyllanthus* using DNA barcoding. In a study by Srirama et al. (2010), psbA-trnH was used to effectively differentiate between sixteen species of *Phyllanthus* . In their study, 95% of *Phyllanthus* specimens collected from vendors throughout southern India were identified as belonging to one of six *Phyllanthus* species (Srirama et al., 2010). Although the official barcoding regions for land plants was decided to be rbcL and matK, it is possible that the psbA-trnH region may prove to be a more effective region in the speciation of *Phyllanthus* plants. At the very least, this region may be used to provide additional DNA evidence in the identification of plants when contrasted with using rbcL and matK alone.

In November 2009, the Consortium for the Barcode of Life reported in Nature that it had chosen rbcL and matK as the approved DNA barcode (Hollingsworth et al., 2009). In accordance with the Consortium for the Barcode of Life's decision, rbcL and matK were used to positively identify *Phyllanthus* specimens used in this study.

Section 5: 2.5.0 Historical Perspectives: Traditional use in India/Brazil

Plants belonging to the *Phyllanthus* genus have a long history of use in the folkloric medicine in countries such as Brazil and India. Although scientific study of *Phyllanthus* genus is fairly recent, it has been used in traditional herbal remedies for over two thousand years due to its demonstrated therapeutic potential. In Brazilian folkloric medicine, the *Phyllanthus* genus is commonly used to treat urinary infections and kidney stones (Calixto, Santos, Filho, Yunes, 1998). In Indian

Ayurvedic medicine, the *Phyllanthus* genus is used to treat ailments involving the stomach, liver, kidney and spleen (Patel, Tripathi, Sharm, Chauhan, & Dixit, 2011).

Subsection 1: 2.5.1 Traditional Use in Brazil

The use of herbal medicines is commonplace in the localities of Brazil (Martins, Pereira-Filho, & Cass, 2011). Brazilians typically have used *P. niruri* in order to prevent and cure urolithiasis, or kidney stones. As a result of its positive effect on kidney stones, certain plants of the *Phyllanthus* genus have been given the common name "quebra-pedra," which translates as "stone breaker" or "shatter stone" (Martins, Pereira-Filho, & Cass, 2011). *Phyllanthus* leaves are typically steeped in water to make a tea that is then consumed orally (Cartaxo, Souza, & de Albuquerque, 2010).

Phyllanthus is one of the more popular herbal remedies on the market, and can be acquired in local markets, pharmacies, and herbal remedy stores. It is typically sold as dried and chopped fragments of leaves, seeds, and stems (Martins, Pereira-Filho, & Cass, 2011). "Quebra pedra" is typically advertised to the locals as a treatment option for urolithiasis and other kidney and bladder afflictions. Unfortunately, the taxonomic similarity of several co-habiting plant species (of both Phyallnthus and other genuses) has led to confusion among suppliers and vendors. As a result, a handful of species of plants are being sold under the same name. One study collected 16 "quebra pedra" samples from 14 sellers in a street market in Porto Alegre, Rio Grander do Sul, Brazil. Even though all samples claimed to be "quebra-pedras," there were seven different species of plant. *P. niruri* was the only plant from the *Phyllanthus* genus of the seven species and the only plant proven to have

therapeutic potential. Researchers found that after being dried and fragmented for selling, *P. niruri* and other Euphorbia products appeared to be more morphologically similar, a possible cause for the inter-species confusion (Aita, Matsuura, Machado, & Ritter, 2009).

Subsection 2: 2.5.2 Ayurvedic Use of Phyllanthus in India

In India, *Phyllanthus* is used to treat a wide range of ailments including migraines, jaundice, gonorrhea, syphilis, skin diseases, malaria, menstrual problems, diabetes, chronic dysentery, liver disease, anorexia, constipation, irritable bowel syndrome, and urinary tract infection (Patel, Tripathi, Sharm, Chauhan, & Dixit, 2011). Herbal remedies are typically prescribed by herbal doctors that follow an alternative system of medicine practiced in India, called Ayurved (Vijendra & Khatri, 2010).

The most common ways of preparing *Phyllanthus* is by boiling plant matter to make a tea that is consumed, or by creating a paste that is applied to the affected area of the skin (Patel, Tripathi, Sharm, Chauhan, & Dixit, 2011). Sometimes, certain parts of the plant are used (such as leaves only or roots only), and other times, the whole plant may be used. For instance, in order to cure jaundice, the traditional remedies suggest grinding *Phyllanthus* leaves into a powder and creating a tea or creating fresh juice from the whole plant and ingesting it 3-4 times per day (Singh, Raghubanshi, & Singh, 2002). Ayurvedic practitioners in India also use *Phyllanthus* to help support healthy liver function and to treat liver diseases, such as hepatitis, jaundice, and liver cancer (Unander, Bryan, Lance, & McMillan, 1993).

Due to its widely-recognized therapeutic effects, *Phyllanthus* is commonly sold in markets in India. In one study, morphological analysis was performed on samples from 25 different markets, and six different species were found even though they were all labeled as the same plant (Srirama et al., 2010). Due to the taxonomic confusion between *Phyllanthus* species and its widespread usage in traditional Indian medicine, more stringent herbal market regulation is necessary.

Subsection 3: 2.5.3 Regulation of Phytomedicines in India and Brazil

The widespread use of herbal medicines, such as *Phyllanthus*, necessitates the creation of guidelines for safety. The World Health Organization (WHO, 2000) has published guidelines to help regulate herbal medicine for national regulatory authorities, scientific organizations and manufacturers. WHO advocates 4 main steps for regulation: (1) assessment of quality; (2) assessment of safety; (3) assessment of efficacy and (4) intended use. Documentation of these parameters would allow for better regulation of the market for traditional herbal medicines. Despite this suggestion, the regulation of *Phyllanthus* has remained minimal at best.

In India, herbal drugs have been regulated since 1940 and are available both through prescription and over-the-counter. All herbal drugs are regulated by the Department of Ayurveda, Yoga, & Naturopathy, Unani, Siddha, and Homeopathy (AYUSH), although herbal drugs are generally not required to provide evidence of safety and efficacy through scientific information (Sahoo, Manchikanti, & Dey, 2010).

Brazil, on the other hand, has a much stricter guideline for herbal medicine. Herbal medicines are considered drugs and therefore are required to show complete documentation of efficacy, safety, and quality control (Calixto, 2005). Furthermore, for older medicinal herbs that are already registered, it is required to assess their safety for 5 years, and efficacy for 10 years. (Calixto, 2000)

The Brazilian Pharmacopeia has approved and identified only *P. niruri* and *P. tenellus* as effective and safe for human consumption (Martins, Pereira-Filho, & Cass, 2011). However, it is incredibly difficult to control the chemical quality of herbal medicine because native medicinal plants are cultivated and collected from many different areas of the country. Furthermore, different species of *Phyllanthus* plants share morphological features, causing confusion between therapeutically active plants and those that are not. Since collectors rarely consult taxonomists or have access to advanced identification methods such as chromatographic fingerprinting or molecular barcoding, it is easy for misidentification of co-habiting *Phyllanthus* species to occur. (Martins, Pereira-Filho, & Cass, 2011).

Section 6: 2.6.0 P. niruri and Liver

Extracts of *P. niruri* have shown therapeutic potential against rising cholesterol levels in the liver and bloodstream. A recent study showed that *P. niruri* could increase high density lipoprotein (HDL) levels by increasing the activity of an enzyme known as lecithin cholesterol acyltransferase (LCAT). The primary role of this enzyme is to synthesize HDL, which is commonly referred to as "good cholesterol." The study also showed that *P. niruri* was able to facilitate the rapid

catabolism of LDL (low-density lipoprotein, known as "bad cholesterol") through LDL receptors in the hyperlipemic environment of cholesterol-fed rats (Khanna, Rizvi, & Chander, 2002). This dual mechanism in which *P. niruri* alters cholesterol levels aptly applies to our project because HCV recently has been shown to have an affinity for the same receptors as low-density lipoproteins (LDL) (Khanna, Rizvi, & Chander, 2002). Because of this result, scientists proposed that the LDL receptor is competitively inhibited by the HCV virus. The rapid binding of beta-lipoproteins is stimulated by *P. niruri* treatment which, in turn, competitively inhibits the involvement of HCV antigens with the LDL receptors that the virus uses to propagate itself and cause damage to the liver.

An important compound that has been isolated from plants of the *Phyllanthus* genus is a lignin called phyllanthin (Sharma). One study investigated the effect of phyllanthin on ethanol-induced cytotoxicity. In this study, rat hepatocytes were treated with ethanol, causing a decrease in cell viability because of membrane integrity impairment and oxidative damage. Phyllanthin was subsequently tested on liver cells and researchers found that its hepatoprotective properties helped improve cell viability. However, it was found that the whole extract (obtained from *P. amarus*) rather than phyllanthin alone, displayed a more rapid restoration of liver cell health and a greater increase in cell viability. This suggests that there are other phytochemicals in the extract that contribute to the hepatoprotective activity of *Phyllanthus* species (Chirdchupunseree & Pramyothin, 2010).

Section 7: 2.7.0 Antiviral activity of Phyllanthus extracts

P. niruri has demonstrated antiviral properties in both *in vitro* and *in vivo* studies. Specific viruses that have been effectively treated include hepatitis B virus (HBV), hepatitis C virus (HCV), and woodchuck hepatitis virus (WBV) (Venkateswaran, Millman, & Blumberg, 1987).

Subsection 1: 2.7.1 Phyllanthus and WBV and HBV

In a landmark study by Venkateswaran, Millman, and Blumberg (1987), the effects of an extract of *P. niruri* were examined on woodchuck hepatitis virus (WBV). Since WBV and HBV have substantial cross-reactivity and significant homology of DNA, WBV can serve as a proxy for human hepatitis B virus (Venkateswaran, Millman, & Blumberg, 1987). This study was conducted with the principal goal of determining if a plant extract such as *P. niruri* could inhibit endogenous DNA polymerase of HBV, which is necessary for the replication of the virus. By inhibiting HBV DNA polymerase, the viral load of the cells could effectively be neutralized. Venkateswaran, Millman, and Blumberg (1987) suggested that the extract had a positive effect via "prevention by delay".

This relationship between hepatocellular carcinoma and HBV showed that fully differentiated liver cells allow complete replication of HBV whereas less differentiated cells do not allow replication. The proliferation of HBV within the differentiated cells leads to cell death caused by immune response, and the less differentiated cells divided and multiplied in response to the differentiated cell death. With such an increased rate of multiplication of cells, chromosomes are more liable to

sustain mutation effects, which could be perceived as cancer by the host organism's immune system. Thus, by decreasing the viral load or inhibiting the entry of virus into liver cells, the death rate of cells would slow, and ultimately delay the immune system response to perceived "cancers". This, however, is just one hypothesis about how decreasing viral load could mitigate the likelihood of liver carcinoma or other diseases (Venkateswaran, Millman, & Blumberg, 1987).

The results of the paper demonstrated that animals administered with the extract showed a significant drop in titer of woodchuck hepatitis antigen, and also that the extract was not effective when administered subcutaneously, but was effective when administered intraperitoneally. This could be an important piece of information to keep in mind when developing extracts for human antiviral studies. After this study, there was a wealth of research done to probe antiviral activity and hepatoprotective activity of *Phyllanthus* extracts.

Subsection 2: 2.7.2 P. niruri and HIV

Extracts from *P. niruri* have demonstrated anti-viral behavior, specifically against HIV replication. A study by Naik and Juvekar (2003) assessed the anti-HIV effects of alkaloidal extracts of *P. niruri* on human MT-4 cell lines. This study sought to characterize the pharmacological profile of this plant as well as to understand the extent of its antiviral properties. To quantify the antiviral effect, a factor called the selectivity index (SI) was employed. SI is a ratio of how toxic *Phyllanthus* extracts were to viruses with respect to how non-toxic extracts were to healthy host cells. A high SI value indicated high toxicity towards cells infected with

virus and low toxicity towards host cells. Compared to the reverse transcriptase inhibitor control drug Azidothymidine, which has a SI value of 2046.11, the SI value of the *Phyllanthus* extract was much lower at 13.34. Although the SI of the *Phyllanthus* extract was comparatively low, this value suggests that the extract still exhibits some selective toxicity towards virus-infected cells. Naik and Juvekar (2003) concluded that *P. niruri*'s alkaloidal extracts significantly inhibited the growth of HIV-1 and HIV-2 on human MT-4 cell lines.

Ogata et al. (1992) identified a specific chemical component from *P. niruri* aqueous extracts after discovering its inhibitory effects against HIV-1 reverse transcriptase. Using nuclear magnetic resonance spectroscopy (NMR), they matched the compound to repandusinic acid A monosodium salt (RA). RA was tested for selectivity and found to be 10 times more effective in binding with HIV-1 reverse transcriptase versus DNA polymerase alpha.

Another active compound, niruriside, was isolated and identified by Qian-Cutrone et al. (1996) from MeOH extracts of *P. niruri* leaves. Niruriside was shown to significantly inhibit the binding of the REV (regulation of virion expression) protein to RRE RNA (REV-responsive element). Qian-Cutrone et al. found that niruriside showed specific inhibitory activity against the binding of REV protein to RRE RNA. REV is responsible for regulating the transport of viral RNA (Gosser et al., 2001). The extraction protocol used in our experiments is an altered niruriside extraction methodology outlined by Qian-Cutrone (1996). Niruriside is classified as a saponin, a phytochemical that is similar to the body's hormones (Chevallier, 1996). It

is a colorless powder with molecular formula $C_{38}H_{42}O_{17}$ and molecular weight 770.2397 g/mol.

There have been various other studies done on extracts of related species in the *Phyllanthus* genus on HIV infection. *P. niruri* shows potential in treating HIV-1 and HIV-2 infection. Further research is needed to determine the proper dosage of active compounds that will inhibit the virus while not harming human host cells. There are numerous other active compounds that could be present in other parts of the plant, which could be tested for possible anti-viral properties.

Subsection 3: 2.7.3 Phyllanthus species activity on hepatitis C

Previous literature has led scientists to conclude that the *Phyllanthus* genus may have an overall positive protective effect on the liver. However, to date, few clinical studies have focused their efforts on the potential effects of *Phyllanthus* species specifically against HCV. Our project aims to resolve some of the shortcomings of these initial studies, including the fact that these few studies tend to focus on the therapeutic effects of *P. amarus* rather than *P. niruri*. Because they are interrelated species, they may display similar chemical properties.

Recent studies show that HCV is currently resulting in more fatalities in the US than HIV. A 2012 article stated that 3.2 million people have chronic hepatitis C virus with over 15,000 people dying from HCV infection in year 2007 (Harmon, 2012). It's apparent that HCV is a rising public health crisis that must be addressed by both research scientists and public health officials.

Hepatitis C is transmitted via blood to blood contact. Prior to 1992, before blood donations were screened for hepatitis C, organ transplants and blood transfusions were common ways of spreading HCV (CDC, 2009). Today, HCV is typically spread through drug use and sharing needles, needlestick injuries in health care settings, and transmission from a pregnant woman to her baby. However, maternal transmission of HCV is rare, and only 4 out of every 100 infants born to mothers with HCV will become infected (CDC, 2009). Although HCV can be transmitted through sexual contact and the sharing of personal care items that may have come in contact with blood, the probability of infection in this manner is relatively low. Hepatitis C is not spread through saliva or breastmilk (CDC, 2009).

The earliest reports of *P. amarus* on HCV in a standardized laboratory setting came from a study on *P. amarus* root clone activity against bovine viral diarrhea virus (BVDV). BVDV was the popular surrogate model of HCV before the recently-developed infectious J6JFH system became standard. BVDV was attractive because it was easy to culture, shared a similar structural organization, and caused chronic long-term infections in their hosts. The two viruses also shared other biological features, such as LDL receptor mediated endocytosis, internal ribosome entry sites (IRES) for translation, and viral enzyme functions (Buckwold, Beer, & Donis, 2003). As a result, the BVDV model was a good approximation of HCV in humans. The study revealed that the application *P. amarus* extracts reduced BVDV-induced cytopathic effect (CPE) in a dose-dependent manner. Therefore, higher concentrations of extracts from *P. amarus* successfully were able to decrease the ability of BVDV to kill the cells. However, this study has a number of shortcomings.

Since these extracts were crude and the concentrations that exhibited positive activity were so high, their results are not clinically relevant because cells would not be able to tolerate such high loads (Bhattacharyya & Bhattacharya, 2003). Also, the BVDV animal model is not as relevant since an infectious human model was established several years later in 2006. Despite these facts, the study may suggest that *P. amarus* and related species may exhibit antiviral effects against HCV.

In 2011, Ravikumar et al. conducted cell culture studies with an infectious model that confirmed a potential active agent. A crude methanolic extract of *P. amarus* roots and leaves was obtained, dried, and dissolved in sterile water with 0.2% DMSO with final concentrations of 1000 µg/mL. Huh7 cells were infected with HCV monocistronic replicon with concentrations ranging from 5-40 µg/mL. *P. amarus* root extract showed significant inhibition of HCV-NS3 protease enzyme and *P. amarus* leaf extract showed considerable inhibition of NS5B in the in vitro assays. Viral enzymes NS3 protease and NS5B RNA dependent RNA polymerase are essential enzymes for viral RNA replication. Both extracts significantly inhibited the replication of HCV monocistronic replicon RNA and HCV H77S viral RNA in HCV cell culture system in a dose-dependent manner. Standard interferon alpha was used as a positive control for decreasing viral replication; when standard interferon was combined with *P. amarus* root, the decrease in viral load was an additive effect. This study marks the potential for *P. niruri* as a potential therapeutic source for hepatitis C; however, concentrations as high as 40 µg/mL are too high to be applied to clinical studies, and the effect of the DMSO vehicle, which was used to dissolve the extracts, was not quantified. It is also difficult to qualify the exact species of *Phyllanthus*

used, but it should be noted that a voucher specimen was stored in their laboratory (Ravikumar et al., 2011)

A clinical trial in February 2011 was also conducted in India, testing *P. niruri* on patients with HCV. Blood samples were taken from 50 HCV patients before and after *Phyllanthus* administration every week for 10 weeks in order to analyze liver profile enzymes, antioxidant enzymes, antioxidant vitamins and lipid peroxidation, which are measures of oxidative stress. As HCV is a major source of oxidative stress, these levels serve as an important diagnostic tool. Based on these tests, it was found that therapy with *P. amarus* increases antioxidants and reduces lipid peroxidation of hepatic cellular and intracellular membranes and protects liver damage due to free radicals in HCV. However, this study had tremendous shortcomings. The dosage per patient was not mentioned, as well as the origin of the plant, and whether or not the plant was stored as a voucher. The preparation of the herbal extract was not specified and the change in viral load was not indicated. Viral load must be measured because it is possible that the extract may decrease the expression of oxidative stress indicators without actually decreasing the viral load. It is only through a significant decrease in viral RNA levels that an effect can be quantified (Nikam, Nikam, Sontakke, & Khanwelkar, 2011).

Subsection 4: 2.7.4 HCV Replication

Current knowledge about the viral replication cycle of HCV is limited because an efficient cell culture system or convenient animal model for studying the mechanisms has yet to be established (Bartenschlager & Lohmann, 2000). However,

since much is known about other closely related viruses in the flavivirus and pestivirus families and about the characterization of recombinant HCV proteins, a likely method of replication can be theorized (Bartenschlarger & Lohmann, 2000). The cycle is outlined in five major steps: 1) penetration of the host cell and injection of genomic RNA from the virus particle into the cytoplasm; 2) translation of the inserted RNA, processing of the polyprotein and formation of a replicase complex associated with intracellular membranes; 3) synthesis of a minus-strand RNA from the original, complementary plus-strand RNA; 4) production of new plus-strand RNA molecules which in turn can be utilized for the synthesis of new minus strands, for polyprotein expression or packaging into progeny virions; 5) release of virus from the infected host cell (Bartenschlager & Lohmann, 2000).

Attachment and entry of the viral particle to the host cell is initiated by interaction between the cell surface receptors of the host cell and a viral attachment protein on the surface of the virion (Bartenschlager & Lohmann, 2000). The E2 glycoprotein on the virus particle binds to the CD81 receptor of the surface of the host cell (Lindenbach et al., 2005). Whether this initial binding is followed by the internalization of the virus particle is an area of research that is still in progress and has yet to form a conclusion on this matter (Bartenschlager & Lohmann, 2000). It has also been observed that members of the Flaviviridae family may enter the cell by binding to low-density lipoprotein (LDL) receptors – the medium of viral genome injection, however, still cannot be concluded from this observation (Bartenschlager & Lohmann, 2000).

Subsequently, the virus particle enters by endocytosis, which is mediated by clathrin-coated pits in the host cell (Moradpour, Penin & Rice, 2007). The endocytic pathway of the host cell leads the virus particle (carried via a vesicle) to the viral genome's direct translation. The protein product of this translation is a polyprotein precursor that is co- and post- transnationally processed by cellular and viral proteases. The final products of this modification are mature structural proteins that will be used for the enveloping of replicated viral genomes (Moradpour, Penin & Rice, 2007).

The positive, single-stranded RNA genome, meanwhile, is complemented by a negative strand to form an RNA intermediate molecule (Bartenschlager & Lohmann, 2000). The negative strand then serves as a template for the production of a large amount of RNA plus strands (Bartenschlager & Lohmann, 2000).

The newly synthesized plus strands of RNA are then encapsidated by the viral proteins that were produced by the aforementioned polyprotein (Bartenschlager & Lohmann, 2000). The specific virion assembly and release mechanism of HCV is largely unknown in this stage of viral replication because systems which would allow for a biochemical amount of virus particles have been largely absent until quite recently (Maradpour, Penin & Rice, 2007). The enzyme NS2 and other non-structural proteins are hypothesized to be involved in this process, but no definitive research has been conducted to verify this. It is also presumed that virions are formed from budding through either the endoplasmic reticulum (ER) of the host-cell or an ER-derived compartment. Recent research has also proposed the possible link between lipoprotein metabolism and viral assembly (Maradpour, Penin, & Rice, 2007).

Section 8: 2.8.0 Antioxidants and Hepatoprotective Activity of P. niruri

The rationale behind our research is that *P. niruri* can be used in the treatment of HCV due to its well-documented activity against liver-related problems and diseases. A bulk of existing literature has focused on the characterization of the antioxidant properties of *P. niruri* and its resulting effects in the liver. In a 2007 study, researchers in India looked at the antioxidant activity of methanolic extracts of five plants from the *Phyllanthus* genus. While extracts from the five different *Phyllanthus* species exhibited different levels of antioxidant activity, their positive activity demonstrated the potential of plants from this genus to be used as natural sources of antioxidants (Buckwold, Beer, & Donis, 2003).

Subsection 1: 2.8.1 Overview of Antioxidant and Hepatoprotective Activity

Antioxidants are defined as molecules capable of inhibiting oxidation of other molecules. Oxidation reactions usually produce free radicals or reactive oxygen species (ROS) which, when produced within a cell, can cause damage or death to the cell. With too many of these molecules within a cell, oxidative stress becomes prevalent. Oxidative stress is simply an imbalance between pro-oxidants (harmful) and antioxidants which leads to oxidative damage. This damage usually manifests itself in the form of DNA, protein, or lipid alteration. Various degenerative diseases have been linked to oxidative stress, including cancer, atherosclerosis, and stomach ulcers, among others. However, it has been recently reported that cells of patients infected with HCV produce an unusual augmentation of oxidative stress due to an accumulation of iron within the liver.

Experimental models suggest that oxidative stress is involved in the pathogenesis of liver diseases (Bjelakovic, et al., 2011). Hepatitis C virus is a blood-borne pathogen that can cause serious liver diseases such as cirrhosis and hepatocellular carcinoma. However, the mechanism through which the virus induces pathogenesis remains unclear. A study conducted by de Mochel et al. (2010) concluded that hepatocyte proteins are likely to act as a persistent, endogenous source of ROS during HCV induced pathogenesis. Another study concluded that the core protein of HCV has been shown to induce the overproduction of ROS within the liver (Moriya et al. 2010). This has significant implications in terms of treatment options for HCV-infected cell populations. Since HCV infection induces oxidative stress and stifles antioxidant activation in the liver, this synergistically exacerbates the effects of oxidative stress on the liver and facilitates hepatocarcinogenesis.

Recent studies have been conducted to determine the efficacy of antioxidant treatments on HCV-infected cells and/or cells overrun by oxidative stress. The results of such studies have surprisingly revealed an array of conclusions. One experiment conducted by Nakamura et al. (2010) demonstrated that the antioxidant species resveratrol (RVT) actually enhanced HCV replication. This molecule is a natural type of phenol produced naturally by several types of plants. Although these findings are still debated and discussed, this elicits the notion that not all types of antioxidants may protect HCV-infected cells against oxidative stress. Bjelakovic et al. (2011) suggested the antioxidants beta-carotene, vitamin A, vitamin C, vitamin E, and selenium as potential chemopreventive agents. However, their results were equivocal; antioxidant supplements of these species had no significant effects on all-cause

mortality or liver-related mortality. On the other hand, Venturini et al. (2010) reported that untreated patients with chronic hepatitis C showed lower antioxidant capacity and higher levels of pro-oxidant activity. This implies that greater oxidative activity could play an important role in pathogenesis and its virulence in the evolution of HCV. Untreated patients could thus be treated with particular antioxidants to combat the accelerated onset of oxidative stress. Finally, a mitochondria-targeted antioxidant known as mitoquinone may decrease necroinflammation in the livers of chronic hepatitis C patients (Gane et al,. 2010). Since mitochondrial oxidative damage is responsible for other chronic liver diseases, further research in this particular direction is warranted.

Subsection 2: 2.8.2 Antioxidants in vitro studies

Fractions and isolates from *P. niruri* have been shown to have antioxidant effects in-vitro. Sabir and Rocha in 2008 demonstrated the antioxidative and hepatoprotective potential of aqueous extracts of *P. niruri*. They induced lipid peroxidation of rat liver cells in-vitro using iron and sodium nitroprusside (SNP). In cells treated with iron or SNP, they found that there was a respective 75% or 85% increase in the formation of therabarbarbituric acid reactive substance (TBARS), a marker of lipid peroxidation which indicates damage to DNA. *P. niruri* extracts showed protective activity against both iron peroxidation (84.7% protection) and SNP peroxidation (61% protection), preventing the increase in levels TBARS.

Chirdchupunseree and Pramyothin (2010) investigated the protective effect of phyllanthin on ethanol induced rat liver cell injury. Pretreatment of hepatocytes using

phyllanthin, a lignan that is a major constituent of the aqueous extract of *P. amarus*, yielded a hepatoprotective effect. The antioxidant effect of phyllanthin was demonstrated by a measured decrease in reactive oxygen species and a restoration of levels of key liver enzymes. However, when pretreatment with phyllanthin alone was compared with pretreatment using the aqueous extract, it was found that the aqueous extract yielded better enhancement, indicating that other phenolic compounds play a role in liver protection.

The protein fraction of *P. niruri* has also been investigated for its antioxidative effect. Sarkar and Sil (2007) investigated the efficacy of the protein isolate of *P. niruri* against thioacetamide toxicity. Thioacedamide leads to the production of reactive oxygen species (ROS) that increase the rate of lipid peroxidation in membranes and can cause the degeneration of DNA. Sarkar and Sil (2007) found that pretreatment of mice hepatocytes in vitro led to a dose dependent increase in cell viability. Interestingly, protein treatment without exposure to thioacetamide produced minimal effect, which suggests that protein isolates from *P. niruri* provides a protective effect rather than an enhancement effect.

Harish and Shivanandappa (2006) found that pretreating rat liver homogenates with crude aqueous and methanolic extracts of *P. niruri* from the fruits and leaves inhibited peroxidation due to iron and ascorbate. Since the leaf and fruit extracts contained differing concentrations of phenols, they expected one extract or the other to demonstrate more potent antioxidant activity. However, they found little correlation between the antioxidant activity and the phenolic content of the extracts, which was contradictory to the claim of Chirdchupunseree and Pramyothin (2010).

Subsection 3: 2.8.3 Antioxidants in vivo studies

In a study conducted in 2006, researchers investigated the hepatoprotective potential of *Phyllanthus* niruri against carbon tetrachloride (CCl4) induced liver damage. CCl4 is an environmental toxin that causes the formation of trichloromethyl and trichloromethyl peroxyl radicals. These radicals are responsible for lipid peroxidation that establishes a condition of oxidative stress. This also results in the generation of reactive oxygen species (ROS) that alters the enzymatic activity and thus cell function. These effects can eventually lead to liver injury, cirrhosis development and carcinogenesis.

Bhattacharjee and Sil (2006) investigated the antioxidant properties of *P. niruri* by using aqueous and methanolic extracts on mice dosed with CCl4. They also isolated protein fractions from the aqueous layer as additional methods of treatment. Serum levels of hepatic integrity related marker enzymes GPT and ALP were measured. Antioxidant enzymes, SOD and CAT, peptide antioxidant (GSH), and lipid perioxidation were also measured. Induced toxicity via CCl4 caused the following changes: increased levels of GTP and ALP, increase lipid peroxidation, reduced levels of SOD and CAT. It was determined that the aqueous fractions of *P. niruri* provided major hepatoprotective activity, and further investigation found that the protein isolates in the aqueous extract was partially responsible for this activity. Separate trials were run where *P. niruri* protein fraction was given to mice before CCl4 and after CCl4. This examined the preventative and curative role of the protein isolate. Results showed that both cases saw increased levels of GPT and ALP as well as restored SOD and CAT to normal levels. Since GSH is one of the major defense

against oxidative stress, treatment using *P. niruri* restored GSH levels and reduced lipid peroxidation. These results show that *P. niruri* can significantly rejuvenate antioxidative defense mechanisms in damaged liver (Bhattacharjee & Sil, 2006).

This study of *P. niruri* in reducing oxidative stress also extends to liver damage induced by a non-steroidal anti-inflammatory drug, nimesulide. In these in vivo studies, where aqueous extract and protein isolate were also used, researchers produced similar results. Nimesulide treatment caused severe deficits in hepatic GSH and antioxidant enzymes such as SOD and CAT. Just like the CCl4 study, treatment with *P. niruri* protein fraction caused recovery of the defense mechanisms(Chatterjee, Sarkar, & Sil, 2006).

Chapter 3: Methodology

Section 1: 3.1.0 Overview

To address our research questions, our methodology includes several interdisciplinary components. First, we germinated and grew our plants with the assistance of the US Botanic Garden (USBG) and the University of Maryland Greenhouse, with seeds obtained from a trusted online retailer recommended by the USBG. After harvesting and obtaining sufficient plant matter, we proceeded with chemical extractions. The specific plants used in the extractions were recorded and genetically characterized by sequencing genes identified as reliable plant DNA barcodes. The extracts were then analyzed in terms of the plant's anti-viral activity in cell culture systems. We quantified cell viability and viral load -- the number of RNA copies in the culture sample -- using real-time polymerase chain reaction (PCR). These parameters determined the efficacy of the treatment.

Section 2: 3.2.0 Plant Care and Observation

Due to the gracious help of the staff at the US Botanic Garden, *P. niruri* was successfully germinated and cultivated for our first and second plant harvests. The seeds were obtained from an online vendor, Trade Winds Fruit. The specific harvesting conditions are listed in Appendix A. On Day 181 are germination, the plants were moved to the University of Maryland Greenhouse. The specific growing conditions and how the greenhouse conditions were regulated are also listed in

Appendix A. The plants were harvested three times (Set 1, 2, and 3) and an alpha numeric labeling system was used to match up plants from different harvests to keep track of the different plant groups. Detailed observations regarding the plant's reproductive structure were described and photographed with a dissecting microscope.

Section 3: 3.3.0 DNA Barcoding

P. niruri is taxonomically confused with other related species (Duke, 2009). DNA barcoding was done on all 19 plant samples to ensure that subsequent experiments will be done on the same species of *Phyllanthus*. Protocols proposed by Kress and Erickson (2007), and the rbcL and matK gene combination proposed by Hollingsworth et al. (2009) were used because they have produced promising results with previous experiments.

The rbcL-a and matK sequences were the target regions that were amplified to generate a barcode for identifying *P. niruri*. We decided to focus our regions of interest to rbcL and matK because these genes, when combined in barcoding studies, showed 72% discrimination of land plant test cases (Hollingsworth et al., 2009). Additionally, the Consortium for the Barcode of Life reported in the scientific journal *Nature* that it had chosen rbcL and matK as the approved DNA barcoding regions for land plants. PCR, as suggested by Kress and Erickson (2007), was used to amplify the target sequences, thus giving us a larger concentration of DNA to work with (Appendix C).

Subsection 1: 3.3.1 Sample preparation

Fresh plant leaves were physically extracted and then lysed with the TissueLyser System (Qiagen). Lysed plant cells were subjected to DNA extraction via the DNeasy Plant Mini Kit (Qiagen, 2006). All 19 plant samples yield concentrated DNA at around 100ng/µl concentrations following extraction. DNA samples were stored in 1x TE (10mM Tris-Cl pH=8.0, 1mM EDTA pH=8.0) at -20°C (Appendix B).

Subsection 2: 3.3.2 Primer Design

We employed two primer sets published by Hollingsworth et al (2009) in order to barcode our samples (Table 2). Extracted DNA samples were subjected to PCR with the following conditions: 95°C for 4 min; [5 cycles: 94°C for 30 sec ; 55°C for 1 min ; 72°C for 1 min]; [30 cycles: 94°C for 30 sec; 54°C for 1 min; 72°C for 1 min]; 72°C for 10 min; 10°C at ∞.

Author	Primer name	Region - Binding	Primer sequence (5'-3')
Erickson	rbcLa_f	rbcL - Forward	ATGTCACCACAAACAGAGACTAAAGC
	rbcL_rev	rbcL - Reverse	GTAAAATCAAGTCCACCRCG
Ki-Joong Kim	3F_KIM f	matk - Forward	CGTACAGTACTTTTGTGTTTACGAG
	1R_KIM r	matk - Reverse	ACCCAGTCCATCTGGAAATCTTGGTTC

Table 1: Primer sets used in our study published by Hollingsworth et al (2009).

Subsection 3: 3.3.3 Sequencing

The PCR products were sequenced by GENEWIZ Inc. Both forward and reverse sequences were sequenced to ensure accuracy. Raw sequences were cleaned with CodonCode to trim ends and locate inaccurate sequence peaks.

Subsection 4: 3.3.4 Sequence Analysis

Sequences were aligned with MUSCLE (EMBL-EBI) with default settings. Our sequencing data was analyzed through two methods: 1) neighbor-joining tree method and 2) BLAST distance method.

Trees were created with SeaView (Pole Bioinformatique Lyonnais) through a neighbor-joining method (Saitou & Nei, 1987). We pulled 61 additional matK *Phyllanthus* sequences and 36 additional rbcL sequences from the National Center for Biotechnology Information (NCBI) database. These sequences were uploaded to

SeaView to reconstruct our phylogenetic tree. Default settings were followed with 1000 bootstrap replicates and Kimura 2-Parameter distances were established.

The distance method was done through performing BLAST nucleotide searches through NBCI. We BLASTed all cleaned sequences and analyzed the top hits. We took into consideration the max identity percentage and max score of each hit, and subsequently classified our samples based on these criteria. All specimens of *P. niruri* used in the biochemical analysis and extractions were compared to this standard.

Section 4: 3.4.0 Extraction

Initially we sought to test the *Phyllanthus niruri* extracts on HIV/HCV co-infection on different cell lines as a proof of principle that the extract could be applied for this particular condition. As a result, we based our extraction protocol on a methanolic extraction outlined in Qian-Cutrone et al. (1996) to isolate a compound from *Phyllanthus niruri* known as nirurside. This compound was found to be active against HIV reverse transcriptase, and previous literature review had indicated *Phyllanthus niruri* was considered to be phytochemically active in the liver, held a history of activity against hepatitis B, and was long used in Brazilian and Ayurvedic medicine. This provided reason for us to choose this particular extraction protocol, because it may have had the potential to be effective on both diseases. However, due to time constraints, we only focused on analyzing extracts on HCV but still used the niruriside extraction protocol as our base.

We modified the Qian-Cutrone extraction protocol to maximize the chances of obtaining active fractions and to better suit our purposes and approach, the details of which will be explained later. In addition, while only the methanolic and chloroform extract were collected in order to isolate niruriside, all fractions from the extraction protocol were saved and analyzed. This meant that both the aqueous and organic fractions were saved, since it was shown by Sabir and Rocha (2008) that aqueous extractions contained phytochemically active compounds which had *in vitro* antioxidant and *in vivo* hepatoprotective activity against paracetamol-induced liver damage in mice.

To begin the extraction protocol, the various *Phyllanthus* were first dried, separated and labeled according to their identities. For the extractions, between 5-20 grams of dried plant matter (anywhere from 10-15 plants), mainly derived from the leaves and the stems, were massed. The alpha numeric code as well as the mass from each individual plant used was also recorded. In addition, some of the plant matter was reserved for future DNA extraction, PCR, and barcoding. All of the plants used for the extraction were from group A, which comprised plants grown and identified as *P. niruri*. Dried plant matter was ground using a mortar and pestle with 5 milliliters of methanol. Since the bark is also considered to be a good source of phytochemicals, the stems were also cut up with scissors to aid efficient mashing. This contrasts with the original protocol which used 100 grams of powdered leaves only; a smaller mass of leaves were used because the cell studies portion did not require such a high concentration of the compound.

After maceration, the plant matter was refluxed with around 50 mL of 100% methanol for 30 minutes. The plant matter was then filtered out and the dark green liquid supernatant was collected in a separate beaker. The plant matter was then refluxed again with 50 mL of 80% methanol, and then with 70% methanol, with the supernatant filtered between each reflux.

The extracts were all combined and then heated on low using a hot plate in order to evaporate the solvent (Extract 1). Extracts were carefully monitored to ensure that they did not burn. This yielded about 350 mg of dark green residue. This residue was then re-dissolved in approximately 50 ml of 90% methanol, and then sonicated to facilitate dissolving. This solution was then partitioned against around 35-45 mL of pure hexane three times. The upper hexane layer was combined with all hexane extracts and evaporated on low heat to yield an oily dark green residue (Extract 2). In addition, there was a precipitate which formed in the interface between hexane and water which was collected and evaporated as well (Extract 2b). The amount of solvent used was scaled down proportionally according to the plant mass used, and a few milliliters more or less solvent was added at times depending on how well the compounds of the extract were dissolving.

The lower aqueous layer was then diluted to 75% methanol with 10 mL of distilled water. This solution was then partitioned against 35-45 mL of CCl_4 three times. The lower CCl_4 layers were combined and evaporated to yield a dark yellow powder (Extract 3). Then the aqueous layer was further diluted to 65% methanol solution with 10 mL of distilled water. This was then partitioned three times against 35-45 mL of $CHCl_3$. The upper methanol layer was evaporated to yield a dark green

powder (Extract 4). The lower $CHCl_3$ layer was combined and evaporated to yield a light green residue (Extract 5). For future studies, small amounts of each extract in their respective solvent were transferred to micro centrifuge tubes for evaporation. This yielded around 5-15 mg of dry extract per each tube which could be re-dissolved for future studies. These five crude extracts were later prepared tested on cell cultures to evaluate their antiviral activities.

Section 5: 3.5.0 Biochemical Analysis and HPLC analysis of fractions

After testing all of these extracts on the cells for their activity, our goal was to analyze the composition of the extracts that reduced the amount of virus and/or increased cell survival. However, even though we know what kinds of compounds may possibly be in the plant based on literature search, we could not definitively say which *specific* compounds are present in the extracts. Moreover, we had no way of knowing which compounds of the original extract moved to each partition, potentially concentrating them and increasing their activity or visa versa.

Thus, we made an HPLC profile for each extract. Through the HPLC, we could compare the phytochemical content across extracts by determining which peaks are present and absent in each fraction and how they change in intensity. Theoretically, all the peaks present in the un-partitioned methanol extract should represent the total compounds which may be present in varying extents in the other extracts. Thus, by comparing the chromatograms with each other it should be possible to determine what peaks are present in each fraction, and later correlate this to activity. Since most of the active compounds contain aromatic rings, we will monitor

for absorbance in the aromatic region at 280 nm. While the HPLC method will not allow for direct identification of the present compounds, it should at least give us a general idea of the distribution of compounds in each fraction.

We also used analytical HPLC to provide UV spectra to compare unpurified fractions to pure fractions. The UV spectra will serve as a map of the contents in the whole sample, enabling us to determine which fractions contain active compounds or whether multiple solvent extracts contain the same active compound (Appendix G). However, again, we could not definitively identify specific compounds.

For this analysis we used the high performance liquid chromatograph LC-20AT model equipped with a UV-(SPD-20A) detector, degasser DGU-20As, a low-pressure gradient unit LC-20AT, a CBM-20A communications bus module, and a C-18 Luna 5u reverse phase column, 150x4.6 mm by Phenomenex. We also employed a Shimadzu Gas Chromatograph GC-2010 equipped with a fused-silica wall-coated capillary column and a Shimadzu AOC-20*i* Auto-Injector controlled by GC Solutions software. The GC portion is then coupled with a Shimadzu 2010S Mass Spectrometer. To begin, first a small amount of sample is vaporized in the injector port and mixed with the inert carrier gas He before being introduced to the column. The column employs liquid stationary phase immobilized on a silica support which is useable between around 30-300°C and interacts with the volatilized analytes, having a higher affinity towards non-polar compound.

In order to give a more detailed breakdown the contents of the extract we ran the extracts diluted in DCM (dichloromethane) through the GCMS (gas chromatography-mass spectroscopy). This allowed us to directly determine the mass

and potentially the identity of the compounds present in these fractions. Thus we may be able to obtain the structure and track the contents of any phytochemically active elements of each extract.

The HPLC extracts were prepared at a concentration of around 1mg/mL in DMSO. Since the actual contents of the extracts are unknown, it will not be possible to determine an exact concentration. To ensure dissolution, the samples will be sonicated and filtered to pellet any undissolved components. Then the sample were diluted in water to 0.5 mg/mL and injected onto the HPLC. The solvent composition was isocratic at 25:75 acetonitrile:water and a flow rate of 1.00 mL/min for 10 minutes for each sample run. The un-partitioned methanol extract was run first in order to determine whether or not any modification needs to be made to the sample procedure. After each run, all of the peaks and their respective areas and retention times were determined and cataloged. The detector was set at 254 nm and 280 nm.

For GCMS, the samples were prepared in a similar fashion but instead using $CH2Cl_2$ as the solvent. Again, sonication and filtration of the samples was done to ensure no precipitate was injected on the column. We ran on a temperature ramp from 100-250°C for fifteen minutes, with the last five minutes holding at 250°C. The sample chromatograms will then be run through the library data to search for any matching compounds at various peaks.

By repeatedly refining the extracts, their chemical compositions will become more defined, thus enabling us to better pinpoint the antiviral activity of specific compounds and identify their chemical structures. If an active compound can be isolated, its structure can be determined through NMR and IR spectroscopy, but this

likely will be reserved for future studies as it does not fit into the scope of this project (Lambert, Shurvell, Lightner, & Cooks, 1998).

Section 6: 3.6.0 Extract Preparation

Plant extracts from five separate extractions, labeled Extract 1 to 5, were dissolved in dimethyl-sulfoxide (DMSO) using a sonicator in an ultrasonic bath. Sonicators break intermolecular reactions between chemicals, which helped break up the sticky extract product in DMSO. The weight of the extract prior to dissolution was measured so that the final concentration could be calculated in micrograms per milliliter. Because the extract was so sticky, it was crucial that all of the extract was dissolved to be able to determine the final concentration accurately, which is why the sonicator was used.

Overall, each extract dissolved in 10-12 mL of DMSO, depending on their ability to be dissolved in DMSO (Table 3). The dissolved extracts were vortexed vigorously, divided into 1 mL aliquots in microcentrifuge tubes, and frozen at -80C. To prepare them for application to cell culture, each extract aliquot was defrosted and dissolved in enough DMEM (Dulbecco's Modified Eagle's Medium) to make 1000 μg/mL stock solution. Six more tenfold dilutions were made from this stock solution with DMEM, including 100 μg/mL, 10 μg/mL, 1 μg/mL, 0.1 μg/mL, 0.01 μg/mL, 0.001 μg/mL.

Extract	**Mass of extract (grams)**	**Mass of extract (micrograms**	**Volume of DMSO solvent added to extract (ml)**	**Concentration after dissolve in DMSO** (μg/mL)
1	0.108	1.08×10^5	10	1.08×10^4
2	0.035	3.5×10^4	10	3.5×10^3
3	0.170	1.70×10^5	10	1.70×10^4
4	0.400	4.00×10^5	10	4.00×10^4
5	0.140	1.40×10^5	12	1.17×10^4

Table 2: Extracts concentrations after diluted in DMSO.

Section 7: 3.7.0 Cell activities studies objective

Using *in vitro* cell culture systems, tested the efficacy of different *P. niruri* extracts on HCV. Specifically, our experiment hopes to elucidate the effect, if any, of *P. niruri* plant extracts on HCV viral load and cell toxicity. By quantifying this effect, we can make conclusions regarding therapeutic potential of the plant, extract fractions most likely to contain active compounds, and whether this plant warrants future research to investigate its medicinal properties.

Subsection 1: 3.7.1 Cell Line

We use the Huh7.5 liver cancer cell line, which begin as uninfected liver cells. In order to generate an infectious cell line, the cells must transfected with a J6/JFH1 genotype 2a virus strain in which JFH stands for Japanese fulminant hepatitis. Once transfected with J6/JFH1 genotype 2a virus, the infected cells are referred to by the

name of the virus, or J6JFH cells. Although the liver is not normally in a cancerous state when infected with HCV, this system is the one of the few in existence that can produce an infectious system. Past systems only had success infecting the cells once, but infected cells could not continue the infection and spread the virus to other cells (Bartenschlager & Sparacio, 2007). Furthermore, because the cell study is *in vitro*, application of the results to therapy may prove difficult. Cells often behave differently *in vivo* as opposed to *in vitro* due to biochemical pathways that cannot be properly represented in cell culture media. Similarly, wild-type HCV strains have not been as well experimented as lab-grown HCV strains so that therapies for *in vitro* strains may not be as effective to *in vivo* strains.

Subsection 2: 3.7.2 Kinetics experiment overview

We conducted a time-course (kinetics) experiment, in which we studied the change in virus levels and cell death over time due to our plant extract. The experiment ran for four days, and we took samples of viability and viral load on 24 hours, 48 hours, and 96 hours. On each day, we determined the viral load (how much virus is present in the cell) and cell viability (how many cells are alive). Viral load was quantified by extracting RNA from the cell-secreted supernatant. Quantitative real-time PCR (qT-PCR) estimated viral sample concentrations through comparison with a standard curve, which contains a set of viral-RNA dilutions in which the number of viral RNA copies is known. As for quantifying cell viability, we used an MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay, which applies a dye metabolized only by living cells. When

cells take up the dye, the medium changes color, and the number of viable cells becomes directly proportional to the degree of color change. This color change was measured by absorbance optical density values from a spectrophotometer (Promega, 2009).

If our extracts were active against the virus, we expected that the viral load would decrease and cell viability would remain high. In order words, the amount of virus in the cell should decrease and the cells should stay alive. An extract may be toxic to the cell, so its anti-viral properties must be balanced with its toxicity to the cell. We tested various concentrations of our extract, based on mass of plant sample per volume of solvent. The initial extracts were dissolved in dimethyl sulfoxide and then diluted in DMEM with 1% penicillin/streptomycin and 10% fetal bovine serum before application to cell culture. Our positive control in the experiment was an agent known to decrease viral load without killing the cells. It is currently prescribed in treatment and known as interferon-alpha. Our negative control had no treatment, which served to confirm that our positive control worked and to provide a baseline to analyze the effect of our treatment. Our second negative control was the DMSO and medium, in order to account for the fact that the observed effect may be due to the DMSO solvent itself rather than the extracts. Through this experiment, we found the optimal concentration for different extracts that is effective in both keeping the cells alive and lowering the viral levels.

Subsection 3: 3.7.3 MTS Assay

To measure cell viability, a MTS assay from Promega was used (CellTiter 96® AQueous One Solution Cell Proliferation Assay System). MTS assay was chosen over MTT assay because MTS produced a soluble compound when reduced by cells. Thus, no other reagent was needed to dissolve the crystals, like those formed in the MTT assay.

Ten 96 well culture plates were used to analyze cell viability at 24 hours, 48 hours, and 96 hours. Three plates were designated as the control plates, one for each time point. The control plates contained DMSO diluted in medium, interferon (10 IU/ml) in medium, or medium over infected and uninfected cells. DMSO diluted medium to act as the DMSO vehicle control. This is to account for the fact that DMSO itself as a solvent for the extracts may have an effect on the viral load or cell viability. By measuring the effect of DMSO and using it as a baseline in data analysis, we could distinguish the effect of the extract from the effect of DMSO. Since not all extracts had the same concentration of DMSO solvent, the levels of DMSO in the DMSO vehicle control corresponded to highest DMSO level concentrations used in extracts. For example, Extract 2 had the highest amount of DMSO, with 28.5 µl of DMSO dissolved in 971.5 µl of medium used to make 100 µg/mL concentration of extract. Similarly, the DMSO control had 28.5 µl of DMSO in 971.5 µl of medium. At 10 µg/mL, the amount of DMSO was diluted tenfold in the extract, and this was also done in the DMSO vehicle control. Essentially for each

extract concentration, a DMSO control corresponded to it with proportional levels of DMSO.

For each time point, two plates were designated as the experimental plates, which contained plant extract dissolved in medium over infected or uninfected cells. Each concentration for each extract was plated in triplicates. One plate was designated for the standard curve, in which cell of known quantities were plated (40,000 cells, 20,000 cells, 10,000 cells, 5,000 cells, 2,500 cells, and 1,250 cells). By having a standard curve, an equation could be developed to link the absorbance values to the number of cells, so that the absorbance values from our experimental samples could be calculated into the number of cells. The absorbance of these cells was measured at day 0. A table of each plate layout is included in Appendix K.

Roughly 5000 cells in 100 µl medium were plated in each well at time zero. After 24 hours, the cells would have attached to the bottom of the plate. At this time, medium was removed and 100 µl of the appropriate amount of extract or controls were added. To test if extracts and interferon had any significant effects together, this 100 µl was divided so 50 µl of extract was combined with 50 µl of 10 IU/ml interferon alpha for concentrations ranging tenfold from 0.001 µg/mL 10 µg/mL.

Quantifying cell viability was done by adding 20 µl of Owen's Reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to each well 3 hours before their designated time for absorbance measurements. This compound is bioreduced to soluble formazan by metabolically active cells. Thus, the reagent was

added at 21 hours for the day 1 plate, 45 hours for the day 2 plates, and 93 hours for the day 4 plates. Owen's reagent was allowed to incubate with the cells for 3 hours to allow color change from yellow to purple to take place. For complete protocol on this cell viability assay, refer to Appendix B (CellTiter 96® AQueous One Solution Cell Proliferation Assay)

Reaction product in each 96 well plate was measured using a microplate reader set at 490nm on a SpectraMax Plus384 Plate reader. The microplate reader was blanked using a solution of medium and Owen's Reagent. Then, each well plate was placed in the reader, which measures the absorbance of 490nm light through each well. Light, at the given wavelength, was shined through each well from the top. A sensor beneath the wells measure the percent of light transmitted through the wells. Data from the microplate reader was analyzed using a SoftMax Pro software. Using this data, a graph could be produced based on Beer's Law (optical density= ecl) Where optical density represents the absorbance, 'e' is the extinction coefficient, 'c' is the concentration of the solution being measured, and 'l' is the path length of the light through the well.

Subsection 4: 3.7.4 Viral Load Assay

A 6-well plate was used for each of the 5 separate plant extractions as well as a control plate with only DMSO and infected cells with J6/JFH genotype 2a virus. On each plate, 10^5 cells were loaded per well. Then, each well was loaded with a

different amount of plant extract: 10 µg/mL, 1µg/mL, 100ng/ml, 10ng/ml, 1ng/ml, and 10 IU/ml IFN-α. The highest concentration (100 µg/mL) was not used because the amount of DMSO needed to dissolve the product was too high, and from viability assays, we found that DMSO had a toxic effect on cells. In addition, concentrations that high are unrealistic for clinical application. The IFN-α well served as a positive control because this compound is known to suppress viral RNA replication. The 5 extractions, their concentrations, and the control samples were plated in triplicates, so that samples could be collected at 24 hours, 48 hours, and 96 hours. By samples, we refer to the collection of supernatant or cell medium and the collection of cellular RNA. All samples were stored at -80 C conditions until their viral RNA could be extracted at a later time.

After viral RNA from supernatant was collected, it was extracted and purified using QIAamp MinElute Spin Kit and following the protocol outlined in QIAamp Viral RNA mini Handbook pages 23-26 following the spin protocol. However, the amount of cells used the extraction was triple of the amount stated in the handbook. The handbook states to use 140 µl of sample, so our tripled amount was 420 µl. The amount of buffer used was also tripled as recommended in the handbook protocol. This tripling of the protocol is required in order to get enough viral RNA. Viral RNA was eluted in 60 ul of Buffer AVE.

Supernatant Viral RNA was then quantified through quantitative real-time PCR, in which a fluorescent probe recognizes the viral RNA sequence. Through

TaqMan® One-Step RT-PCR Master Mix Reagents Kit, viral RNA extracted from the supernatant is converted to complementary DNA (cDNA). The probe attaches to the cDNA viral RNA sequence, and upon the sequence of the cDNA's complementary DNA strand, Taq polymerase will run into the probe. The probe will be cleaved, causing it to fluoresce. The amount of light released from the probe corresponds to the concentration of viral RNA, and compared to a standard curve of known RNA copies, the amount of viral RNA can be interpolated (Appendix B).

Section 8: 3.8.0 Data Analysis methods for cell data

In order to analyze the cell data, the number of viral copies per cell had to be calculated because it was later discovered that the extracts influenced the number of cells significantly. Due to this effect, simply comparing the number of viral load between extracts and control would not be an accurate representation of the effect of the extract. To calculate the number of viral copies per cell, the MTS data was integrated with the viral load data.

5000 cells were plated in the MTS assay and 100,000 cells were used in the viral load assay, so the changes in the number of cells observed in the MTS assay was multiplied by a factor 20 in order to estimate the possible number of cells that would be present in the viral load assay. For example, if the number of cells had changed from 5000 to 6000 cells, in the viral load assay, we can estimate the the cells would behave the same way, and the number of cells would change from 100,000 to 120,000 cells. This calculation served as the number of cells.

For the viral load, based on the quantitative real-time PCR protocol used, 8 ul of the extracted viral RNA from each extract was measured for viral load. These 8 ul came from an elution of 60 µl. Each 60 µl elution came from 420 ul of viral supernatant, which came from 1.5 ml of viral supernatant in the viral load assay. Once the viral load was scaled up to account for all of these factors, the total amount of viral RNA in 1.5 ml in the total number of cells could be calculated by simple division.

Sample	A: Number of cells measured by MTS assay	B: Number of cells in viral load assay = A*20	C: Viral load in 8 µl measured from real-time PCR	D: Viral load in 60 µl = C * 60/8	E: Viral load in 1.5 mL supernatent = D*1.5/4.2	F: Viral load/cell = E/B
Extract 1 0.001 µg/mL	4596.1	91,922.5	175,543	1,316,572.5	4,702,044.6	51.1

Table 3: Data calculations for viral load per cell

The viral load per cell was calculated three times due to the fact that triplicates were used in the experiments. After the above calculations were completed, the percent difference in viral copies/cell was calculated via the percent change formula ((experimental value - control value)/(control value) * 100%). The standard error was also calculated and used in all data sets. The student t-test was used throughout for tests for significance between different samples (alpha = 0.05).

Chapter 4: Results

Section 1: 4.1.0 Plant Structure

High resolution pictures were taken with an AxioCamHR microscope camera (Carl Zeiss) to elucidate the physical structure of *P. niruri*. We were able to capture structures of the male flower, female flower, cross-sections of the capsule, and leaves. We used these images to compare the plants grown for this study with the documented taxonomy of known *P. niruri*.

Overall, we observed from maturation of the capsule that male and female flowers were coupled, and more mature capsules were located closer to the stem as shown in Figure 1(e). When the capsule reached a certain maturation point, the male flower was no longer present. In addition, as the plant matured the stem became woodier, especially towards the root end of the plant. Female flowers were observed to have five or six petals, with a single flower in the center that consisted of a pedicel, three-parted ovary (Figure 1c, f, g). The petals in female flowers were white with a dark green stripe down the center with a lighter green stripe on either side of the dark green stripe; the flower petals came to a triangular point, in comparison to male flowers which were rounded (Figure 1a). The capsule was very small, approximately 2 mm in length. A few male flowers were observed to have five petals although it is known that six petals were also possible. The characteristics of the male flowers were hard to elucidate due its small size, even under the microscope; it was observed

that the stamens are yellow. Three leaves were measured for the length, and they were about 5-6 mm (Figure 1d).

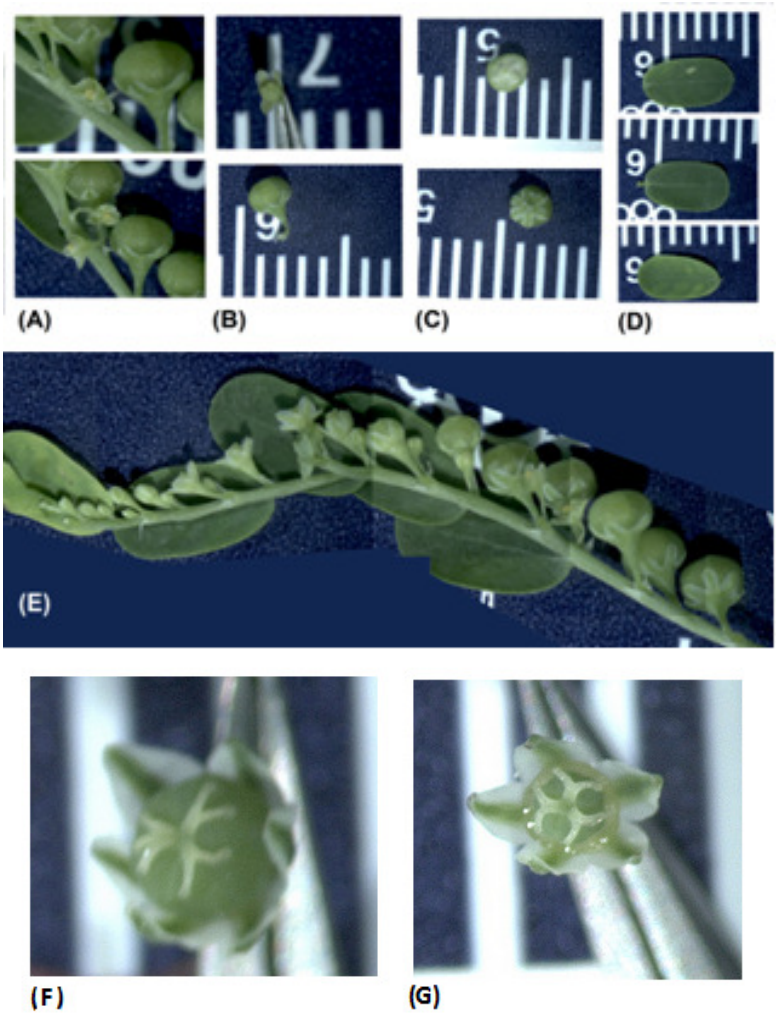


Figure 1: Major physical features of *P. niruri* leaf and reproductive structure were photographed and documented with an AxioCamHR microscope camera (Carl Zeiss), in order to provide physical descriptions linked to the DNA barcode. The following structures were identified (a) male flower (b) female flower (c) capsule cross-section (d) leaves (e) branch of *P. niruri* (f) female flower with six petals and (g) female flower with five petals.

Section 2: 4.2.0 DNA Barcoding

Subsection 1: 4.2.1 PCR and alignment

Both matk and rbcl primer sets were successful in the amplification of extracted DNA samples. All 19 samples produced distinct bands at 1kb for the matk primer set. Similarly, 18 of the 19 samples produced distinct bands at 700bp for the rbcl primer set. Only sample number 49 failed to amplify rbcL with the standardized PCR conditions.

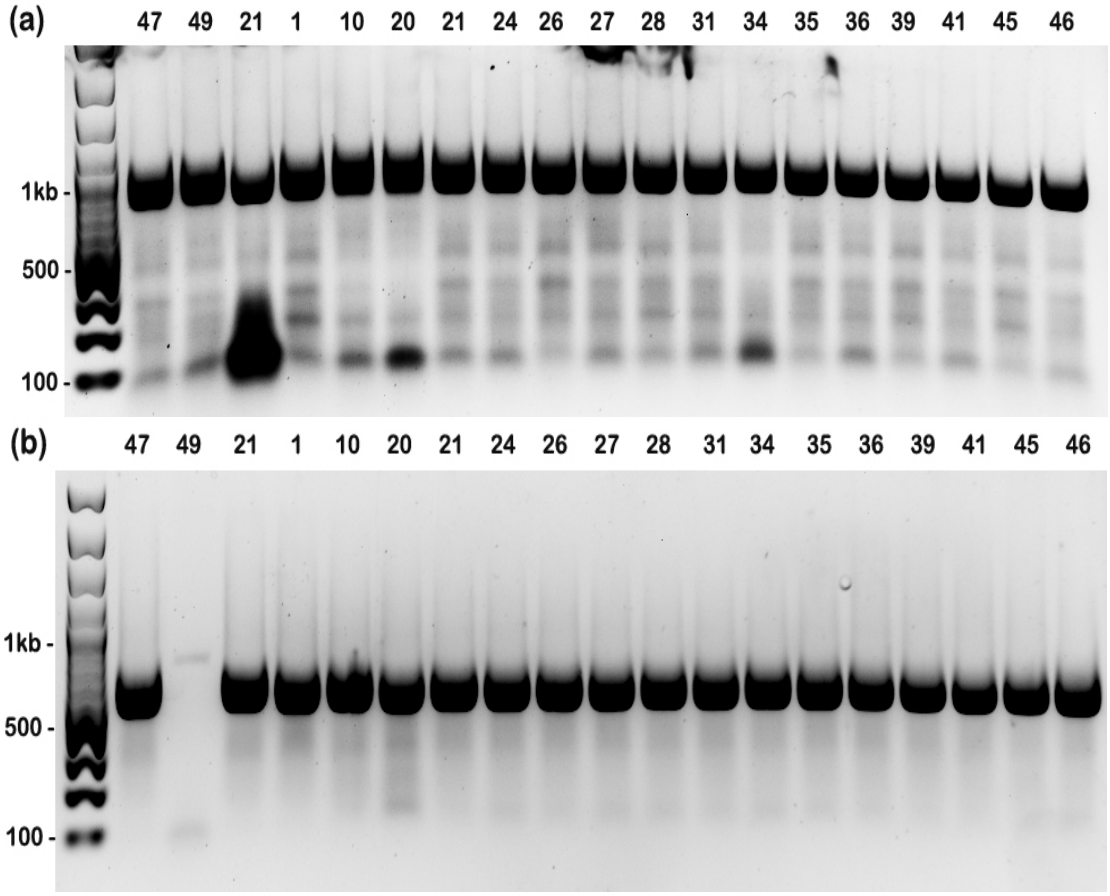


Figure 2: Successful PCR amplification of 19 *P. niruri* gDNA samples with (a) matk and (b) rbcl primer sets. Sample 49 in (b) failed to be amplified.

Sample 49 failed at other test conditions for rbcl, thus we proceeded with tree analysis with only matk region for this sample (Figure 2b). Matk sequences revealed 5 segregating sites while there were no segregating sites in the rbcL sequences.

Subsection 2: 4.2.2 Tree-based assignment shows matches with P. niruri and P. amarus

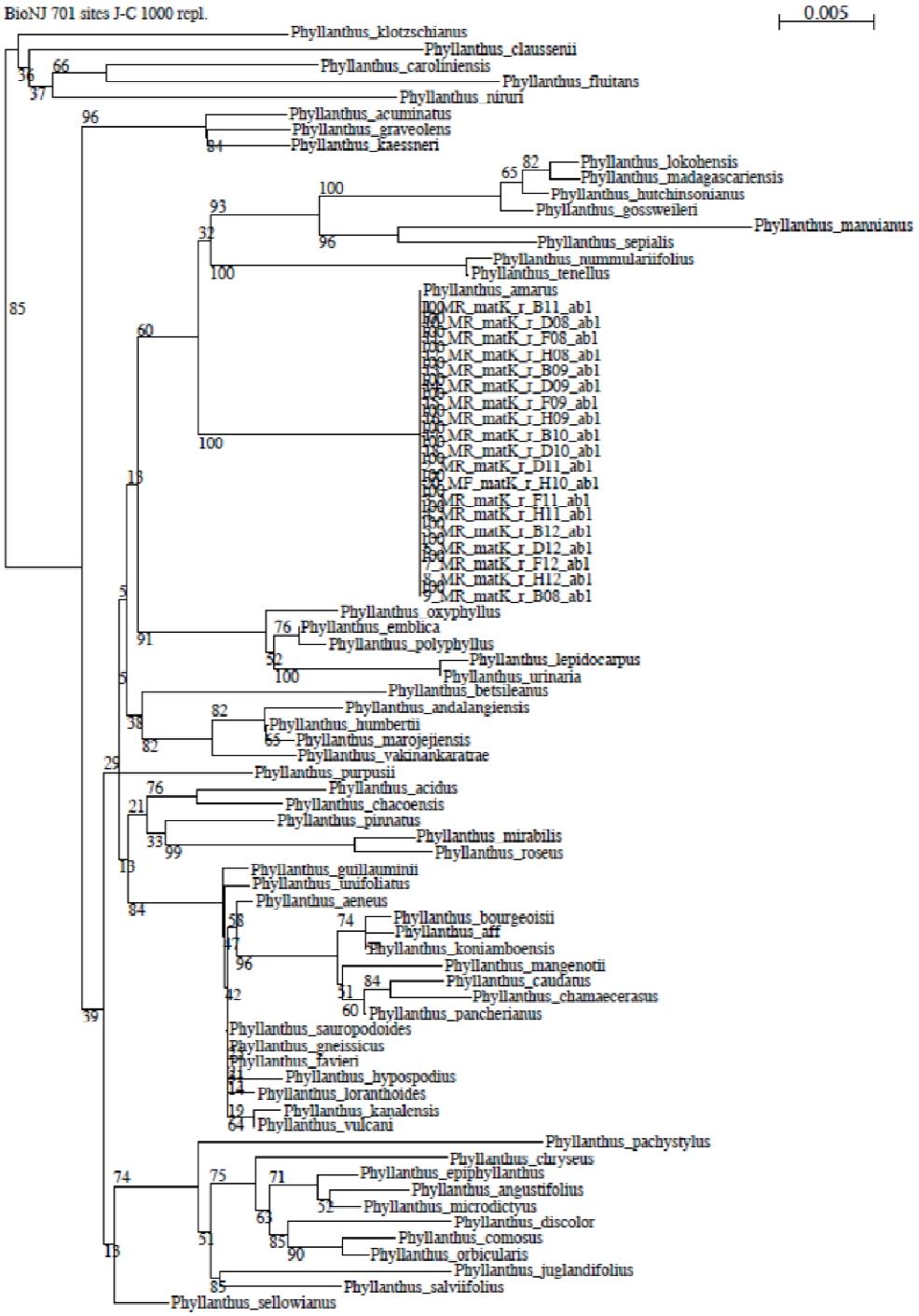


Figure 3: Phylogenetic tree of Phyllanthus matK sequences show that all 19 samples most closely align with P. amarus. The tree was created with SeaView with 1000 bootstrap replicates and default distance settings.

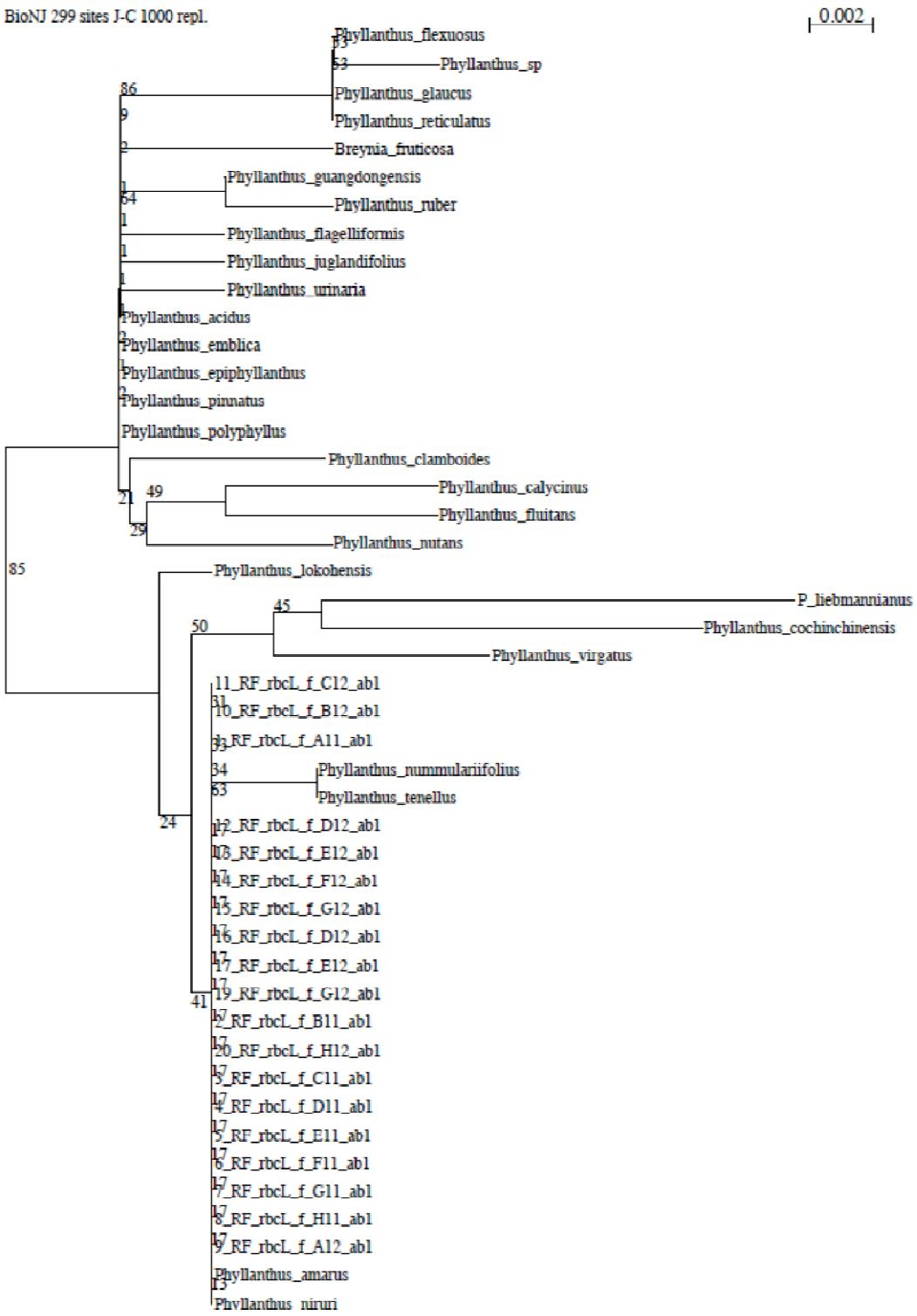


Figure 4: Phylogenetic tree of Phyllanthus rbcl sequences show that all 19 samples closely align with Phyllanthus amarus and P.niruri. The next closest neighbors are P. nummulariifolius and P. tenellus. The tree was created with SeaView with 1000 bootstrap replicates and default settings.

All 19 matk sequences were mapped most closely with P. amarus (Figure 3). P. niruri was mapped on a completely separate branch rooted at the base of the tree. The next closest related ancestors to our samples are P. tenellus and P. nummulariifolius. P. niruri was mapped most closely to P. fliutans, P. caroliniensis, P. claussenii, and P. klotzschianus.

The 19 rbcL sequences were mapped to P. amarus and P. niruri (Figure 4). The next closest tree neighbors are P. nummulariifolius and P. tenellus. These were done with 1000 bootstrap replicates using default distance methods.

Subsection 3: 4.2.3 Distance-based assignment shows matches with P. niruri and P. amarus

The BLAST (NCBI) distance based approached showed our rbcL sequences to be most similar to P. niruri voucher PS0215MT01 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene from the chloroplast (Figure 5). A neighbor joining method was used to create the subsequent tree of the top BLAST hits with a max sequence difference of 0.75. The next closest neighbor of our samples as mapped by rbcL is Phyllanthus amarus ribulose-1,5 -bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast.

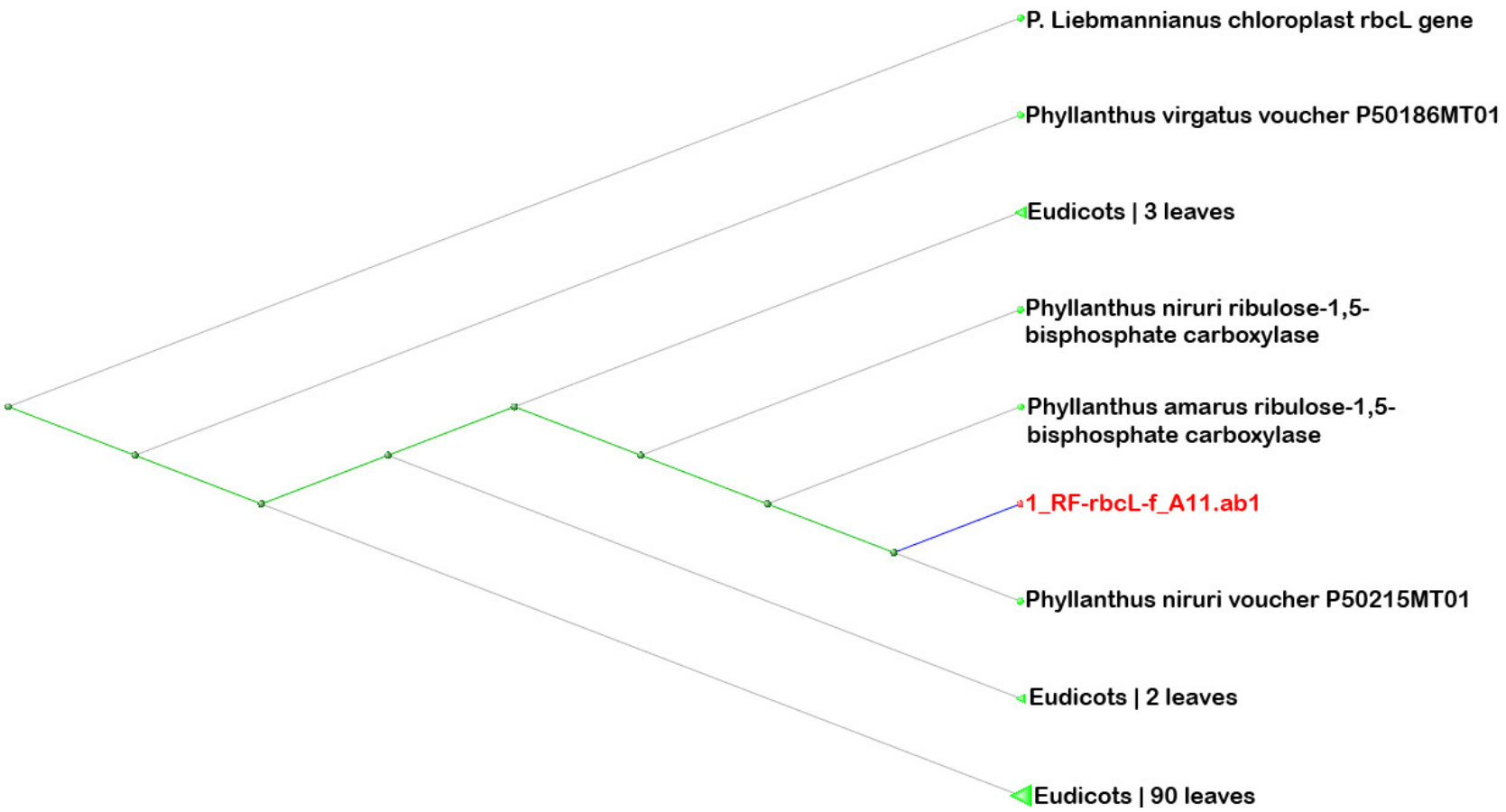


Figure 5: Neighbor-joining tree of Phyllanthus rbcL sequences shows that all 19 samples most closely align with P. niruri voucher PS0215MT01 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast. The next closest neighbor is Phyllanthus amarus ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast. The tree was generated through BLAST (NCBI) pairwise alignments using a max sequence difference of 0.75.

The matK sequences were found to be most similar to P. amarus maturase K (matK) gene, partial cds (Figure 6). The next closest neighbor is P. amarus voucher Philcox 7651 trnK gene, partial sequence and matK gene, complete cds. No P. niruri hits were observed with the default BLAST settings. P. niruri hits were not observed with a maximum score higher than 1363 or over 96% maximum identity.

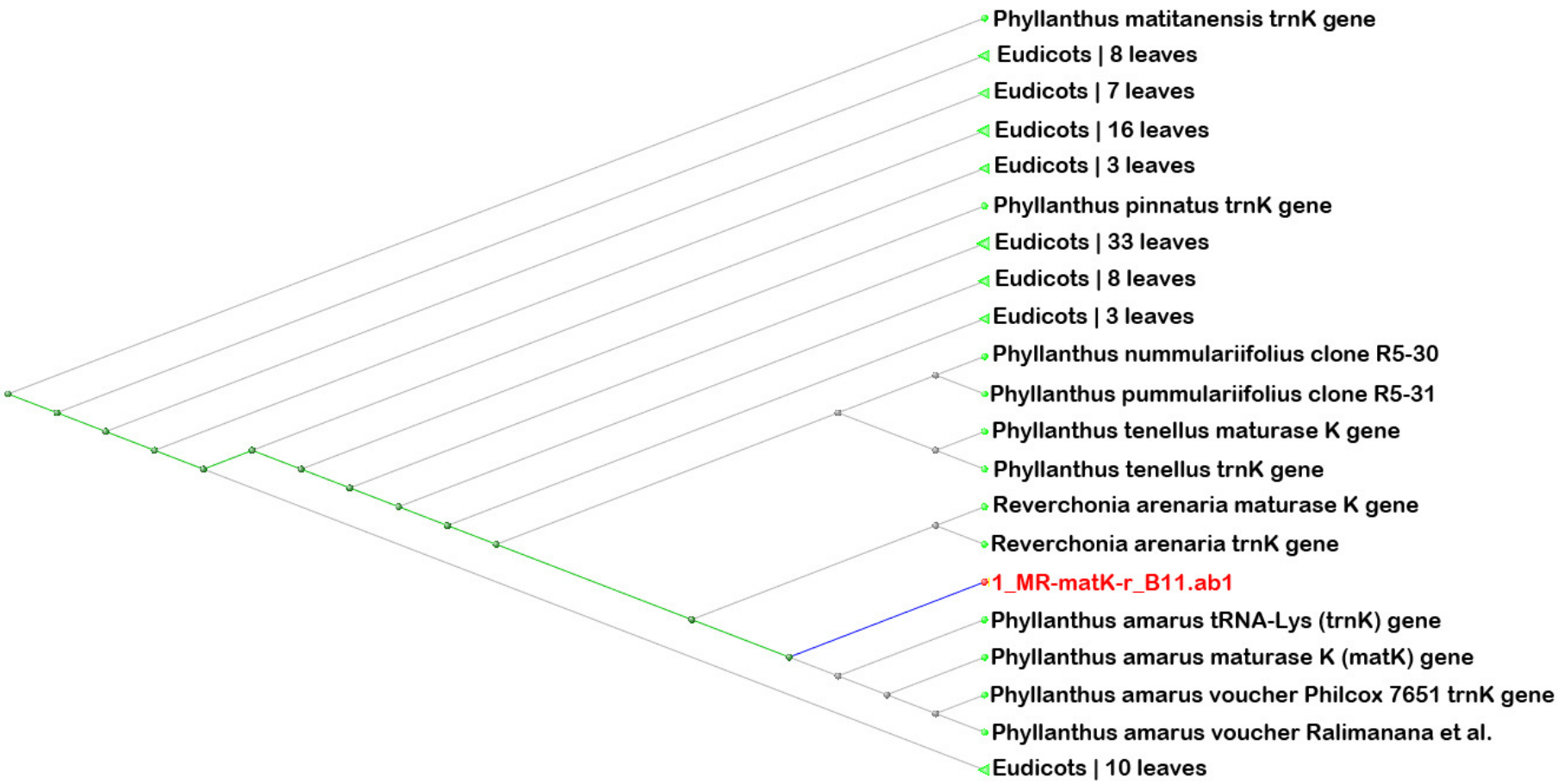


Figure 6: Neighbor-joining tree of Phyllanthus matK sequences show that all 19 samples most closely align with P. amarus maturase K (matK) gene, partial cds; plastid. The next closest neighbor is P. amarus voucher Philcox 7651 trnK gene, partial sequence and matK gene, complete cds; chloroplast. The tree was generated through BLAST (NCBI) pairwise alignments using a max sequence difference of 0.75.

Both the tree-based and distance-based methods revealed similar results as reflected by their phylogenetic trees.

Section 3: 4.3.0 Extractions and HPLC analysis of fractions

The HPLC data chromatograms are included in Appendix L. It provided interesting information about the composition of each fraction. Table 4a and 4b summarizes the results found.

Table 4a : Below is the summarized data for the HPLC reports. Each set of peak areas are grouped relative to retention times (Rt) approximately. The table is split into two to fit.

Detector (nm)	Sam.	Rt (min)	Area	Rt (min)	Area	Rt (min)	Area	Rt (min)	Area	Rt (min)	Area
254	1	1.136	42847	2.156	69826			2.756	325108		
280											
254	2			2.284	14494			2.752	13210		
280				2.279	30314						
254	3					2.486	12929				
280						2.481	14888				
254	4					2.631	455804	2.81	862804		
280						2.633	374112	2.829	91643	3.037	802205
254	5					2.631	49069	2.97	37647	3.092	18730
280											

Table 4b:

Dect. (nm)	S	Rt (min)	Area	Rt (min)	Area	Rt (min)	Area	Rt (min)	Area
254	1	3.189	264974	3.59	206447				
280									
254	2					3.829	350523	8.324	7141653
280		3.256	353447					8.338	165890
254	3			3.632	113080	3.971	46013		
280		3.235	105624	3.566	143199				
254	4								
280									
254	5	3.226	108684						
280		3.302	478741						

Each extract displayed a different chromatographic profile and overall peak distribution. The information yielded from the detector at 280 nm showed that there are very few compounds with aromatic systems in our samples, as these peaks were minimal. Most informative was the detector data at 254 nm which gave a distinct profile of each extract. It is important to note that similar retention times across peaks likely means that the compound contained in that peak was present in multiple extracts. This is likely due to a lack of preference for one solvent over the other during the partitioning process. In addition, the retention times, which should be identical for each compound, were not entirely conserved. This could mean that the

peaks were of different compounds, or simply that the large amount of compounds shifted the over all retention time.

Unfortunately, the composition of the extracts was not ideal for running on GCMS and we were unable to get any significant data from this trial. This was either due to the fact that CH2Cl2 could not dissolve enough sample to merit detection, or more likely that the boiling points of most of the compounds were above the level where they could be separated on GCMS. This data was not included in the report.

Section 4: 4.4.0 The effect of P. niruri extracts on HCV viral copies per cell

In order to analyze the effects of *P. niruri* extracts on hepatitis C replication, the viral copies per cell was calculated for HCV-infected cells with treatment and without treatment. Because we found that the number of cells changed due to the extract (the data for which will be shown in Figure 11a-c), the viral load per cell was a more useful indicator to compare samples than the viral load alone. Based on the percent change in the viral copies per cell between treated and untreated cells, it was possible to identify the most effective concentrations for Extracts 1 to 5 and compare their effect with that of standard interferon, which is displayed in Figure 7. This percent change is also referred to as the percent suppression.

The suppression of HCV over time does not change very much for Extract 1 at 0.001 μg/mL and Extract 2 at 0.001 μg/mL. As seen in Figure 7, suppression from Extract 1 at 0.001 μg/mL ranges from 67.4% to 79.4% from 24 to 96 hours, and while Extract 2 at 0.001 μg/mL experiences a wider range from 67.0-88.5%, it is not much larger. Other extracts, however, show different behavior. Both Extract 3 at 1 μg/mL

and Extract 4 at 10 μg/mL exhibit strong suppression early that does not last over time, but the slope of this decline is different between these two extracts. Extract 3 experiences a slower initial decline, changing from 70.3% to 66.7% suppression from 24 to 48 hours. However, by 96 hours, the suppression effect dropped by half. Extract 4 at 10 μg/mL exhibits the strongest suppression of HCV viral copies per cell at 24 hours reaching 99.35% suppression, but the effect drops off more quickly. Suppression is 59% at 48 hours and later falls to 42% by 96 hours. Very high suppression is also seen in extract 5 at 10 μg/mL and standard interferon alpha (10 IU/ml), but their behavior varies from Extracts 3 and 4. In these samples, the effect of suppression is not observed initially, showing 8-12% suppression at 24 hours, but it increases over time, reaching 99.94% and 93.70% suppression, respectively, by 96 hours.

At 24 hours, Extract 1, Extract 2, and Extract 3 do not demonstrate significant changes in HCV suppression among concentrations. The suppression for these extracts narrowly ranges from 62.7%-82.7% consistently across these extracts, which may indicate that a wider range of concentrations may be need to have been tested in order for a behavior pattern to be more clearly qualified. For extract 4, at 10 μg/mL, there is initial strong suppression at 99.35%, but this effect drops drastically to 15.3% as the concentration decreases to 1 μg/mL before increasing back up to 60-70% suppression. This could be an indication of solubility issues, a fluke, or a error in the instrument while reading this time point. However, as concentrations decreased, activity returned in a dose-dependent manner. For Extract 5, the suppression varies

widely, such that no pattern can be reasonably demonstrated, which may indicate particular flaws with reading this particular time point (Figure 7) .

At 48 hours, in general, the effect across concentrations of all extracts is between 53.9%-87.2%, except for Extract 5 0.001 μg/mL. This concentration had 36.2% suppression against HCV although there is large variation in this data point. There seems to be little variation in the activity across concentrations, and although extracts do experience dips in activity across concentrations at certain points, the error bars overlap too closely to be able to say a discernible difference exists between samples. Extract 1 dips in its effect on HCV viral suppression at 0.01 μg/mL, which like the extracts at 24 hours, may be an indication of solubility problems Extract 5 at 0.001 μg/mL has significant error in its reading, which may be due to larger variation in the number of cells for the calculation of number of viral copies per cell.

At 96 hours, across concentrations of each extract, we begin to see the behavior of the extracts more clearly elucidated over time. For Extract 1, in general, the suppression had not changed significantly from 48 hours or 24 hours, staying within approximately 60-80% suppression. This may indicate that the effect may be longer-lasting. At 0.01 μg/mL and 0.1 μg/mL for Extract 1, there is a dip in the effectiveness of the extract. This may be an indication of solubility issues, a fluke, or an error in the instrument while reading this time point, since as concentrations increased, activity returned in a dose-dependent manner. For Extract 2, similar behavior occurs at 1 μg/mL, but it exhibits the opposite behavior as Extract 1. As concentrations decreased, the suppression increased again in a dose-dependent manner. For Extracts 3 and 4, the suppression of HCV was strong initially, but over

time by 96 hours, the effect was not sustained. At 96 hours, these two extracts are the weakest of all five extracts to suppress the number of HCV viral copies per cell, and nearly half the strength of Extract 5. For Extract 5, it displays the most clear dose dependent effect that as concentrations decreased, the antiviral effect of the extracts increased from near complete suppression at 10 μg/mL to no suppression at 8% at 0.001 μg/mL.

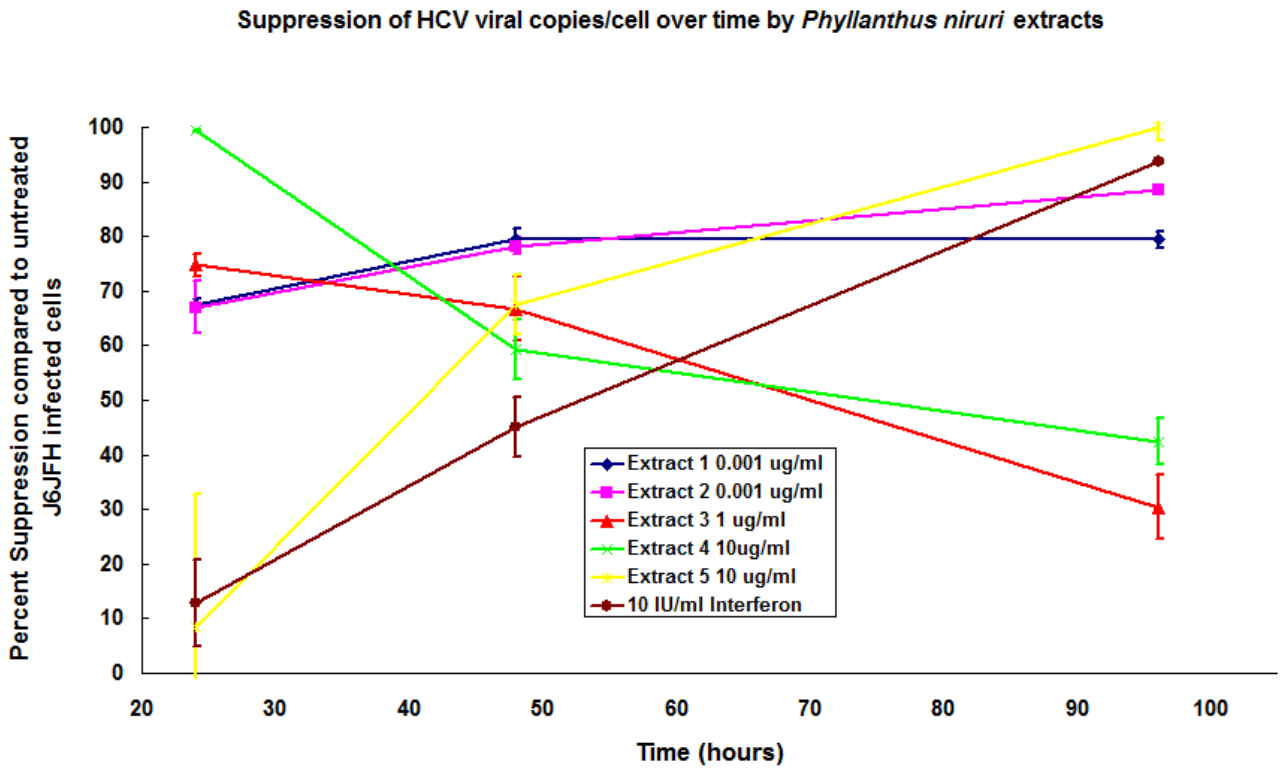


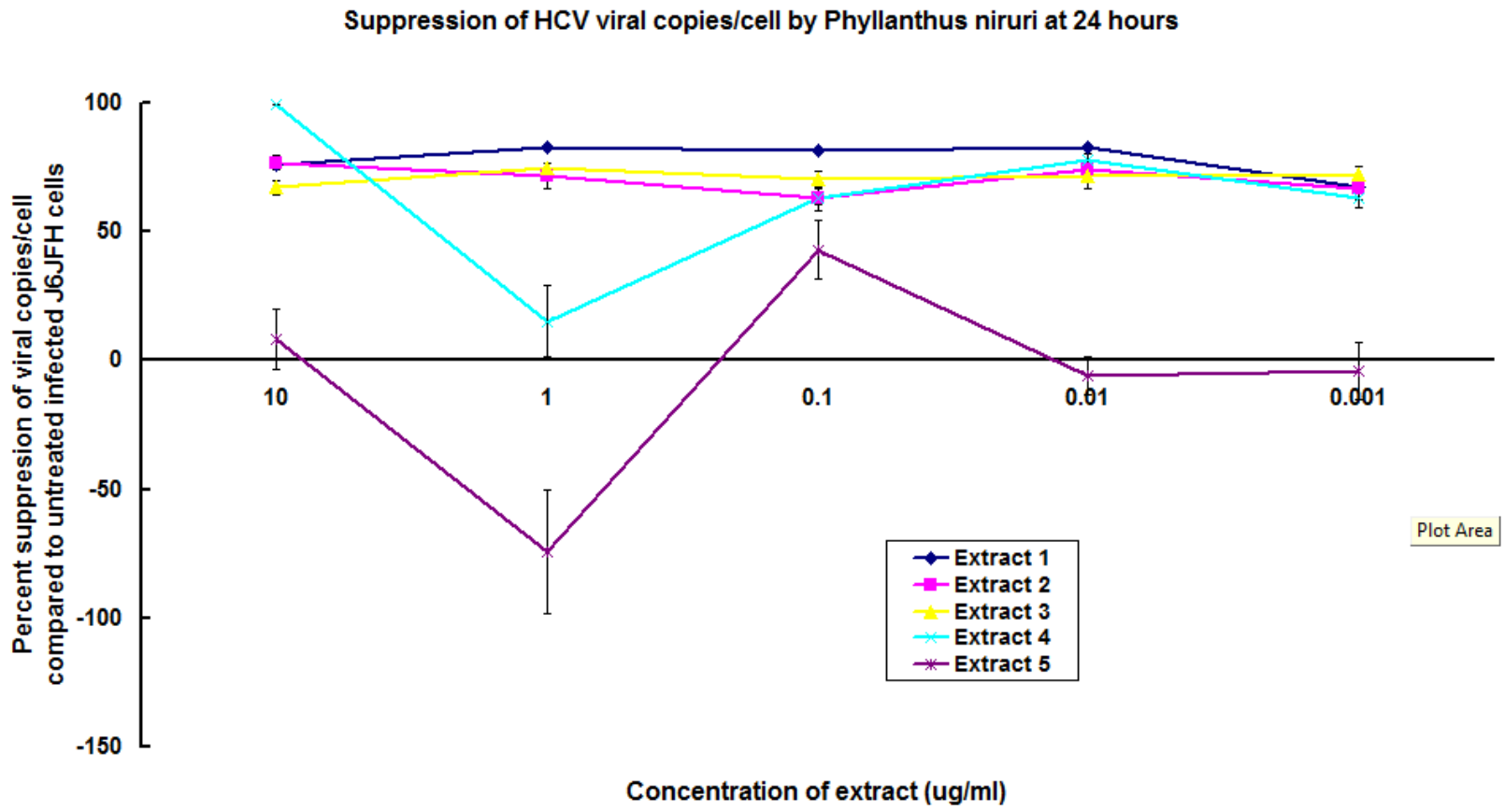
Figure 7: HCV-infected Huh7.5 cells were treated with *P. niruri* extracts for four days. The number of cells and viral load was measured at 24, 48, and 96 hours with MTS assay and quantitative real-time PCR respectively, after which the viral copies per cell was calculated. Based on the percent change in the viral copies per cell between the extract-treated cells and untreated infected cells, the following results were obtained for extracts 1-5 and standard interferon. Only the most effective concentrations from each extract are displayed. Different extracts differ in their effect on the number of HCV viral copies per cell.

Subsection 1: 4.4.1 The effect of P. niruri extracts on HCV viral copies per cell over concentrations at each time point

It was also possible to look at the changes in suppression over extract concentrations at each time point, as seen in Figure 8a-c. At 24 hours, Extract 1, Extract 2, and Extract 3 do not demonstrate significant changes in HCV suppression among concentrations. The suppression has a narrow range from 62.7%-82.7% across these extracts, which may indicate that a wider range of concentrations should have been tested in order for a more significant dose-dependent pattern to emerge. For Extract 4, at 10 μg/mL, there was initial strong suppression at 99.35%, but this effect drops drastically to 15.3% as the concentration decreases to 1 μg/ml before increasing back up to 60-70% suppression. This could be an indication of solubility issues, a fluke, or an error in the instrument while reading this time point. However, as concentrations decreases, activity returns in a dose-dependent manner. For Extract 5, the suppression varies widely, such that no pattern can be reasonably demonstrated, which may indicate particular flaws with reading this particular time point.

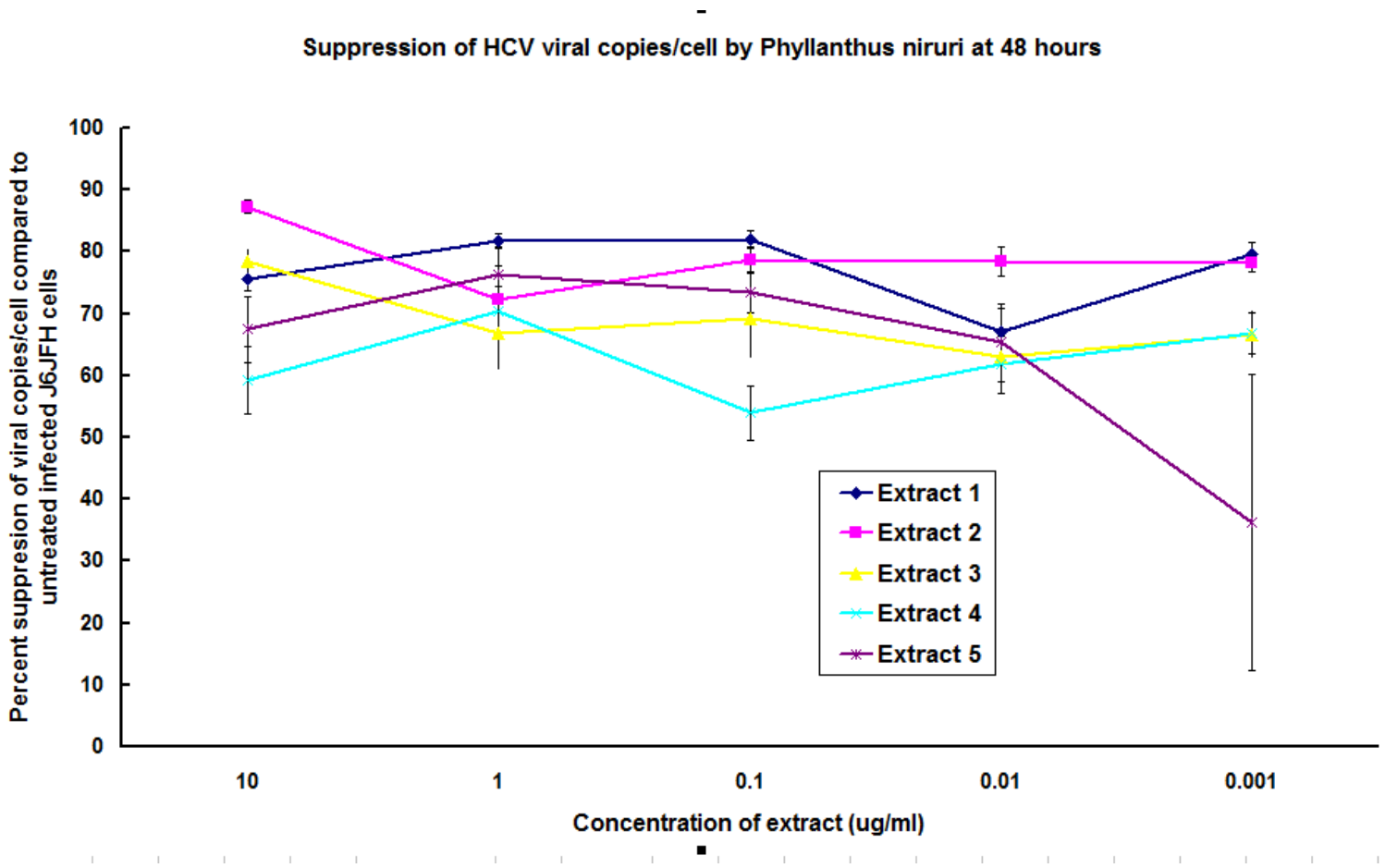
At 48 hours (Figure 8b), there seems to be little variation in the activity across concentrations. Although extracts do experience dips in activity across concentrations at certain points, the error bars overlap too closely to be able to say a discernible difference exists between samples. Extract 1 dips in its effect on HCV viral suppression at 0.01 μg/mL, which like the extracts at 24 hours, may be an indication of solubility problems Extract 5 at 0.001 μg/mL has significant error in its reading, which may be due to larger variation in the number of cells for the calculation of number of viral copies per cell.

At 96 hours (Figure 8c), across concentrations of each extract, we begin to see the behavior of the extracts more clearly elucidates over time. For Extract 1, in general, the suppression had not changed significantly from 48 hours or 24 hours, which may indicate the the effect may be longer-lasting. At 0.01 μg/mL and 0.1 μg/mL for Extract 1, there is a dip in the effectiveness of the extract. Since concentrations increase as activity returned in a dose-dependent manner, this may be an indication of solubility issues or an error in the instrument while reading this time point. For Extract 2, similar behavior occurs at 1 μg/mL, but it exhibits the opposite behavior as Extract 1. As concentrations decreases, the suppression increases again. For Extracts 3 and 4, the suppression of HCV was strong initially, but over time by 96 hours, as indicated here, the effect was not sustained. At 96 hours, these two extracts are the weakest of all five extracts to suppress the number of HCV viral copies per cell, and nearly half that of Extract 5. For Extract 5, it displays the most clear dose dependent effect, that as concentrations decreases, the antiviral effect of the extracts increases from near complete suppression to no suppression at 8% at 0.001 μg/mL.



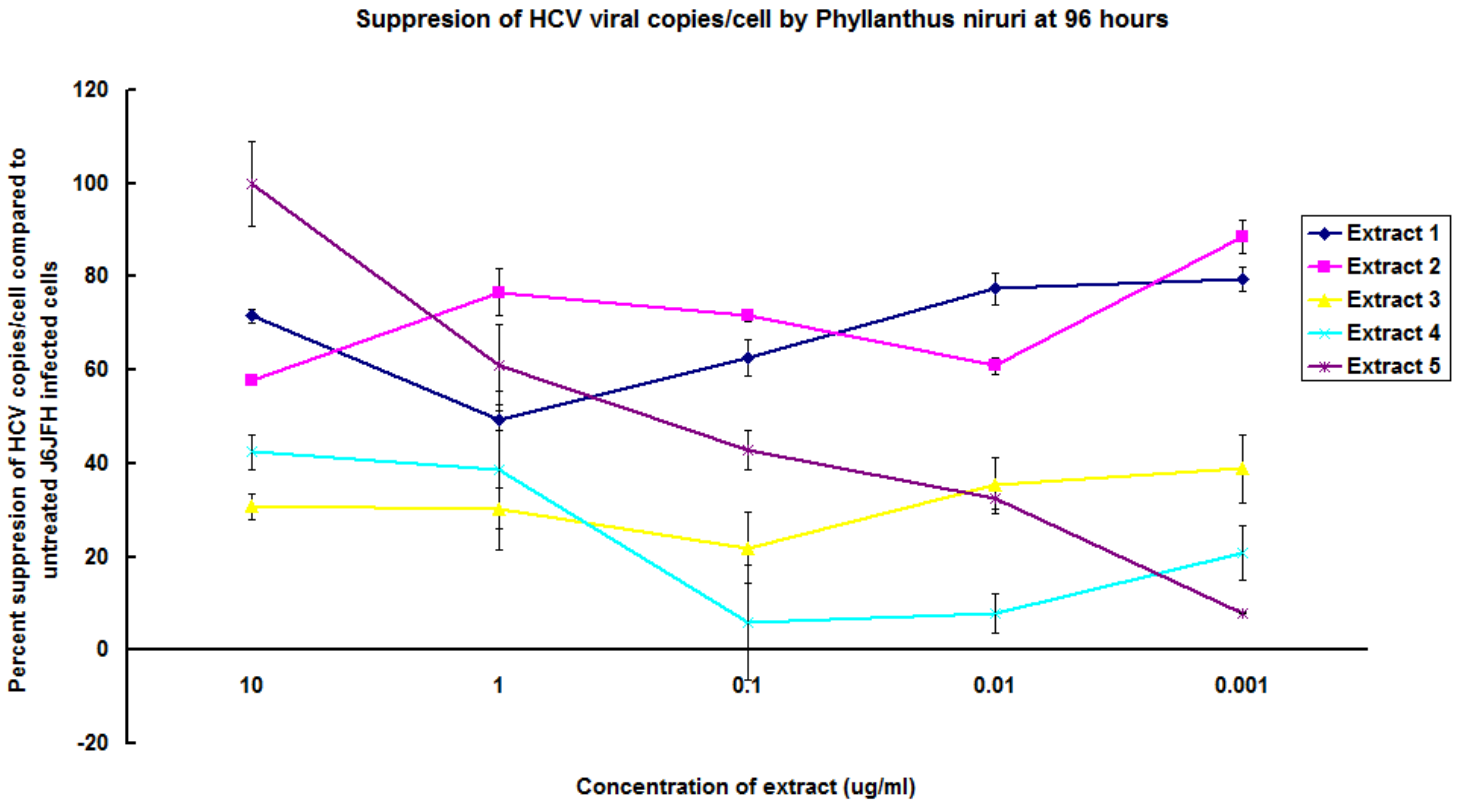
a.

Figure 8a-c: ***Suppression of HCV viral copies/cell by P. niruri at different time points***. The suppression of viral copies per cell changes over concentrations at a single time point. The behavior of different extracts is presented at 24 hours (8a), 48 hours (8b), 96 hours (8c).



b.

Figure 8a-c*: Suppression of HCV viral copies/cell by P. niruri at different time points.* The suppression of viral copies per cell changes over concentrations at a single time point. The behavior of different extracts is presented at 24 hours (8a), 48 hours (8b), 96 hours (8c).



c.

Figure 8a-c*: Suppression of HCV viral copies/cell by P. niruri at different time points.* The suppression of viral copies per cell changes over concentrations at a single time point. The behavior of different extracts is presented at 24 hours (8a), 48 hours (8b), 96 hours (8c).

Subsection 2: 4.4.2 The effect of P. niruri on Huh7.5 cells survival

In order to assess the toxicity of the extracts on cell cultures, all concentrations of extract are tested on Huh7.5 uninfected liver cancer cells. The data for toxicity came from the MTS assay. Two trials of the MTS assay are done. In the first trial, the MTS assay experimental design failed to include the standard curve at day 0 and other controls, namely purely untreated cells (Huh7.5 cells and J6JFH cells) with medium only. However, this first trial allows for assessment of what effects the extracts have with respect to DMSO, and a change in the number of cells was not expected. These initial results indicated that there was a change in the cell number from the DMSO control, and that there was enough reason to believe *Phyllanthus* showing activity and not the DMSO. Thus, the second trial conducted includes untreated controls.

Based on the second trial of the MTS assay, we find that the effect of *P. niruri* extracts on Huh7.5 cells survival is not significant ($p > 0.05$ for all concentrations tested) at 24 hours to increase the viability of Huh7.5 cells, except for Extract 1 and lower concentrations (0.001 μg/mL and 0.01 μg/mL) of Extract 2 after 24 hours where $p < 0.05$. Results from the five extracts on uninfected Huh7.5 cell cultures for 24 hours are shown in Figure 9a. All extracts, with the exception of Extract 4, have an increase in percent growth for treated Huh7.5 cells versus untreated cells. Extract 4 showed a decrease in cell viability that increases gradually as concentrations increase. Overall, at 24 hours incubation, the percent change for each extract stays relatively consistent over the range of concentrations.

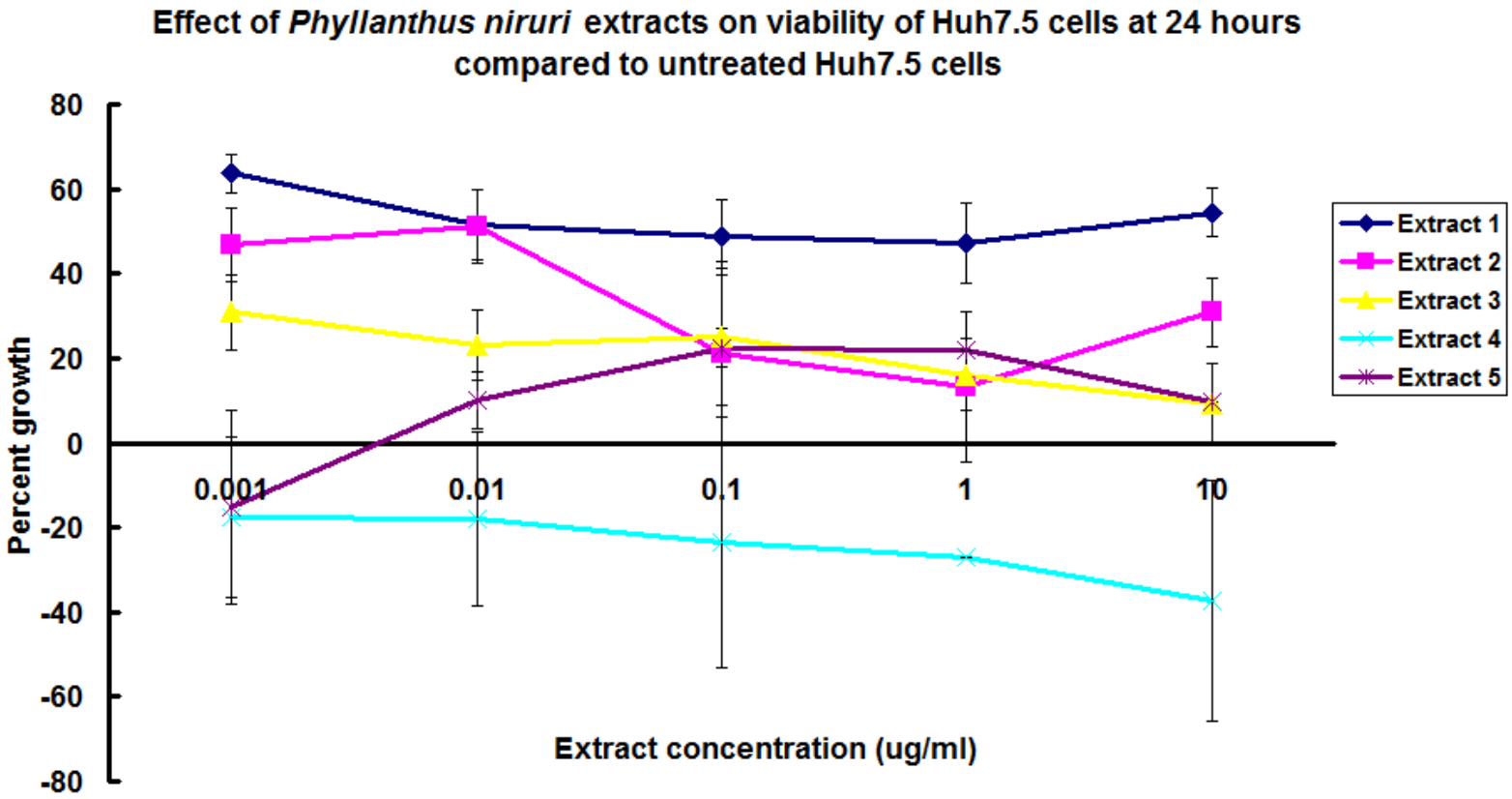
For 48 hours, the ability of extracts to increase the viability of Huh7.5 cell survival varied greatly. Extracts 1, 2, and 3 showed no great significance as the p-

value varies spontaneously across different concentrations. Extract 4 significantly increases cell viability in concentrations of 1 μg/mL, 10 μg/mL, and 100 μg/mL. Four out of six concentrations tested for Extract 5 are significant according to $p < 0.05$. In Figure 9 below, Extract 1 and 4 have positive percent increase for treated cells versus untreated cells. Extracts 2 and 3 show no negative changes in cell viability at low concentrations while at high concentrations, there is a negative percent change in treated cells with respect to non-treated cells. Extract 5 shows a negative percent change at low concentrations of 0.001 μg/mL and 0.01 μg/mL, while positive percent changes in cell growth in concentrations of 0.1 μg/mL and greater.

In the 96-hour Huh7.5 cell cultures, Extracts 1 and 2 significantly increase cell viability at high concentrations (10 μg/mL) for Extract 1 and 10 μg/mL for Extract 2. Extract 4 was significant in affecting cell viability for 0.001 μg/mL as well as 10 μg/mL and 100 μg/mL. Extract 5 only proved significant at 10 μg/mL. All other concentrations for extracts were found to be insignificant in regards of cell viability. In the 96-hour Huh7.5 graph (Figure 9c), all extracts had a positive effect on cell growth in treated cell cultures in concentrations of 10μg/mL and lower. At 100μg/mL, all extracts significantly decreases cell growth to various degrees, possibly due to the higher levels of DMSO. Extract 2 and 3 had the largest effect on cell growth at 100μg/mL, with a decrease in cell growth in treated vs. untreated difference of 83% and 82% respectively.

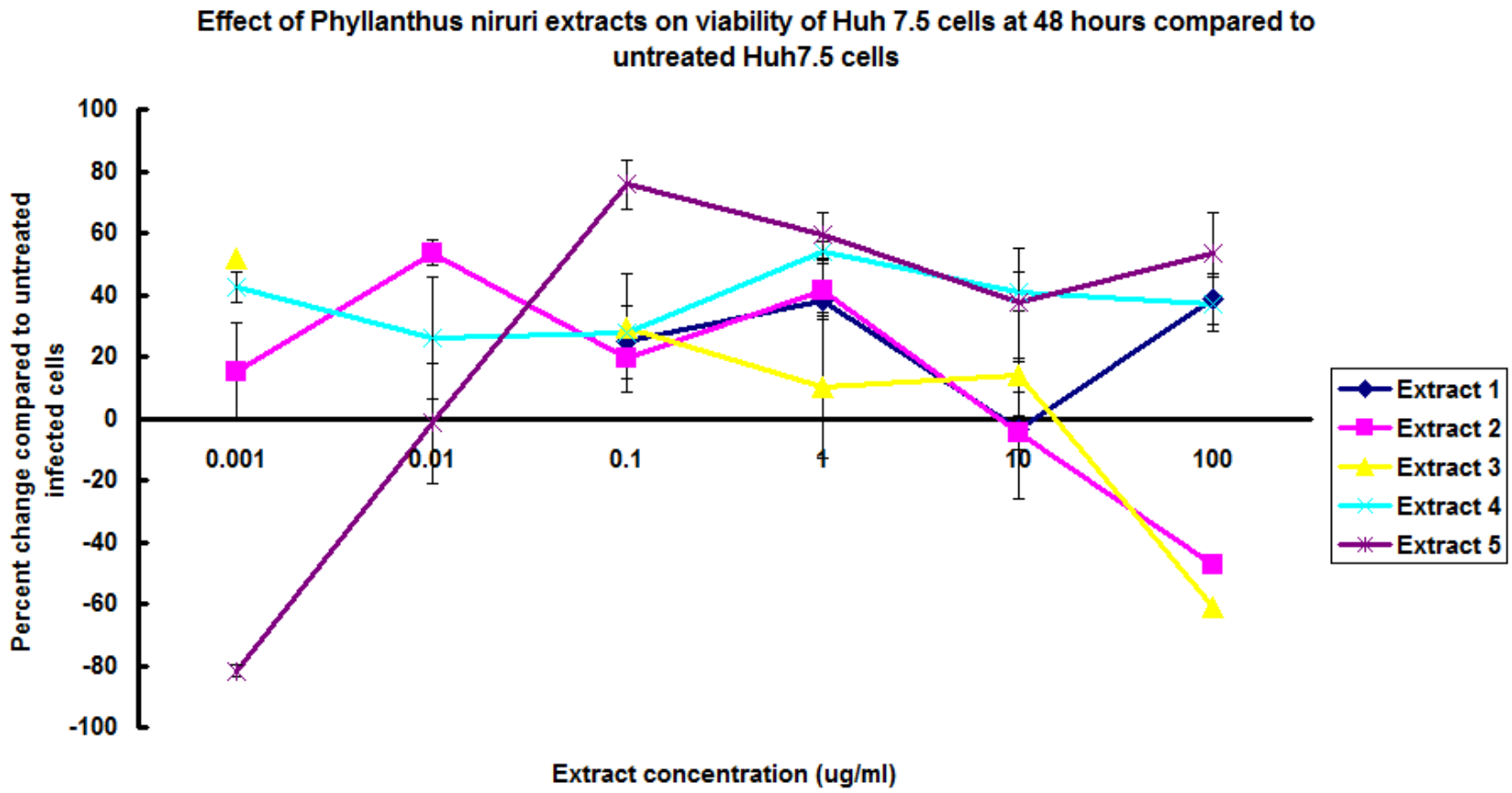
Overall, over time, the effect of extracts on cells did not seem to have a negative impact on the number of cells. Either the number of cells was often comparable or significantly greater under the influence of the extracts, but often not

less than the number of cells in the untreated sample, unless the extract was at very high concentrations with higher levels of DMSO. This relationship can perhaps most easily be seen with Figure 10a-e, which shows the change in the number of cells over time due to the effect of different concentrations of a single extract in comparison to untreated cells. Quadratic regression curves show that the number of viable cells is either comparable or greater than untreated cells, since both the curves and error bars overlap frequently.



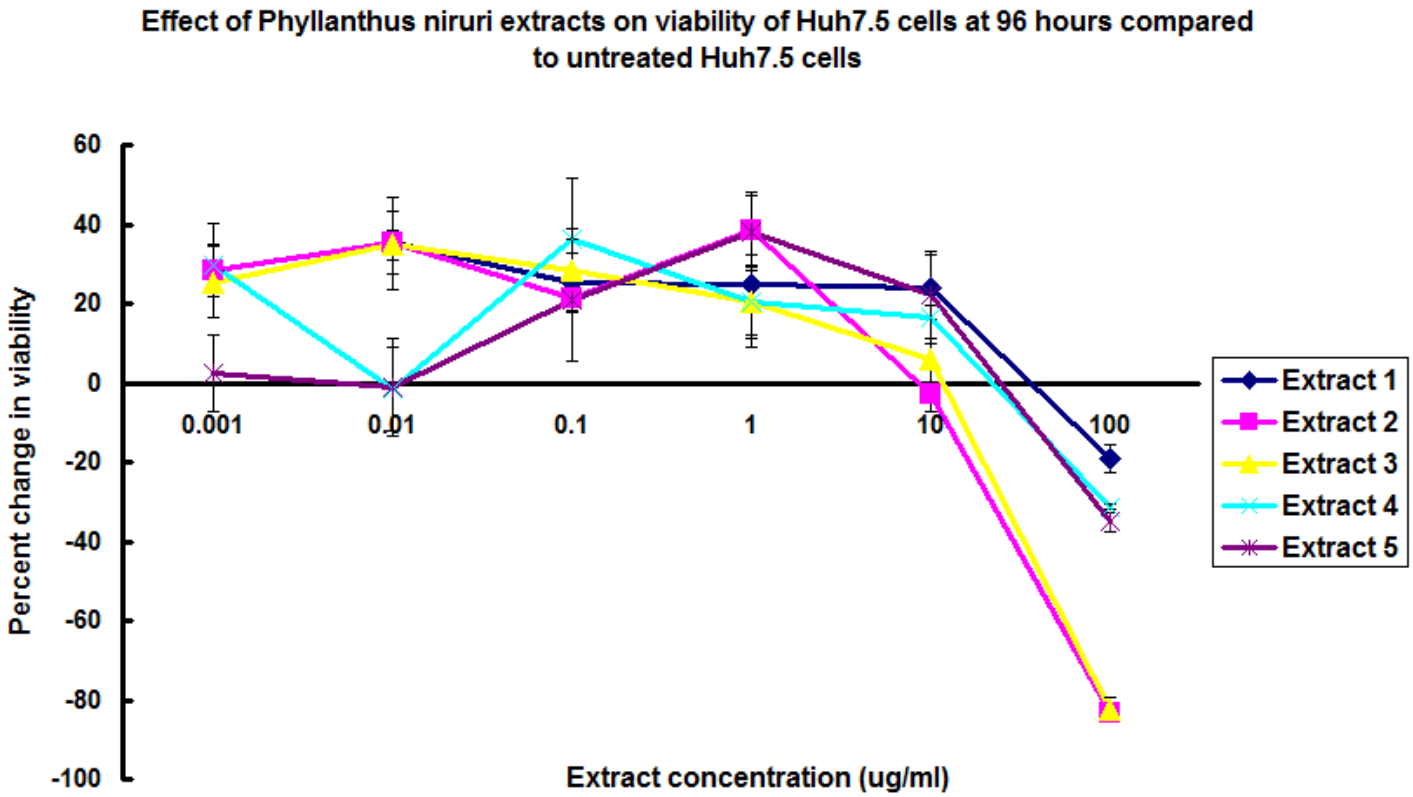
a.

Figure 9a-c: Percent change in cell growth between uninfected treated and uninfected untreated Huh7.5 cells when treated with *P. niruri* extracts 1, 2, 3, 4, and 5 in concentrations of 0.001, 0.01, 0.1, 1, 10, and 100 µg/mL for (a) 24 hours, (b) 48 hours, and (c) 96 hours. Percent growth expresses increase or decrease of the treated cultures compared to the non-treated cells.



b.

Figure 9a-c: Percent change in cell growth between uninfected treated and uninfected untreated Huh7.5 cells when treated with *P. niruri* extracts 1, 2, 3, 4, and 5 in concentrations of 0.001, 0.01, 0.1, 1, 10, and 100 µg/mL for (a) 24 hours, (b) 48 hours, and (c) 96 hours. Percent growth expresses increase or decrease of the treated cultures compared to the non-treated cells.



c.

Figure 9a-c: Percent change in cell growth between uninfected treated and uninfected untreated Huh7.5 cells when treated with *P. niruri* extracts 1, 2, 3, 4, and 5 in concentrations of 0.001, 0.01, 0.1, 1, 10, and 100 µg/mL for (a) 24 hours, (b) 48 hours, and (c) 96 hours. Percent growth expresses increase or decrease of the treated cultures compared to the non-treated cells.

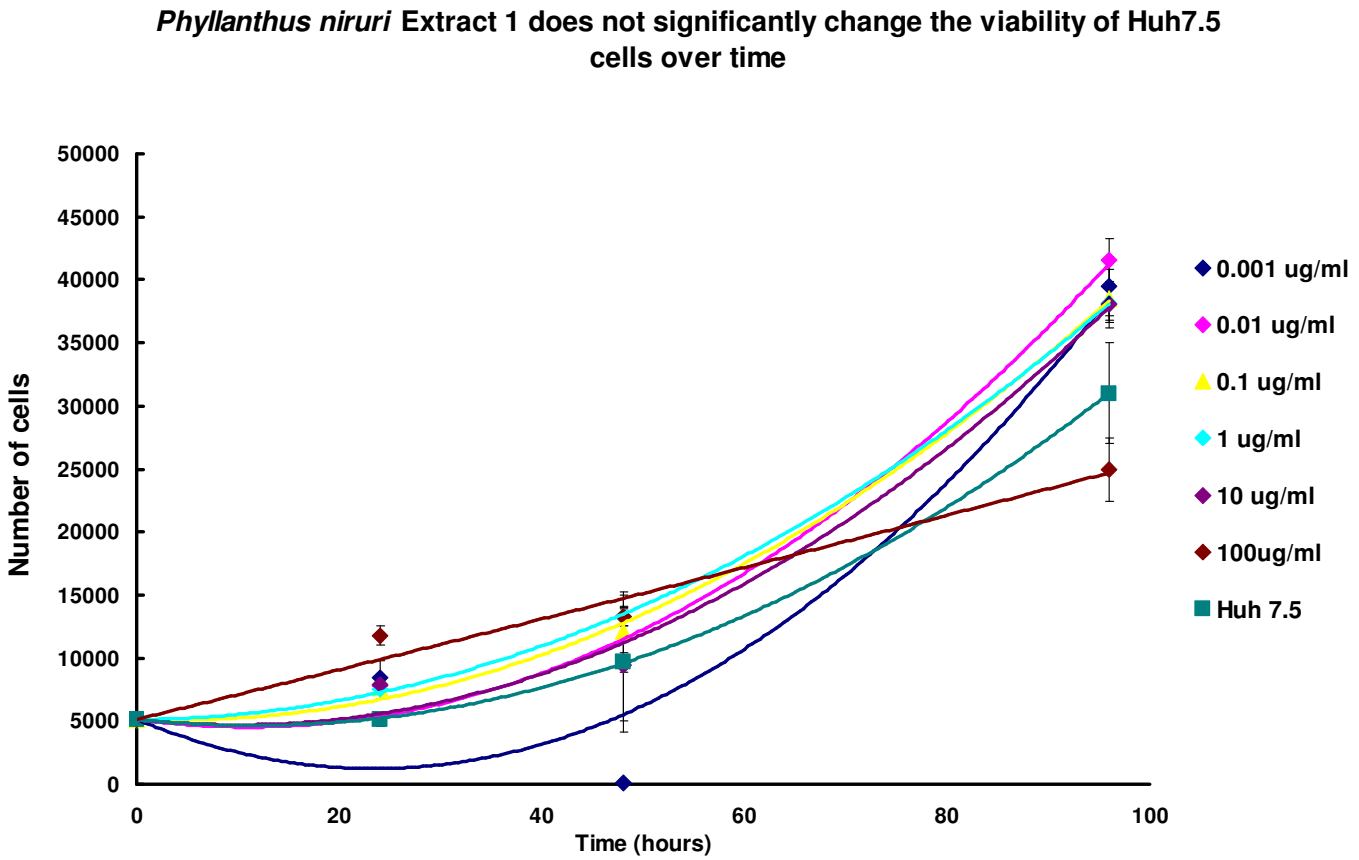
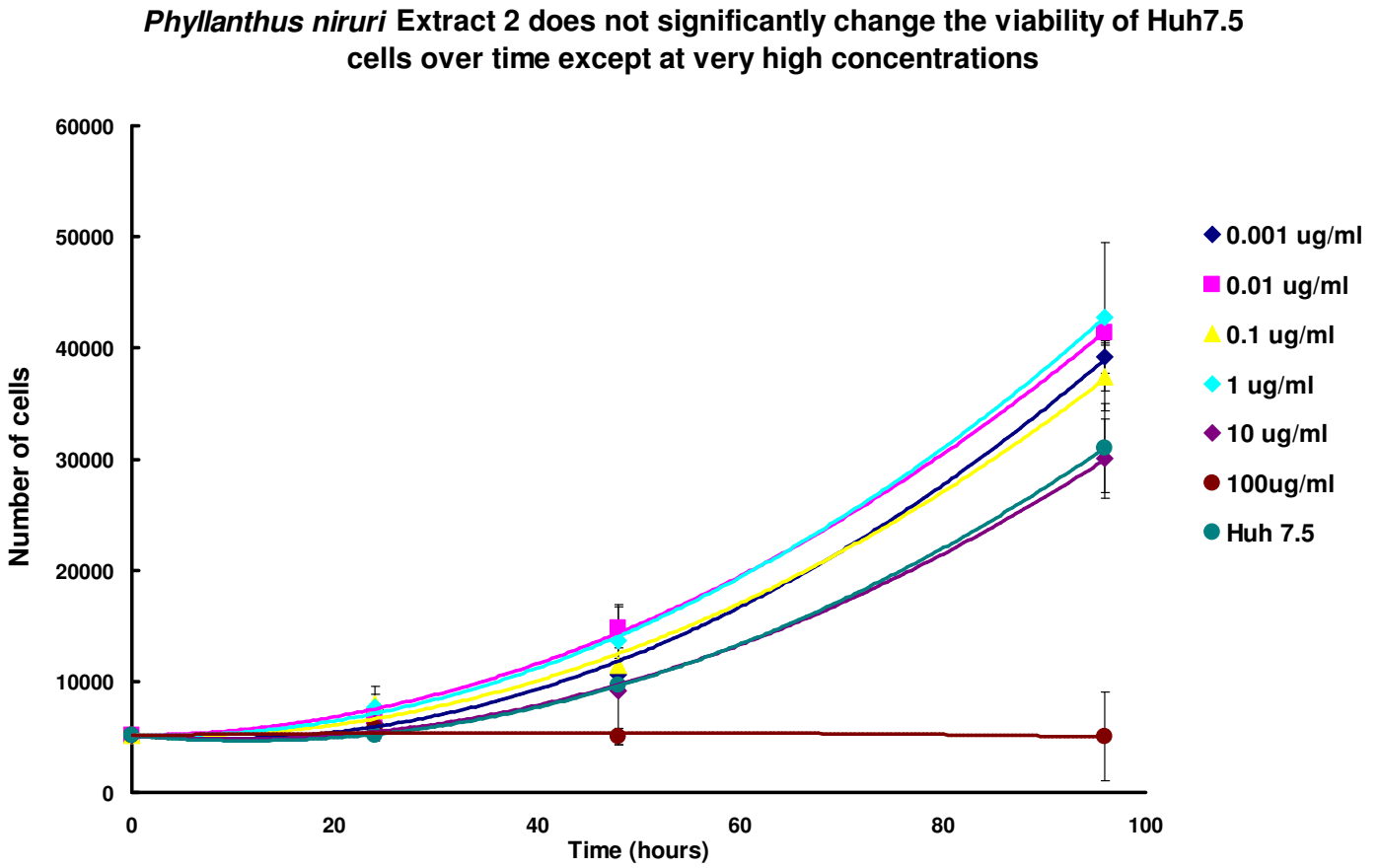
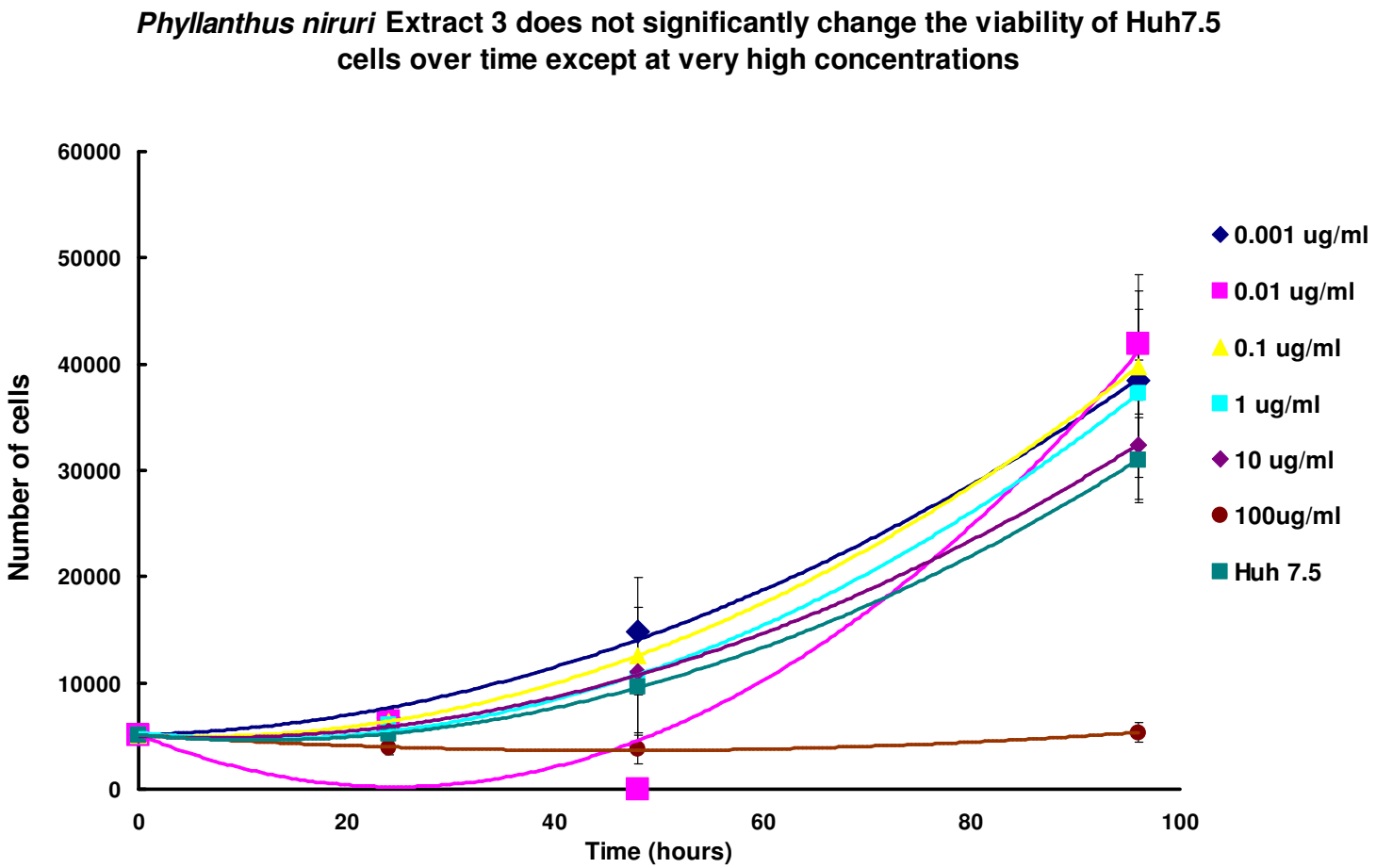


Figure 10a-e. Change in the number of cells over four day treatment of Huh7.5 cells with *Phyllanthus niruri* extracts. Huh7.5 cells were treated with different concentrations of *P. niruri* ranging 0.001, 0.01, 0.1, 1, 10, and 100 µg/mL, and the number of cells was compared to untreated Huh7.5 cells. A quadratic regression curve was drawn to analyze the change in cells over time. The extracts tested included (a) Extract 1, (b) Extract 2, (c) Extract 3, (d) Extract 4, and (e) Extract 5.



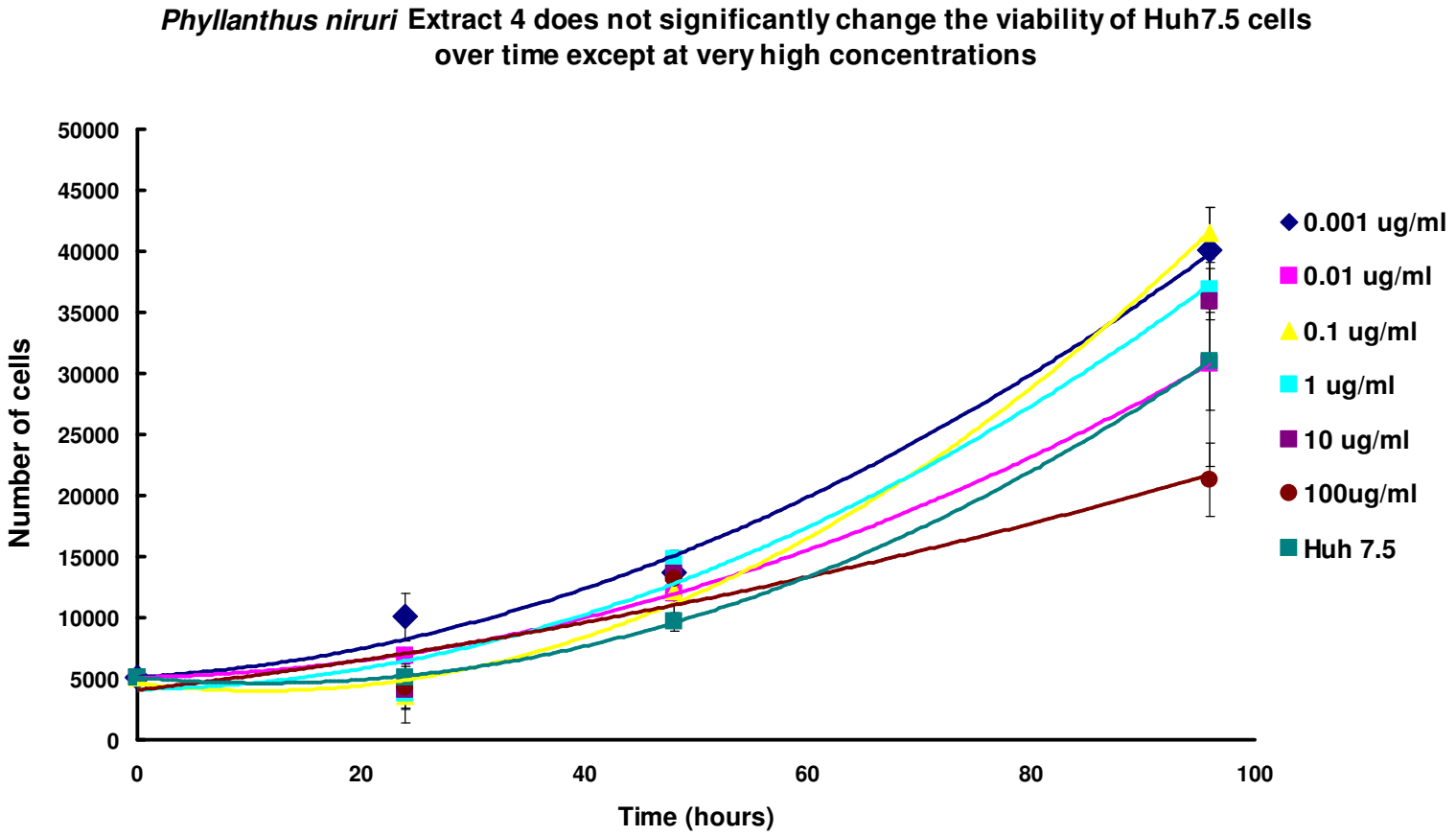
b.

Figure 10a-e. Change in the number of cells over four day treatment of Huh7.5 cells with *Phyllanthus niruri* extracts. Huh7.5 cells were treated with different concentrations of *P. niruri* ranging 0.001, 0.01, 0.1, 1, 10, and 100 µg/mL, and the number of cells was compared to untreated Huh7.5 cells. A quadratic regression curve was drawn to analyze the change in cells over time. The extracts tested included (a) Extract 1, (b) Extract 2, (c) Extract 3, (d) Extract 4, and (e) Extract 5.



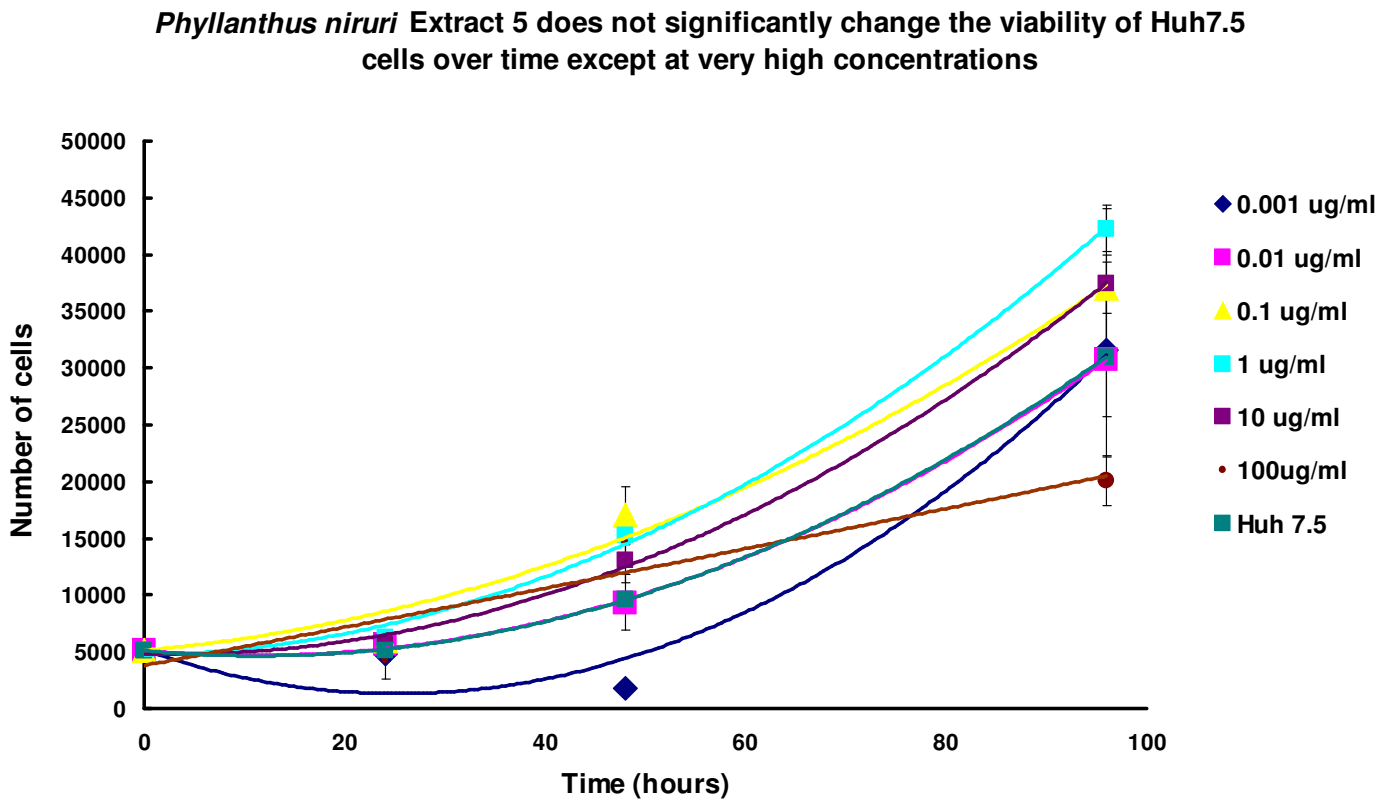
c.

Figure 10a-e. Change in the number of cells over four day treatment of Huh7.5 cells with *Phyllanthus niruri* extracts. Huh7.5 cells were treated with different concentrations of *P. niruri* ranging 0.001, 0.01, 0.1, 1, 10, and 100 µg/mL, and the number of cells was compared to untreated Huh7.5 cells. A quadratic regression curve was drawn to analyze the change in cells over time. The extracts tested included (a) Extract 1, (b) Extract 2, (c) Extract 3, (d) Extract 4, and (e) Extract 5



d.

Figure 10a-e. Change in the number of cells over four day treatment of Huh7.5 cells with *Phyllanthus niruri* extracts. Huh7.5 cells were treated with different concentrations of *P. niruri* ranging 0.001, 0.01, 0.1, 1, 10, and 100 µg/mL, and the number of cells was compared to untreated Huh7.5 cells. A quadratic regression curve was drawn to analyze the change in cells over time. The extracts tested included (a) Extract 1, (b) Extract 2, (c) Extract 3, (d) Extract 4, and (e) Extract 5



e.

Figure 10a-e. Change in the number of cells over four day treatment of Huh7.5 cells with *Phyllanthus niruri* extracts. Huh7.5 cells were treated with different concentrations of *P. niruri* ranging 0.001, 0.01, 0.1, 1, 10, and 100 µg/mL, and the number of cells was compared to untreated Huh7.5 cells. A quadratic regression curve was drawn to analyze the change in cells over time. The extracts tested included (a) Extract 1, (b) Extract 2, (c) Extract 3, (d) Extract 4, and (e) Extract 5

Subsection 3: 4.4.3 The effect of P. niruri extracts on J6JFH cells

For infected cells, treatment with *P. niruri* extracts results in an increase in the number of cells. The results are reported in Figure 11a-c. This effect was most apparent at 24 hours (Figure 11a), where the increase was statistically significant ($p < 0.05$) for all concentrations tested for Extract 3 at 0.01 μg/mL. For extract 1, increases in concentrations of extract were correlated to an increase in the number of cells. Extract 2 does not seem to vary in its effect on the number of cells over concentrations, staying in a narrow range from a 59.9%-73.9% increase in the number of cells. Extract 3 varied in its effect over concentrations that neither increases or decreases in a particular direction as concentration increased. Extract 4 had the strongest effect on the number of cells, ranging from a 96.3% increase in the number of cells at 1 μg/mL to as much as a 306% increase in the number of cells at 100 μg/mL. Extract 5 has the second strongest effect, ranging from 87.4-117% increase in the number of cells. The increase was also particularly high for very high extract concentrations for Extracts 1 and 4. It was for this elevation in the number of cells that the number of viral copies per cell is used as a means to compare the effectiveness of samples to lower viral load, rather than just the viral load alone.

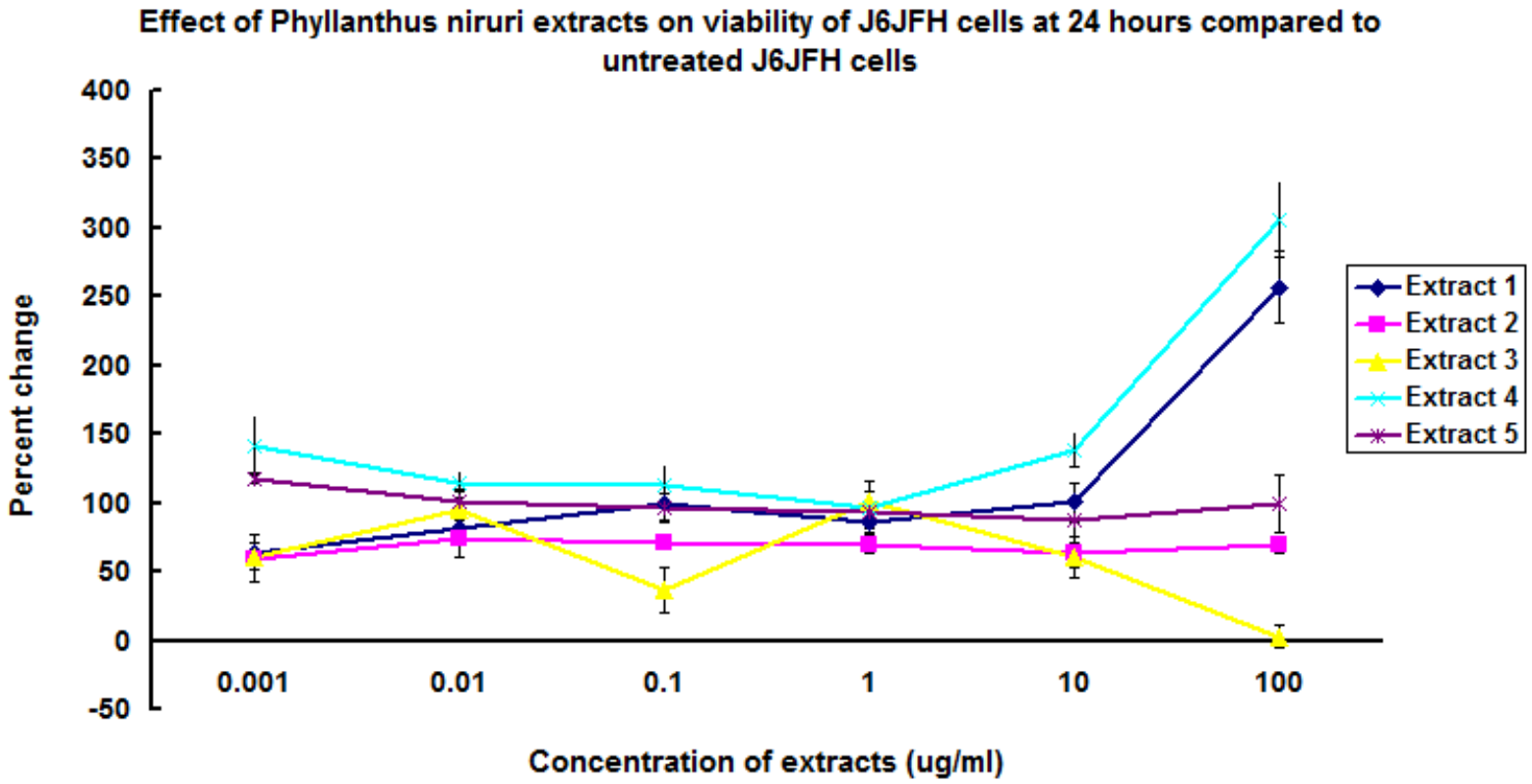
At 48 hours (Figure 11b), *P. niruri* extracts increase the number of cells significantly ($p < 0.05$) in most extracts with some exceptions, notably at 0.01 μg/mL of Extract 1, 1 μg/mL of Extract 2, and 0.001 μg/mL in Extract 5. In Extract 3, only the lower concentrations of the extract demonstrated a significant effect on cell viability, while all concentrations of Extract 4 continued to have strong effects on cell proliferation. For Extracts 1 and 5, the positive effect on cell viability seemed to increase with concentration and the effect appeared to plateau at lower

concentrations. The effect in Extract 2, 3, and 4 seemed to decrease as concentrations of the extract rises. The effect in Extract 4 decreases at lower concentrations and levels off at concentrations of 0.1 μg/mL and greater. Overall, Extract 5 has the greatest positive percent increase in cell growth of treated J6JFH infected cells versus non-treated cells.

Results from 96 hours (Figure 11c) differs from that of 24 and 48 hours. Extracts 1, 2, and 3 shows a gradual decrease in percent change between treated and untreated cells. Extract 5 data depicts an initial decline in cell viability for lower concentrations of 0.001 and 0.01 μg/mL and then an increase in cell viability for concentrations 0.1, 1, and 10 μg/mL. For all extracts, a concentration of 100u g/ml results in a significant reduction in cell viability. The largest negative percent change belongs to Extract 2 and 3 with values near 77% and 72% decrease respectively. For 96 hour cells, not many extract samples significantly ($p < 0.05$) increases number of cells in culture. The only samples that have significant p values were Extract 2 at a concentration of 10 μg/mL and Extract 3 at a concentration of 1 μg/mL.

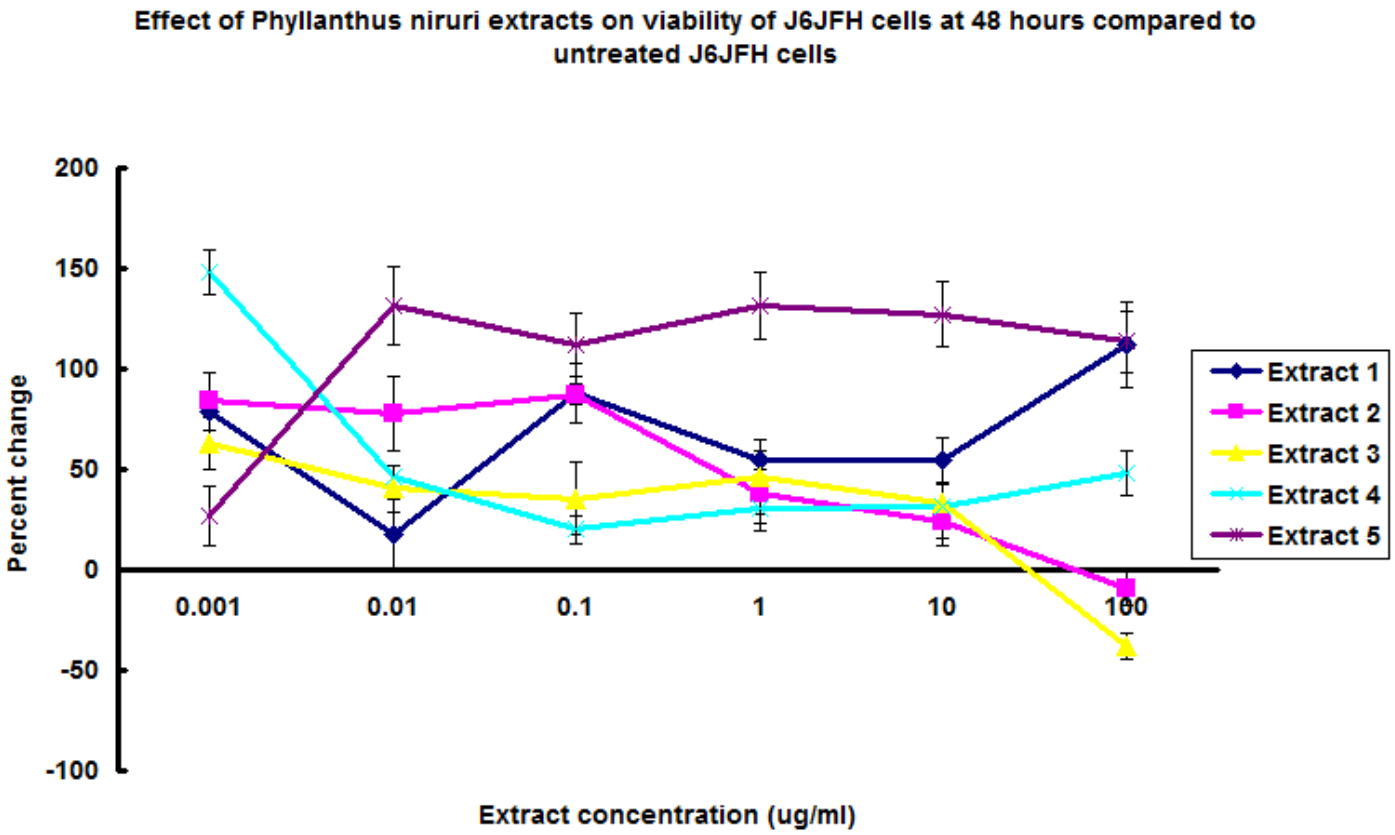
Overall, over time, like with the uninfected samples, the effect of extracts on cells did not seem to have a negative impact on the number of cells. In fact, more often than not, the number of cells was often significantly greater under the influence of the extracts for infected cells, unless the extract was at very high concentrations with higher levels of DMSO. This contrasts with the uninfected cell sample, which only saw comparable and occasional increases in cell proliferation. The increase in the number of cells be most easily be seen with Figure 12a-e, which shows the change in the number of cells over time due to the effect of different concentrations of a

single extract in comparison to untreated infected cells. Quadratic regression curves show that the number of viable cells is either comparable or greater than untreated cells, since both the curves and error bars overlap frequently.



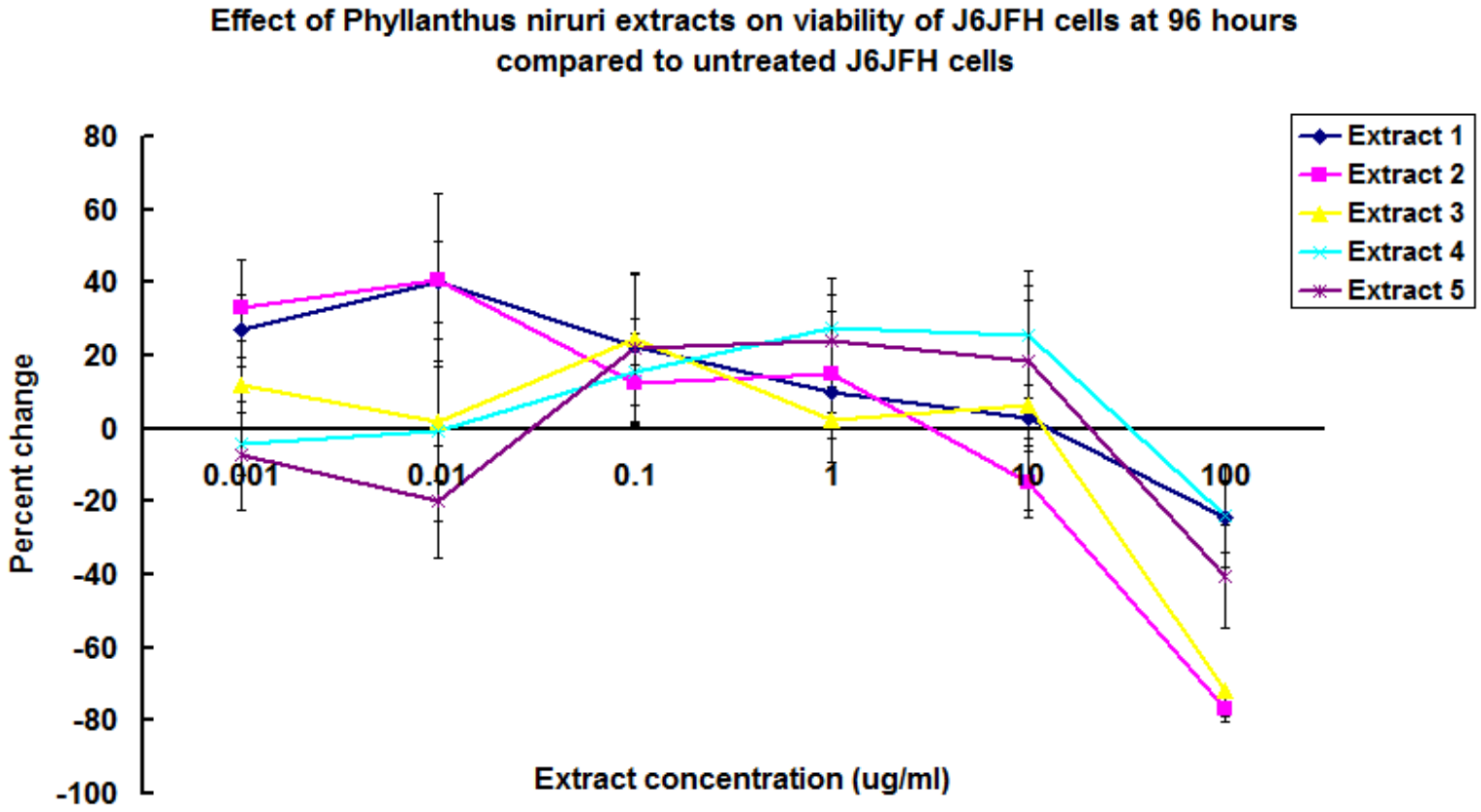
a.

Figure 11a-c: (a) *P. niruri* extracts significantly ($p < 0.05$ for all concentrations tested) increase the viability of J6JFH cells after 24 hours. (b) Extracts result in moderate increase in cell viability for most extracts for 48 hours. (c) Extracts treated 96 hour cells show decrease in viability at high concentrations.



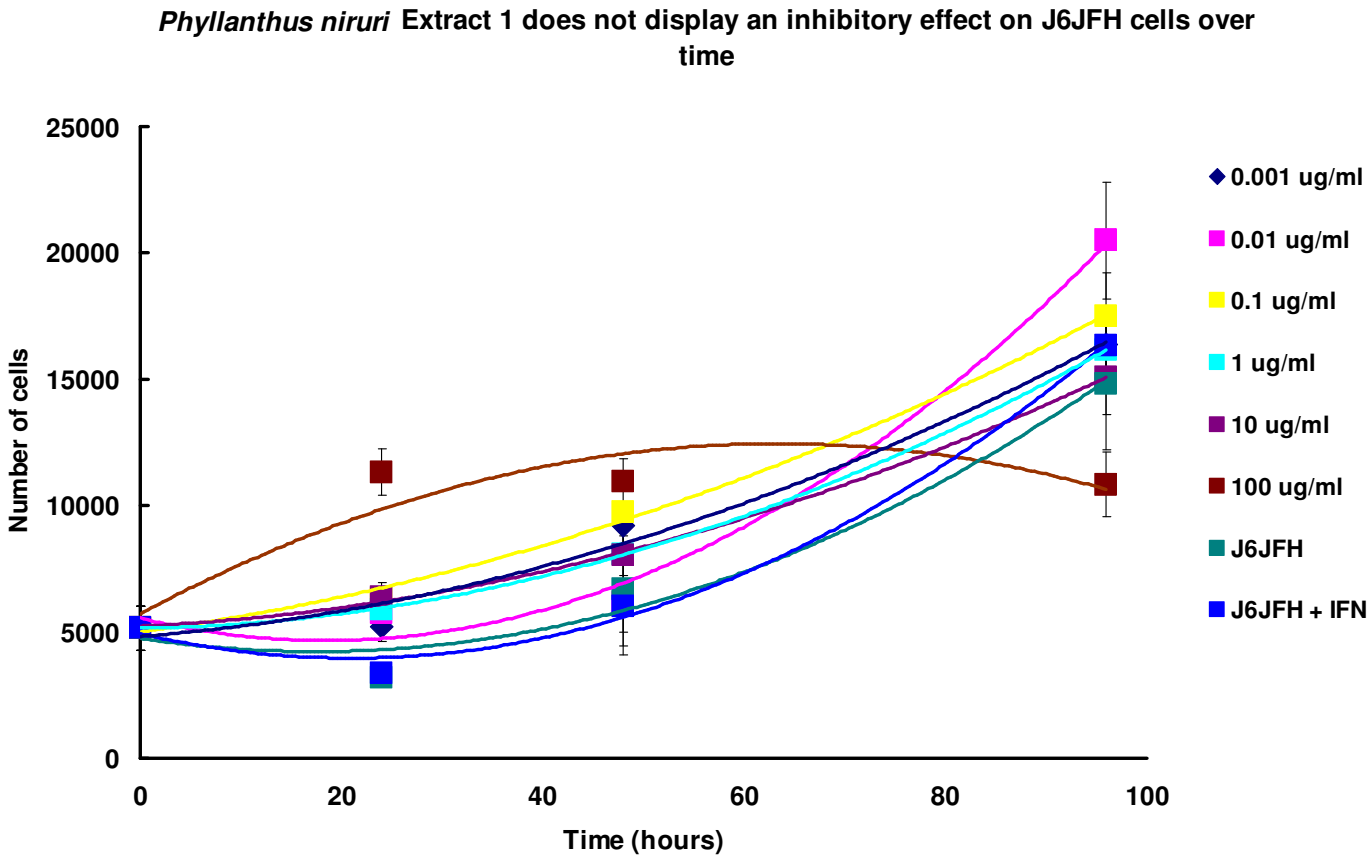
b.

Figure 11a-c: (a) *P. niruri* extracts significantly ($p < 0.05$ for all concentrations tested) increase the viability of J6JFH cells after 24 hours. (b) Extracts result in moderate increase in cell viability for most extracts for 48 hours. (c) Extracts treated 96 hour cells show decrease in viability at high concentrations.



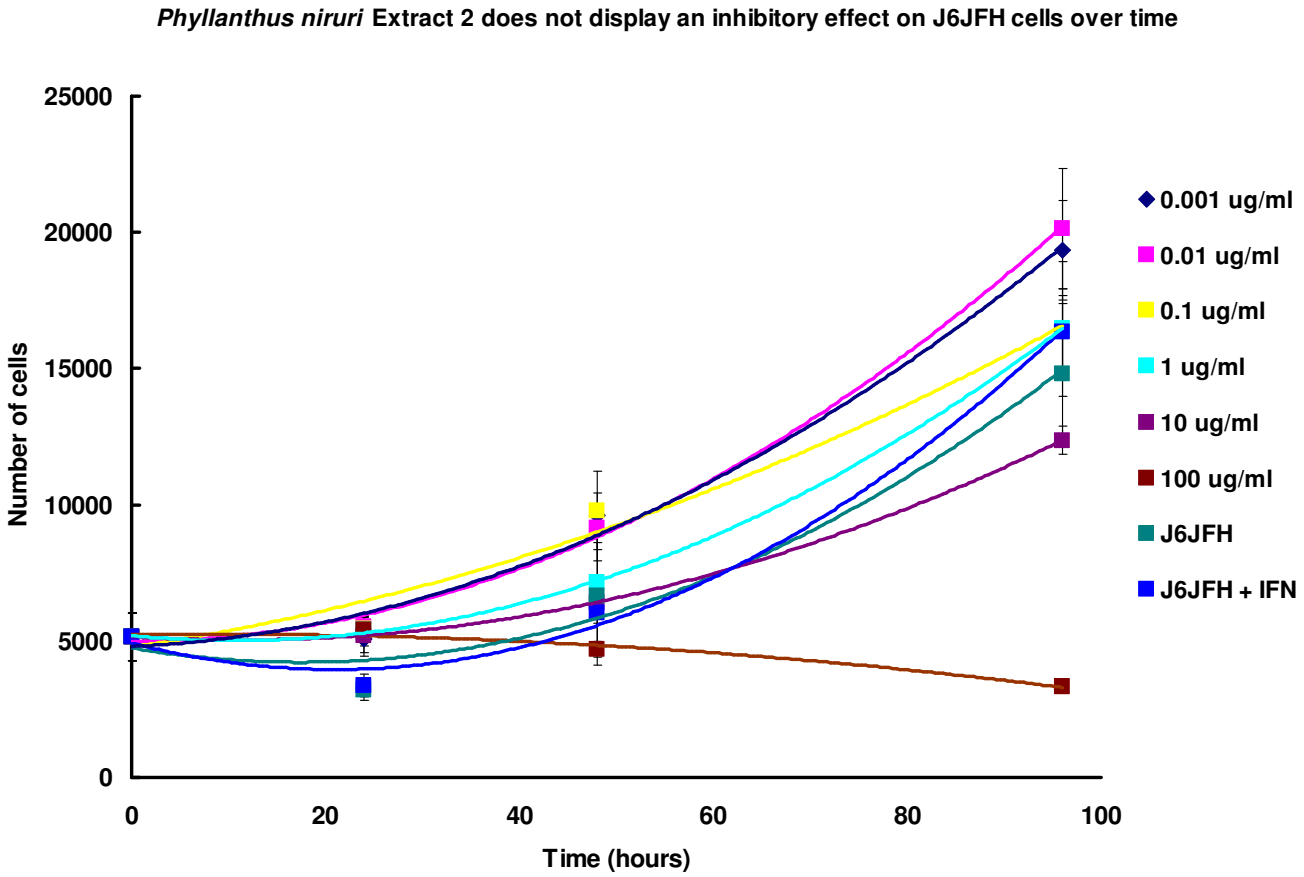
c.

Figure 11a-c: (a) *P. niruri* extracts significantly ($p < 0.05$ for all concentrations tested) increase the viability of J6JFH cells after 24 hours. (b) Extracts result in moderate increase in cell viability for most extracts for 48 hours. (c) Extracts treated 96 hour cells show decrease in viability at high concentrations.



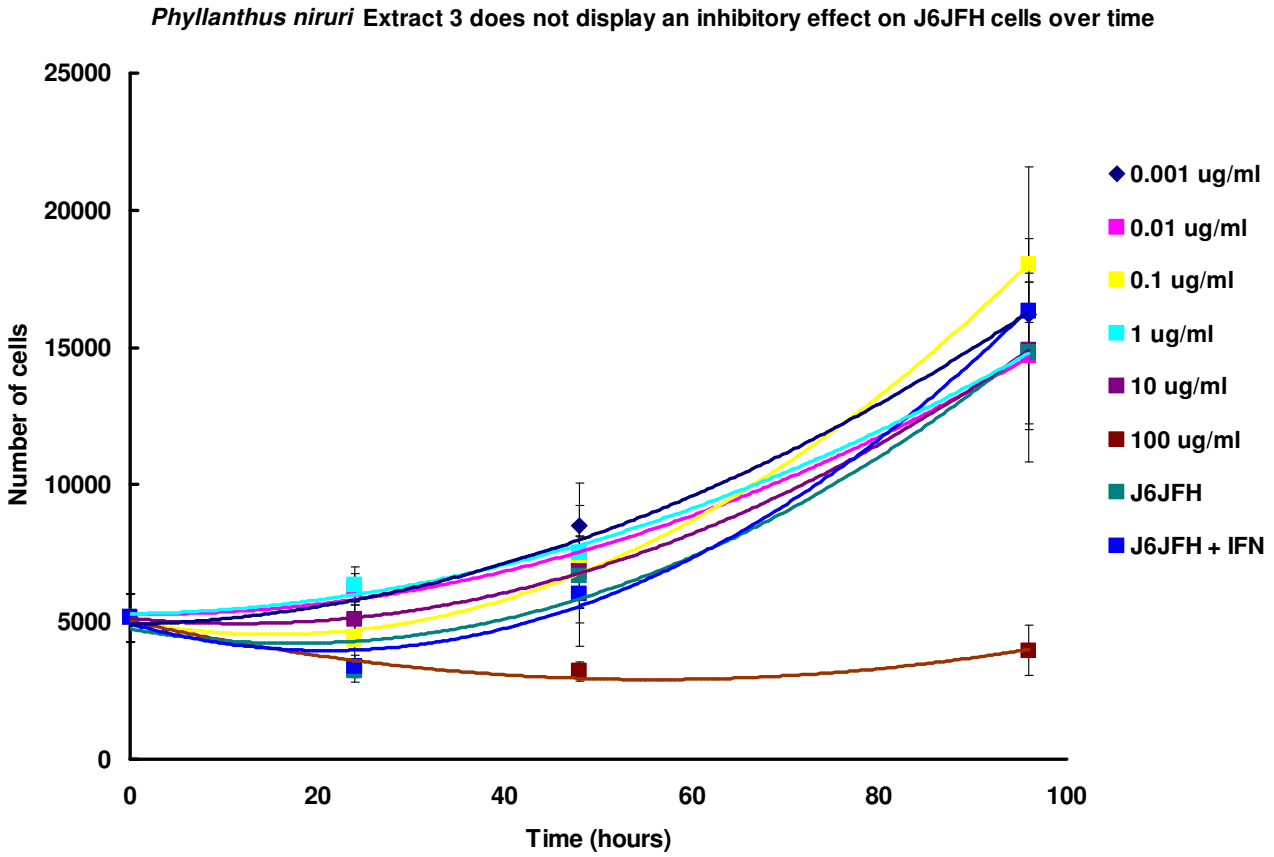
a.

Figure 12a-e. Change in the number of cells over four day treatment of J6JFH cells with *Phyllanthus niruri* extracts. J6JFH cells were treated with different concentrations of *P. niruri* ranging 0.001, 0.01, 0.1, 1, 10, and 100 µg/mL, or 10 IU/ml of standard interferon, and the number of cells was compared to untreated infected J6JFH cells. A quadratic regression curve was drawn to analyze the change in cells over time. The extracts tested included (a) Extract 1, (b) Extract 2, (c) Extract 3, (d) Extract 4, and (e) Extract 5.



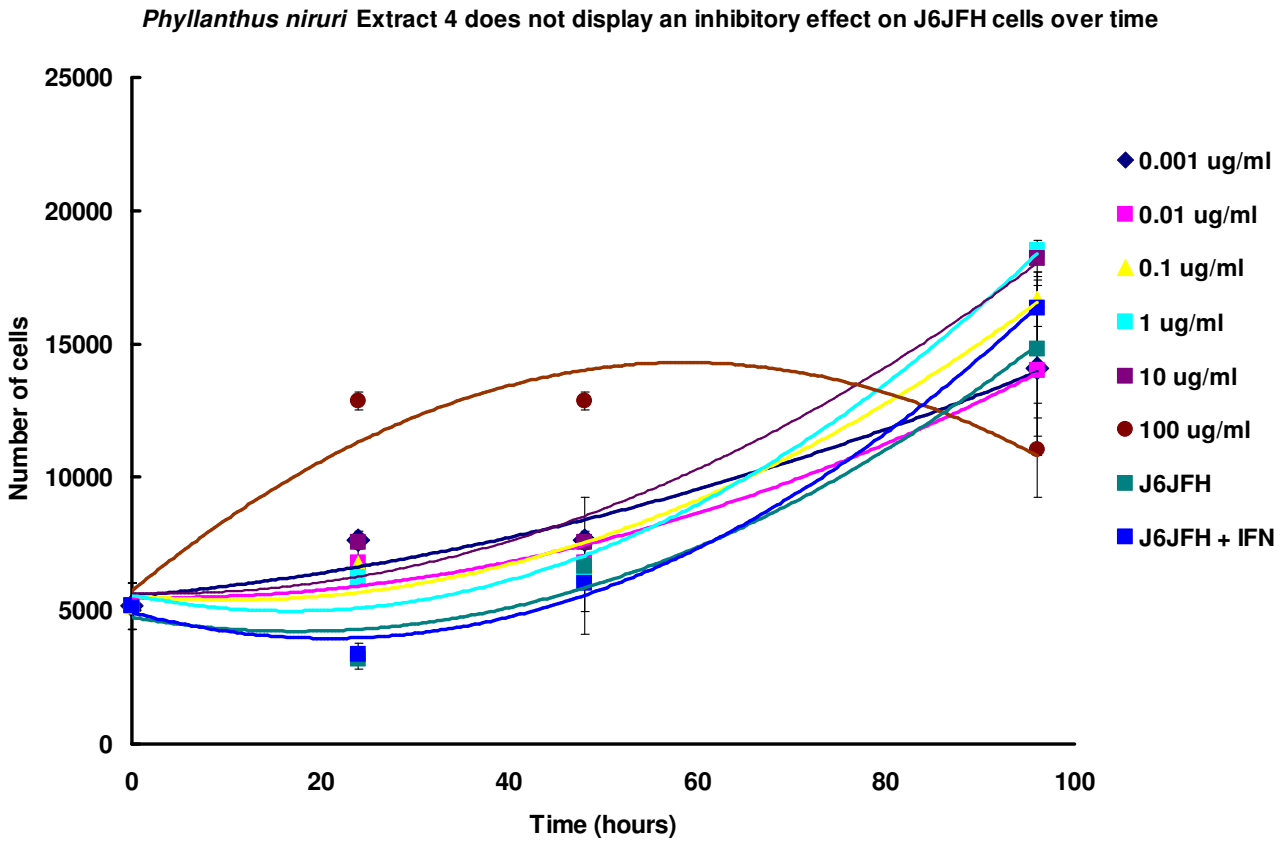
b.

Figure 12a-e. Change in the number of cells over four day treatment of J6JFH cells with *Phyllanthus niruri* extracts. J6JFH cells were treated with different concentrations of *P. niruri* ranging 0.001, 0.01, 0.1, 1, 10, and 100 µg/mL, or 10 IU/mL of standard interferon, and the number of cells was compared to untreated infected J6JFH cells. A quadratic regression curve was drawn to analyze the change in cells over time. The extracts tested included (a) Extract 1, (b) Extract 2, (c) Extract 3, (d) Extract 4, and (e) Extract 5.



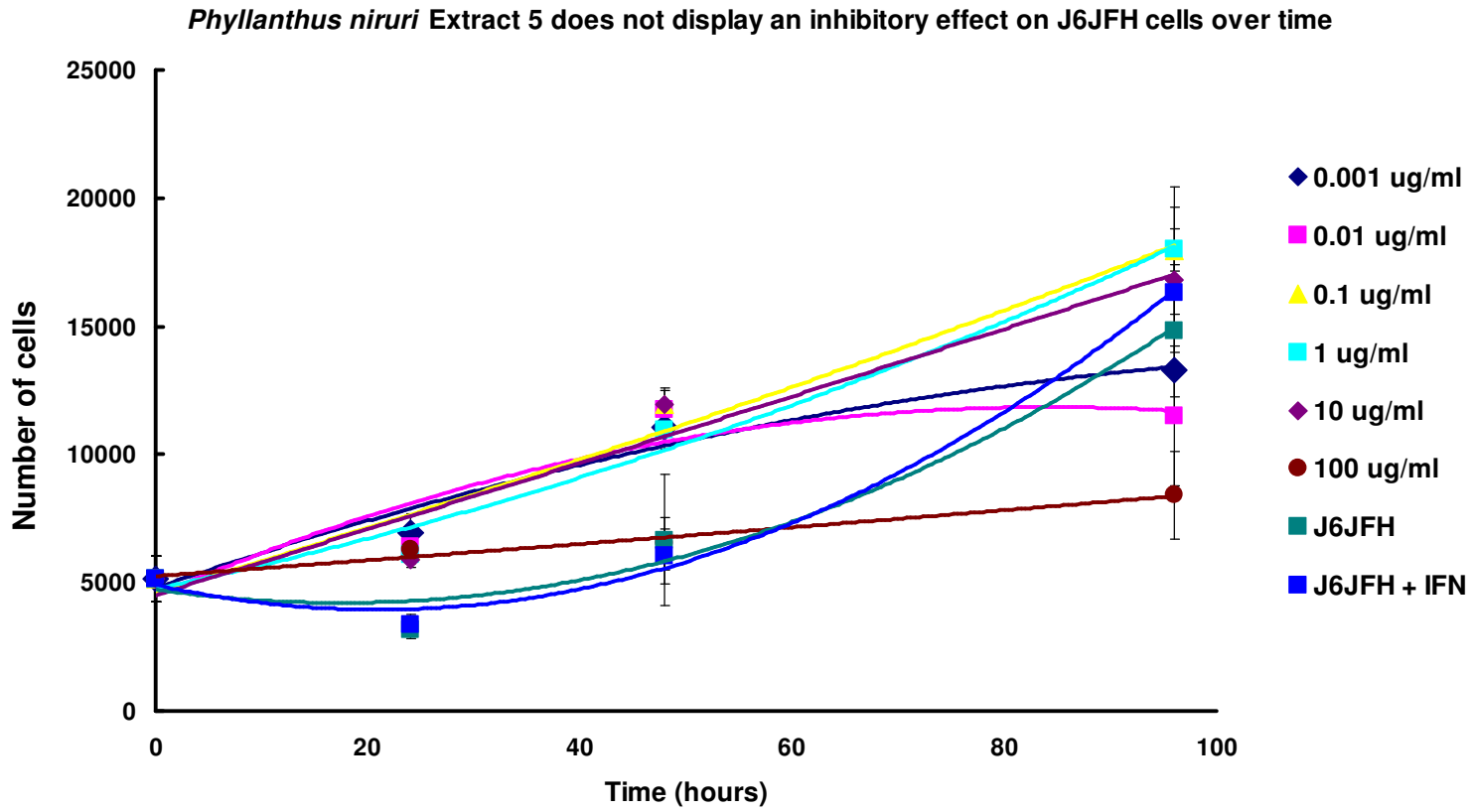
c.

Figure 12a-e. Change in the number of cells over four day treatment of J6JFH cells with *Phyllanthus niruri* extracts. J6JFH cells were treated with different concentrations of *P. niruri* ranging 0.001, 0.01, 0.1, 1, 10, and 100 µg/mL, or 10 IU/ml of standard interferon, and the number of cells was compared to untreated infected J6JFH cells. A quadratic regression curve was drawn to analyze the change in cells over time. The extracts tested included (a) Extract 1, (b) Extract 2, (c) Extract 3, (d) Extract 4, and (e) Extract 5.



d.

Figure 12a-e. Change in the number of cells over four day treatment of J6JFH cells with *Phyllanthus niruri* extracts. J6JFH cells were treated with different concentrations of *P. niruri* ranging 0.001, 0.01, 0.1, 1, 10, and 100 µg/mL, or 10 IU/ml of standard interferon, and the number of cells was compared to untreated infected J6JFH cells. A quadratic regression curve was drawn to analyze the change in cells over time. The extracts tested included (a) Extract 1, (b) Extract 2, (c) Extract 3, (d) Extract 4, and (e) Extract 5.



e.

Figure 12a-e. Change in the number of cells over four day treatment of J6JFH cells with *Phyllanthus niruri* extracts. J6JFH cells were treated with different concentrations of *P. niruri* ranging 0.001, 0.01, 0.1, 1, 10, and 100 µg/mL, or 10 IU/ml of standard interferon, and the number of cells was compared to untreated infected J6JFH cells. A quadratic regression curve was drawn to analyze the change in cells over time. The extracts tested included (a) Extract 1, (b) Extract 2, (c) Extract 3, (d) Extract 4, and (e) Extract 5.

Subsection 4: 4.4.4 The effect of P. niruri extracts is still significant with respect to the DMSO vehicle control

Based on statistical tests, as outlined in Appendix I, we determined that the effect of *P. niruri* extracts is still significant with respect to the DMSO vehicle control. This was important in order to distinguish if the observed effect was due to our extracts or the solvent it was dissolved in. We looked at the difference between the viral copies per cell produce due to our extracts and produced due to DMSO by calculating the p-value. Lower p values mean that the extracts had a significantly lower number of viral copies per cell than DMSO.

Subsection 5: 4.5.0 The IC50 and IC90 determined from the cell viability curves at each time point

IC50 is the half maximal inhibitory concentration for Extracts 1-5 at three time periods of incubation are given in the table below. The lowest IC 50 values correspond to Extract 4 at all incubation times. Extract 1 showed a low IC50 value of 0.95 μg/mL for 96 hour incubation. Extract 5 also has low IC50 values for 48 hour and 96 hour incubation. Overall, 96 hour incubation requires lower concentration of extract to inhibit 50% viral activity compared to 24 hour and 48 hour incubation.

IC90 is defined as the concentration of extract needed to inhibit 90% viral activity. A number of our samples did not reach 90% inhibition of viral activity. The lowest IC90 concentration corresponds to Extract 2 at 96 hours incubation. Extract 5 also had a low value, 7.6 μg/mL. All other extracts indicated by an IC90 value >10μg/mL means that the IC90 lies at concentrations higher than the ones tested.

Table 5: 50% Inhibitory Concentration (IC50)

Extract/Time point	24 hours	48 hours	96 hours
Extract 1	<0.001µg/mL*	<0.001µg/mL	0.95 µg/mL
Extract 2	<0.001µg/mL	<0.001µg/mL	<0.001µg/mL
Extract 3	<0.001µg/mL	<0.001µg/mL	>10 µg/mL**
Extract 4	0.34 µg/mL, 5 µg/mL	0.1 µg/mL	>10 µg/mL
Extract 5	<0.001µg/mL	0.006 µg/mL	0.6 µg/mL

* <0.001µg/mL indicates that the IC50 is lower than concentrations tested
** >10 µg/mL indicates that the IC50 is higher than concentrations tested

Table 6: 90% Inhibitory Concentration (IC90)

Extract/Time point	24 hours	48 hours	96 hours
Extract 1	>10 µg/mL*	>10 µg/mL	>10 µg/mL
Extract 2	>10 µg/mL	10 µg/mL	0.001 µg/mL
Extract 3	>10 µg/mL	>10 µg/mL	>10 µg/mL
Extract 4	9 µg/mL	>10 µg/mL	>10 µg/mL
Extract 5	>10 µg/mL	>10 µg/mL	7.6 µg/mL

* <0.001µg/mL indicates that the IC50 is lower than concentrations tested
** >10 µg/mL indicates that the IC50 is higher than concentrations tested

Chapter 5: Discussion

Section 1: 5.1.0 Plant structure

Overall our plant proves to be consistent with the general structure associated with Euphorbiaceae. It is hoped that by describing the physical features associated with the plant and linking it back to the barcode that it may serve to better identify whether *P. niruri* used in other studies is the same plant.

According to the Euphorbia PBI Project, flowers of the Euphorbiaceae family are unisexual and simple in structure (Euphorbia Project). The photos of our *Phyllanthus* plants matched these descriptions. We observed both male and female flowers, geologically separated on the plant stem. The two sexes of flowers are located close in proximity, possibly an evolutionary advantage to ease reproduction. Euphorbiaceae family flowers are also characterized by the presence of an involucre, base like structure to which both male and female flowers are attached. We did not observe an involucre in our magnified photos, however due to the limit of our magnification and the small size of all anatomical structures; it is possible we could not see some parts of the *Phyllanthus* plant.

Flower morphology varies in this family of plants, so it is also possible that the involucre is significantly reduced in size or underdeveloped in this species of *Phyllanthus* or just in this particular specimen. The female flowers of the Euphorbiaceae family have a pedicel, a three-parted ovary, and are without petals.

We observed, on *Phyllanthus*, that female flowers do indeed have a pedicel attached to a three-sectioned ovary (seen in the cross sectional images). Each female flower has a three pronged style that each separates into two tips; this is a typical feature of female flowers of the Euphorbiaceae family. The male flowers we observed were very small and we could not identify all the parts. However, the photos do indicate that they are simple flowers, which is again indicative of family Euphorbiaceae.

Despite a few minor discrepancies, our photo does provide ample support that *Phyllanthus* niruri falls under the Euphorbiaceae family based on plant morphology, specifically an examination of the male and female reproductive organs.

Section 2: 5.2.0 Genetic Barcoding

Subsection 1: 5.2.1 Our samples are of the same species

DNA barcoding shows *Phyllanthus niruri* and *Phyllanthus amarus* can be successfully distinguished from other *Phyllanthus* species with rbcL and matK regions. There were 5 segregating sites observed during matK alignment where were not significant during mapping. Moreover, no segregating sites were observed for rbcL alignment. In both mapping approaches, the closest matches were *P. niruri, P. amarus,* or either. Our data suggests that all of our samples are of the same species.

The closest neighbors that showed slight similarity to our samples were *P. tenellus* and *P. nummulariifolius.* These neighbors were observed in both matK and

rbcL mappings. However, they were only seen through the tree-based method and not the distance based method.

Subsection 2: 5.2.2. Our samples are P. niruri and P. amarus

Distance-based mapping for rbcL showed significant matches between our samples and *P. niruri* voucher. The next closest match of *P. amarus* only has 83% coverage compared to 92% coverage with *P. niruri* rbcL gene. Furthermore, the *P. amarus* hit only scored 894 compared to 985 from *P. niruri.* There is a significant drop off in score following the best match of *P. niruri.* This suggests that all of our samples are likely to be *P. niruri.*

Distance-based mapping for matK showed significant matches between our samples and *P. amarus* matK gene. The next two closest hits are both from *P. amarus* with similar max scores of 1515 and 1509 respectively compared to the best hit score of 1515. The top three hits have 96% query coverage and 99% max identity. *P. niruri* genes did not return as hits through default settings of BLAST. This suggests that our plant may be *P. amarus* as well as *P. niruri.* It is likely that since these two species have been so taxonomically confused in the past, researchers uploaded these DNA fragments without a clear understanding on how to identify their samples.

Section 3: 5.3.0 HPLC analysis of data

The HPLC data indicates that the composition of the extracts varied widely between the fractions (Table 4a-b). This indicates that the chemical composition between fractions is likely similarly varied. However, there were a few anomalies in the overall data. To begin, extract 2, which was the hexane partition had a large peak around 8 that was absent from fraction one. This is unusual, due to the fact that all peaks in fractions 2-5 should be contained in some capacity in fraction one. This peak was not located anywhere in fraction one. Other deviation in specific retention times is more explainable by the fact that increased or decreased separation can be seen depending on the total composition of the fraction, but an entirely new peak was surprising to see. This could indicate that some sort of decomposition or reaction went on in this fraction to account for the appearance of a new peak.

Also, initially when we ran the HPLC we injected the fractions in pure DMSO at 50:50 ACN:water and saw no separation. Thus we lowered the acetonitrile concentration to 25%. This gave use slightly better separation, but we realized it was likely the increased amount of DMSO that was detrimentally affecting the separation. Thus we diluted our samples into water. This significantly increased the separation, but lead to broad peaks due to the now high solvent polarity. Ideally, we would re-run the samples in water with the original 50:50 acetonitrile:water solvent ratio.

In addition, the GCMS data unfortunately was not as useful as we thought it would be. The boiling points of the compounds in the extracts are simply too high to

allow evaluation through GCMS. Instead, a procedure such as LCMS would lead to the best method for analysis. Also, it may be useful to instead use a soft ionization method like electrospray ionization to prevent fragmentation and then comparing the molecular ion masses to a table of known phytochemical compounds in *P. niruri*.

Section 4: 5.4.0 The effect of P. niruri extracts on HCV viral copies per cell shows different kinetics across the time points

Overall, *P. niruri* extracts have a significant potent effect on HCV viral copies per cell, as compared to the effects of interferon alpha. Different extracts exhibit varying activities across the time points which indicate different metabolic stabilities and suggesting possible different mechanisms of action. Each of the extracts exhibits varying kinetics over time, suggesting different mechanisms of action in addition to different metabolic stability in vitro.

The initial methanolic extract, labeled extract 1, maintained a relatively high level of viral suppression at the lowest concentration of 0.001 μg/mL maintaining viral suppression between 62.7%-79.4% over the full course of the study from 24 hours to 96 hours (Figures 8a,c). This indicates that the effect of the extract is prolonged, likely due to greater stability which allows the concentration in vitro to remain above active levels.

Viral suppression in hexane fraction, Extract 2, was similarly maintained from 24 to 96 hours staying between 67.0-88.5% even at the lowest concentration of 0.001

μg/mL (Figures 8a,c. Similar to Extract 1, this indicates that there is a strong and immediate suppression of the HCV viral load which is not changed, and if anything seems to increase, over time. This likely means that the active compounds found in extracts 1 and 2 are not degraded below their active concentrations over time. This is an important factor to take into consideration with future studies as compounds which are degraded in vitro may be even more quickly when moved into *in vivo* models.

In contrast, the CCl_4 layer, extract 3, did not experience maintenance of activity over the course of the study. Even at the ten-fold higher concentration of 1 μg/mL, the viral load suppression dropped from 70% suppression at 24 hours to 30.4% at 96 hours (Figures 8a,c). While this initial level of suppression was comparable to the level of suppression seen in the 0.001 μg/mL concentrations of both extracts 1 and 2, it is clear that they do not follow the same time course of activity. The most likely explanation for this is simply that the active compounds in extract 4 are not metabolically stable in this system. It may be helpful to determine the time point at which this extract has maximal activity through a more intensive time course study over the first 24-36 hours to track the activity. However, while this decrease in activity over time is not ideal, it can be compensated for in vivo through more frequent administration of doses.

More dramatically, the final methanolic fraction, extract 4, dropped from the near total suppression of viral load of 99.4% at 24 hours to 42.4% suppression at 94 hours for the largest tested concentration of 10 μg/mL (Figures 8a,c). Again, this very

high antiviral activity, which was found for these compounds at the highest tested concentration, was not sustained for the full time course. This means that they start off with strong antiviral effects that are not sustained across the full 96 hrs. Again, this may simply be an indication that the compound is metabolisms in the medium below its active concentration. However, there may also be additional mechanistic effects at play. The inhibitory compounds may be acting later in the viral replication cycle, such as inhibiting the release of mature virions to immediately cause the strong decrease in viral load or deactivate HCV virions before they can infect the cell. However, this effect may not be maintained over time as the compounds degrade.

Finally, the chloroform extract indicated that a reversal of the type of viral suppression found in extracts 3 and 4 at its highest concentration of 10 μg/mL. Extract 5 (at 10 μg/mL) actually started with a low level of viral suppression of 8% at 24 hours which increase drastically to near fully suppression of 99% at 96 hours (Figures 8a,c). This indicates a very interesting mechanism in which the anti-viral activity is more long term. More interestingly, this was comparable to the effect of interferon-alpha at 10 IU/mL which also started with low suppression of 12.1% at 24 hours that increase to 93.7% suppression at 96 hours. Even though extract 5 dose take a longer time to work, it does result in a viral suppression level equivalent if not higher than that of the currently approve treatment with interferon.

One part of the data that we did not expect to see with extract 5 was seen in Figure 8a, which shows the change in viral RNA suppression over concentration of

extract at 24 hours. Extract 5 does not seem to have a predictable pattern over concentration, and jumps rather erratically from a possible strong 80% increase in viral to a 50% decrease in viral load and then back down to no change in viral load compared to control. This time point most likely needs to be repeated.

This may suggest that the extract may be interfering with another process of HCV viral replication that is delayed, such as inhibiting integrase, reverse transcriptase, or protease. The HCV RNA genome consists of a single stranded RNA molecule (~9600 nucleotides) which encodes proteins crucial to the replication of HCV (Ravikumar et al., 2011). These include some structural proteins and the nonstructural proteins: NS2, NS3, NS4A, NS4B, and NS5B (Bartenschlager, 2005). As NS2 and NS3 in particular play a critical role in HCV replication, inhibiting their function would greatly suppress viral load in HCV-infected cells. Ravikumar et al. (2011) investigated the effect of *P. amarus* root and leaf extracts on NS3 protease and NS5B RNA dependent RNA polymerase, and found that there was significant inhibition. NS3 was inhibited more by the root extract, while HS5B was inhibited more by the leaf extract. Addition of root extract with interferon alpha showed an additive effect in the inhibition of HS5B.

Subsection 1: 5.4.1 P. niruri does not have a significant effect on cell toxicity in uninfected Huh 7.5 cells and does not display an inhibitory effect on J6JFH cells

Our results indicate that *P. niruri* extracts exhibit little toxicity on uninfected Huh 7.5 cells and infected J6JFH cells, the liver cancer cells used as a model for

HCV infection. In the 48 hours and 96 hours of the assay, there was little impact on cell number, suggesting that extracts are nontoxic at the concentrations tested. The only toxicity seen was at 100 μg/mL concentrations of the extracts at 96 hours, which is likely due to the fact that the DMSO concentrations were too high at 2.5%. Concentrations this high were also not used to test the effect of the extract on the viral load, and are also not clinically applicable.

One limitation we had is that some particular samples did not show an absorbance value, and were calculated to have a negative cell number of cell number close to zero, such as in Figure 10c and 10e. However, it is not likely that the extracts were toxic, since the number of cells in later time points for the same treatment condition was very high, but perhaps that the cells were not seeded probably.

Moreover, the concentrations that we tested were pharmaceutically viable and also led to a suppression of viral load. Our concentrations were well within the range of concentrations cited in the literature. Ravikumar et al. (2011) treated Huh7 cells with *P. amarus* leaf and root extracts at concentrations up to 250μg/mL, and no toxic effects were found. As our maximum concentration (10 μg/mL) was well below the concentrations that Ravikumar et al. (2011) used, we can infer that our experimental design would not induce hepatotoxicity.

This result is a positive indication for the overall success of this cell model, and is further supplemented by a study conducted by Lee, Jagannath, Wang, & Sekaran (2011). This group found an anti-metastatic effect of *Phyllanthus* extracts on A549 human lung and MCF-7 breast cancer cell lines. Extracts of four *Phyllanthus*

species (*P. niruri*, *P. urinaria*, *P. watsonii*, and *P. amarus*) were used and they reported IC50 values ranging from 50 - 180 µg/mL for methanolic extracts and 65 - 470 µg/mL for aqueous extracts. It was shown that *Phyllanthus* was capable of inducing apoptosis in conjunction with anti-metastatic effect due possibly to polyphenols present in its extracts (Lee, Jagannath, Wang, & Sekaran, 2011).

However, this could have proven to be difficult for our model, since it utilizes liver cancer cells in order to get a sustainably infective cell line to mimic in vitro HCV infection. However, since this anti-cancer activity was found in lung cancer cells, it is likely that it would exhibit a different oncogenic pathway from the Huh7.5 liver cancer cells utilized in this study. While this also does not eliminate the potential of *P. niruri* as a cancer therapeutic, it does allow continued use of the Huh7.5 as a proper model for in vitro HCV infection without worry of masking the anti-viral or cell survival effects of the HCV infected cells due to the killing of the cancer cells.

Although the origin of the proliferative activity observed by some extracts on the J6JFH cells remains unclear, they may lie in the consideration of direct anti-viral activity. Previous literature offers some insight into these mechanisms, though not specifically for HCV-infected cell lines. For example Qian-Cutrone et al. (1996) showed viral replication inhibition of HIV through the action of niruriside. Niruriside inhibited reverse-transcriptase, the key enzyme used to synthesize DNA from an RNA template. Moreover, Venkateswaran, Millman, and Blumberg proved as early

as 1987 that *P. niruri* had the ability to inhibit DNA polymerase in Woodchuck hepatitis virus (WBV) and HBV.

While these results indicate that the extracts are not toxic to the liver cancer cells (as shown by the absence of toxicity to the cell line), they may in fact have a hepatoprotective effect, a finding reported by Sarkar and Sil (2007). Sarkar and Sil (2007) investigated the hepatoprotective role of *P. niruri* protein isolates against thioacetamide. Thioacetamide is metabolized into thioacetamide sulfoxide, a ROS responsible for the degradation of biomembranes and DNA that subsequently leads to cell death. Pretreatment reduced the production of malodialdehyde (MDA), an indicator of reduced oxidative stress in cells. Treatment with protein isolate alone (without treatment with thioacetamide), did not result in an increase in MDA levels, suggesting that *P. niruri* has a protective effect rather than a restorative effect against ROS induced cell toxicity. Since the viral infection through HCV can cause significant oxidative stress on the tissue and cells, it may be that *P. niruri* could promote cell proliferation through a protective process.

However, these studies also do not fully answer questions about more global toxicity in-vivo. Manjrekar et al. (2008) reported the formation of dense eosinophilic casts in the kidneys and testes of rats that were treated with *P. niruri*. These casts were dense protein masses that could lead to kidney failure and infertility (Manjrekar et al., 2008). While this is relatively new and surprising data given the wide spread use of this plant for various ailments including urinary problems (Patel, Tripathi,

Sharro, Chauhan, & Dixit, 2011), it does indicate that there may potentially be other toxic effects to the plant which should be studied more in depth before these extracts are considered for human trials.

Subsection 2: 5.4.2 IC50 and IC90 values reveal effectiveness of extracts 1-5 as a dose dependent response on the inhibition of viral copies per cell

IC50 and IC90 are measurements of drug effectiveness. The value for IC50 (Table 5) is the concentration of extract needed to inhibit 50% of viral activity *in vitro* while IC90 (Table 6) is concentration required for 90% inhibition. Tables 5 and 6 are repeated below for the ease of interpretation.

Table 5: 50% Inhibitory Concentration (IC50)

Extract/Time point	24 hours	48 hours	96 hours
Extract 1	<0.001µg/mL	<0.001µg/mL	0.95 µg/mL
Extract 2	<0.001µg/mL	<0.001µg/mL	<0.001µg/mL
Extract 3	<0.001µg/mL	<0.001µg/mL	>10 µg/mL
Extract 4	0.34 µg/mL, 5 µg/mL	0.1 µg/mL	>10 µg/mL
Extract 5	<0.001µg/mL	0.006 µg/mL	0.6 µg/mL

Table 6: 90% Inhibitory Concentration (IC90)

Extract/Time point	24 hours	48 hours	96 hours
Extract 1	>10 µg/mL	>10 µg/mL	>10 µg/mL
Extract 2	>10 µg/mL	10 µg/mL	0.001 µg/mL
Extract 3	>10 µg/mL	>10 µg/mL	>10 µg/mL
Extract 4	9 µg/mL	>10 µg/mL	>10 µg/mL
Extract 5	>10 µg/mL	>10 µg/mL	7.6 µg/mL

More effective drugs have lower IC values because lower concentrations are needed to achieve a certain level of viral inhibition. While it is possible to get increased activity with higher dose of drugs, this is not ideal due to the increase of negative side effects. A high concentration is neither ideal nor probable for drugs because it is much more likely that high doses would be detrimental to cells. In addition, high concentration of extracts would require larger amounts of DMSO, which may be toxic to cells at high concentrations thus masking any anti-viral effects or cell rescuing effects. In addition, the higher concentrations indicate amounts that would never be feasible in actual pharmacological studies, as it would not be possible to get a dose that large systemically in an *in vivo* model.

Each set of time points showed variability in the IC50 values. As discussed in section 5.4.1, extracts 1 and 2 have sustained activity across the three time points, while 3 and 4 decrease over time and 5 and six increase over time. In our data of IC50 values for 24, 48, and 96 hours incubation, we expect the less concentration of extracts for longer incubation times because the extract has had more time to affect viral activity. Most of the compounds have an IC50 that is lower than the actual tested concentrations. This indicates that in order to find the true IC50 value it would be necessary to test lower dilutions of the compound.

This is supported by our data as all extracts with the exception of extract 2 have significantly lower IC50 concentrations compared to 24 and 48 hour times.

Extracts 4 and 5 were also very effective at 48 hour incubation, which could indicate that these two extracts are more potent than extracts 1 and 3.

IC90 values indicate drugs are effective enough to reduce viral activity by 90%. Many of our samples did not reach this percentage of viral inhibition, and thus their IC90 concentrations are probably higher than the values that we tested. Out of the 5 extracts, only extract 2 had a significantly low IC90 value for 96 hour incubation time. We would expect low IC90 values to occur at longer incubation times for the same reason as for IC50. It is not unexpected that many samples did not have an IC90 value because the IC50 values were not all significant thus we predicted that the extracts would not be able to inhibit viral activity much above 50%. It was also not expected to see such a large difference between the IC50 and the IC90 values. However, the mechanistic and metabolic stability of the compounds will play a large role in the actual IC50 and IC90 dependence over time. This may be altered by repeated treatment of the cells at multiple time points.

Subsection 3: 5.4.3 Connections to HPLC Data

Our most active extracts based on the cell data were extracts 4 (24 hrs) with an IC50 of 0.34 μg/mL and extract 5 (96 hrs) with an IC50 of 0.6 μg/mL. However, extract 4 did not have long term sustained activity, despite the fact that it was one of the only extracts able to reach almost complete viral load suppression. In contrast, extract 5, which also had near total viral suppression at 96 hours, had very low activity at 24 hours.

Extract 4 is the residual methanolic extract after having been partitioned with hexane, CCl_4, and $CHCl_3$. Extract 5 is the final chloroform partition. These two extracts were also assumed to contain the largest amount of niruriside which functions to inhibit HIV reverse transcriptase activity (Qian-Cutrone et al., 1996). Based on the HPLC data these two extracts share peaks in common at around 2.6 minutes and 2.8 minutes, which were detected by both the 280 nm and 254 nm detectors. In addition, at around 3.0 minutes there was a peak found only in extracts 4, 5 and 1 (which was the original extract thereby containing all peaks.)

These results may indicate that some of the HCV active compounds are localized in the methanolic and chloroform fractions. These fractions may be an ideal place to begin future studies into the identification of active compounds. Previous literature studies indicate that the methanolic extracts may contain active compounds (Qian-Cutrone et al., 1996). In addition, Ravikumar et al. (2011) indicate potential compounds which are found in these species of *Phyllanthus.* It is possible that one or more of these previously identified compounds may be responsible for the observed activity.

Chapter 6: Conclusion

P. niruri is a historic medicinal plant best known for applications ranging from the treatment of kidney stones to the protection of liver cells from toxins. This study sets out to explore the therapeutic potential of *P. niruri* in the treatment of hepatitis C viral infection, which specifically targets the liver. This project was approached through a three-part methodology. The methodology consisted of genetic barcoding of individual plant samples, chemical extraction of those plants, and cell culture studies using the obtained extracts. The preliminary results of the plant extract efficacy on HCV infected cell viability and HCV viral load suppression were measured and analyzed. These were then accompanied by a genetic barcode record to indicate the consistency of the plant species among the chosen specimens.

The extraction protocol resulted in five chemically unique extracts, whose basic profiles were analyzed using HPLC. These extracts were chosen with support from literature to provide the greatest chance of obtaining an active fraction. While they do not represent the only valid and active extractions seen in the literature, they provided a set of unique extracts whose activity could be tracked through the various partitions. In addition, the HPLC profile provided a general outline for the distribution of potentially active compounds across the extractions. Even so, more research may be necessitated into furthering and refining the HPLC profiles and potentially looking into the actual active compounds which may already have been identified in the literature.

To determine the efficacy of *P. niruri* as an HCV antagonist five concentrations, ranging from 0.001µg/mL to 100µg/mL, of each extract were each

tested individually for their ability to suppress HCV viral load and improve cell viability in the infected cell line Huh 7.5. Analysis showed that all of the extracts increased cell viability significantly, ranging from 36.9% to 138.7% for infected cells at 24 hours and 19.9% to 147.9% at 48 hours. The effect seems to decline at 96 hours, where the effect ranges from a 20% drop in viability to an 40.6% increase. Additionally, little to no change in viability was observed on the uninfected control cell line when exposed to each of the extracts. This supports the idea that these *P. niruri* extracts contain active compounds that directly or indirectly diminish HCV's ability to infect and kill the cells, as opposed to causing an inherent proliferation of all liver cancer cells of this type.

In addition, the viral suppression results were equally promising. One of the five extracts, Extract 5, produced greater HCV viral suppression over a four day period than standard interferon, one of the few current treatments approved by the FDA for HCV infection. Two other extracts, 3 and 4, showed strong initial suppression in the first 24 hours, but around 50% of this suppression was sustained over the entire four day period. Of the two extracts that surpassed standard interferon alpha, extract 4 showed a maximum efficacy at 10 μg/mL with 99.4% suppression at 24 hours while 5 had its maximum efficacy at 10μg/mL with 99% suppression at 96 hours all of which are considered viable concentrations for clinical use.

The results of the genetic barcoding using the matK and rbcL chloroplast regions indicated that all of the specimens extracted and subsequently tested in cell culture were the same species. Furthermore, the results indicated that the plants tested are most likely *Phyllanthus niruri*, and that the closest relative is *Phyllanthus amarus.*

In future studies specimens used for experimentation should be harvested from a single lineage of plants descended from a single original plant. This will eliminate the need to verify that all of the specimens used are from the same species.

This study highlights the need for further research on the potential applications of *P. niruri* in treating HCV. The results of this study themselves are limited in their scope, and do not delineate a mechanism for the increased viability or viral suppression observed. The exact method through which this activity is obtained is not directly clear through our research. Whether this proliferative effect is a result of anti-oxidant effects, or the direct inhibition of viral replication, or even prevention of viral entry into the cells is still unclear and mandates further research. Moreover, they do not suggest which compounds within the extracts are responsible for the associated effects, or whether it is a combination of compounds responsible. Yet, these preliminary results are important in establishing a motive for further, more in-depth research into this plant's phytochemicals and their medicinal properties, especially against HCV.

Further studies should be completed which confirm whether or not the cell viral load matches that with the supernatant. It would also be valid to examine any synergistic effects seen when combining the extracts with one another, or with standard interferon-alpha itself or qualitative studies should be used to identify possible active compounds within each extract responsible for the anti-HCV effects. Many of these compounds may have been previously identified in the literature, thus it may be necessary to only isolate the compound as opposed to doing a thorough structural identification through HPLC or Mass Spectrometry. Additionally, any

identified active compounds should then be studied in association with HCV to determine the mechanism by which the HCV virus replication or viability is hindered. Finally, we recommend studies that manipulate the conditions presented in this study, such as the solvent used to dissolve the extract, in order to determine factors that may maximize the effect of the extracts, and minimize and residual effect from the DMSO vehicle.

Appendices

Appendix A: Plant Care

In total, we have three sets of plants grown and harvested under specific conditions.

1. **Set 1** (A1-50, B1-5, C1-3)
    a. **Planting**
        i. The US Botanic Garden (USBG) offered to help grow the plant with seeds obtained from an online retailer, Trade Winds Fruit (http://www.tradewindsfruit.com/). The plants were germinated on March 23, 2010 +/- 10 days.
    b. **Growing conditions**
        i. The plants were grown at the US Botanic Garden Greenhouse.
    c. **Harvest and Drying Conditions**
        i. On day 96 (June 26, 2010), the plants were harvested. The plants were air dried for 10 days, in the dark, under 60-70% humidity, at a temperature of 18C-22C.
2. **Set 2** (A 100 - 199, B1-5, C1-3)
    a. **Harvesting and Drying Conditions**
        i. On day 179 (September 17, 2010), the plants were harvested from the USBG Greenhouse. The plants were air dried for 20 days (?), in the dark, under 50% humidity, at a temperature of 15.5C - 18.3C.
3. **Set 3** (A200-299, B1-5, C1-3)

a. **Growing conditions**

i. On Day 181 (9/20/10), the plants were moved to greenhouse lab space at the University of Maryland College-Park, graciously offered by Dr. Betty Morgavan, the research greenhouse complex director and manager. We were also informed that spider mites grow on our plant.

ii. On Day 352 (3/10/11), we noticed that the plants didn't look very healthy. The leaves and stems were yellowing and the leaves were always closed up on the flowers, rather than opened up as we had seen in the US Botanic Garden. After consulting with experts at the US Botanic Garden and Dr. Morgavan, we realized that salts had been building up in the soil of the plants so we replaced the soil. After the soil change, the plants looked more healthy, fresh, and green. This soil contained 4 parts PX1 (a bark based soilless soil) to 1 part gran-i-grit (crushed granite).

iii. On Day 380 (4/7/11), we noticed that we have West Florida thrips growing on our plant. In 8 samples (one branch size of 2-3 inches) taken, we found 1 thrip per sample on 2 samples.

iv. On Day 395 (4/22/11), we noticed that the plants grow in the presence of fungus gnats, which come around when the soil is wet, as they like to lay their eggs in wet soil and their larvae

cannot exist unless the soil is wet. This usually occurs when seeds are planted due to the soil having to stay wet for germination. The damage from fungus gnats comes from the larvae chewing on roots of very young plants, which is not a a problem with your plants according to the Greenhouse Director, Dr. Morgavan.

v. Periodically during the growing period, we re-potted many plants so that their growth wouldn't be stunted by their pot and lack of nutrients

b. **Harvesting and Drying Conditions**

i. This set of plants were grown under greenhouse conditions were harvested on Day 345 (3/3/11).

ii. The plants were air dried for 7 days, in the dark, at ~40% humidity, and at 18C +/- 2C. Plants were dry to the touch after day 4, but were left until day 7 then harvested into Ziplock bags. Each plant was bundled with a rubber band and hung upside down in a basement room. Plants were not exposed to sunlight nor light of any kind. The humidity was 40% and remained consistent throughout the drying period.

Greenhouse conditions

The greenhouse was regulated at the following conditions for our plant:

1. <u>Shading/Curtains</u>

a. In the greenhouse, the curtains close when it is too hot or if the sun level too high, detecting the light intensity in the form of foot candles (fc)

b. If it is cool outside and not as sunny, then the shades will open.

c. If it is too hot or too sunny, the shades will close all the way.

2. Temperature and Humidity

a. The greenhouse is maintained at 26*C and 50% humidity.

3. Watering

a. Medium watering worked best, and greenhouse staff watered the plants when needed. Some that were root-bound that dried up very quickly.

4. Soil

a. The plants need gravely and lime soil. However, the plants are growing on LC1 soil, which is a peat base, soil-less soil with no organic matter, so it doesn't hold fertilizer. This type of soil is hard to over-fertilize and once the soil is dried out, it is very difficult to re-wet the soil. Heavier soil types tend to hold more water. This type of soil also results in soil compaction, which stresses the plants and caused pink tinge on leaves to appear. The plants were not fertilized.

5. Lights

a. The greenhouse is set to maintain the light range between 10 -30 fc. In addition, the shade curtains will close when the outside light foot

candles gets above 35 foot candles OR the outside temperature gets above 30C.

b. The lights were HID lights, which turn on at 6 AM and off at 8 PM, if it is not too sunny.

c. If the sun is below 10 foot candles (i.e. cloudy), the light will be on.

d. If the sun is too bright at 30 foot candles, light turns off

Appendix B: DNA extraction protocol

(Qiagen, 2006)

DNA extractions were performed on 20 mg dry samples using the DNeasy Plant Mini Kit (Qiagen, 2006). Prior to extraction the samples were lysed using the TissueLyser System (Qiagen) with glass beads. Extraction proceeded following the manufactureres protocol.

Appendix C: PCR Protocol

(Fermentas Life Sciences, 2007), (Ottesen, personal communication, October 2009)

Make sure that the preparation of the PCR mixture is done over ice. The quantity of each component depends on how many PCR reactions we will be setting up. We will be preparing a 20ul reaction mixture. The final concentration of each component should be:

Master-mix	3.0µl
10mM dNTP's	0.8µl
100 µM Forward Primer	0.1µl
100µM Reverse Primer	0.1µl
Bioline Biolase taq (5U/µl)	0.2µl
DNA	1.0µl
H_2O	14.8µl

After all components are assembled, gently vortex the sample and briefly centrifuge to collect all drops from walls of tube. Place the mixture in the thermocycler which will start the amplification process at 95°C for 3 min. Then proceed at 94°C for 30 sec, 55°C for 1 min, and finally 72°C for 1 min. This cycle will be repeated 5 times. Then the reaction will go back up to 94°C for 30 sec, 54°C for 30 min, and finally 72°C for 1 min with a final extension of 10 minutes at 72°C. The reaction will hold at 10°C until we are ready to take out the PCR product.

For matK PCR, 40 cycles instead of 30 cycles will be used at an annealing temperature of 49°. Furthermore, DMSO at 5% total volume is added.

Appendix D: Basic cell maintenance *(Zhang, personal communication, June 2009)*

The purpose for basic cell maintenance is to keep up a cell line to allow for optimal cell survival.

For HIV cell culture system for HIV, a proper media must be made in which cell will grow. To make the B-cell media, combine 435 mL RPMI, 50 mL 10% FBS, 5 mL 1% Pen/Strep, and 10 mL 2% Glutamine. HIV infects white blood cells, which do not replicate; they are produced by the body. Thus, new cells must be acquired for each assay.

For J6JFH cell culture system for HCV, a proper media must be made to allow for optimal cell survival. To make the hepatitis C media, add 445 mL DMEM, 50 mL FBS, and 5 mL Penicillin/Streptomycin.

Protocol for splitting liver cancer cells (Zhang, personal communication, June 2009)

Cells must be split because over time, the cells will grow to the capacity of the plate and have no place else to grow. At that point, the cells will begin to die.

1. Filter medium and trypsin to remove any contaminating bacteria and viruses in the media. The filter has 0.2 micron pores.
2. Add 10-12 mL medium to each flask that the cells will be split into.

3. Label each flask with the passage number (number of times the cell line has been split), desired split ratio i.e. 1:4 ratio from the original stock, name of the cell line, and date
4. Aspirate out medium from original stock.
5. Add 2 mL of trypsin to wash the cell ask to remove any dead cells.
6. Aspirate out the 2 mL of trypsin.
7. Add another 2 mL of trypsin to detach the cells from the bottom of the flask
8. Cap the flask and tip the flask back and forth to evenly distribute the trypsin
9. Incubate the flask at 37°C for 2 minutes
10. Take the flask out and tap the flask on the side. It should be visible whether or not the cells have detached from the flask walls.
11. Add 4 mL of medium and resuspend the cells well with a pipette. Add the appropriate amount of cells to each flask and place in the 37°C incubator until needed. For example, given that the total volume of trypsin and medium at this step is 6 mL, and the desired splits are 1:3, 1:3, 1:6, and 1:8, aliquots would be as follows: 1:3 flask will have 2 mL of the original cell stock; 1:3 flask will have 2 mL of the original cell stock; 1:6 flask will have 1 mL of the original cell stock; 1:8 flask will have 0.75 mL of the original cell stock

Appendix E:Materials for cell culture

Laboratory space in NIH

Dr. Shyam Kottilil has graciously allowed us to conduct our studies in his lab at NIH.

The laboratory is equipped with basic laboratory tools and specific machines such as:

- Applied Biosystems Real-time PCR 7500 machine and software
- Millipore Guava ViaCount Cell Viability machine and software
    - Guava instrument cleaning solution (ICF)
    - Guava ViaCount Reagent

Reagents

- 1% FBS (Fetal Bovine Serum albumin)
- Penicillin/Streptomycin antibiotics
- Recombinant human interferon alpha 2 (Hu-IFN-α2; Hu-IFN-α2b)
- T-20 (HIV conventional treatment)
- RPMI Medium 1640 1x
- Glutamine
- DMEM Medium
- Trypsin
- 98-100% Ethanol
- J6JFH-2a virus standard
- J6JFH-2a virus probe
- J6JFH-2a viral RNA reverse and forward primers

Kits and cell lines

- Cell lines which can be obtained at NIH
    - J6JFH HCV cell culture cells
    - MDM (monocyte-derived macrophages) HIV cell culture cells
- Ambion RNAqueous - 4PCR Kit
- Qiagen Viral RNA Extraction Kit
- 96-well optical reaction plates for PCR
- MiccroAmp, Optical 8-cap strip

Appendix F: Viral RNA extraction

Viral RNA will be extracted from the cell supernatent. The volume for ethanol, supernatant sample volume, and buffer was tripled in order to obtain a high enough viral RNA concentration.

Follow the manufacturer's instructions for QIAamp Viral RNA Mini kit (Qiagen, 2010).

Appendix G: HPLC Protocol (Markom, Hasan, Daud, Singh, & Jahim, 2007), (Murugaiyah & Chan, 2007b)

1. If our most active compounds end up to come from the less polar solvent extracts, we would likely want a **C4 column*** or C8 at the most, where as aqueous extracts would be better separated on a C18 column.

2. The most consistent size for natural product analytical HPLC was 250 mm × 4.6 mm i.d. and 4 µm particle diameter.
3. 1-2 µg of purified extract can be dissolved either in DMSO or a comparable non-reactive solvent (such as acetonitrile) so that no particulates remain then 20 µL will be injected into the column. You do not want the injected volume to be too concentrated otherwise you risk clogging the highly expensive column.
4. The column will then run with a gradient of two solvents with 0.1% HPLC grade Trifluro acetic acid (TFA), which improves the peak shape.
5. We will run an isotonic gradient of acetonitrile (B) and water (A), at around 25:75 volume ratio over a 10 minute period. The flow rate should be held constant at 0.5-1.0 mL/min.
6. As the parts are separated on the column, they will pass through a UV detector at the end. There are two ways to collect spectra depending on the lamp available to you, at a set wavelength, in which 254 nm and 280 nm would be the most efficient one for optimal absorbance of most compounds. The second choice would be to collect PDA spectra, which shows the intensity and amount of absorbance of what is passing the detector across all of the wavelengths it can detect. While the PDA spectra does give far more information, it is often far more than is needed and can complicate the run.

Appendix H: Real-time PCR reaction components ***(Applied Biosystems, 2010)***

Master mix prep for samples	1 reaction
2x Master mix	12.5 µl
40x RNase inhibitor	0.625 µl
3.2 nM forward primer	1.25 µl
3.2 nM reverse primer	1.25 µl
2.5 nm probe	1 µl
Water	0.375 µl
Sample	25 µl

Standard samples	1 reaction
2x Master mix	12.5 µl
40x RNase inhibitor	0.625 µl
3.2 nM forward primer	1.25 µl
3.2 nM reverse primer	1.25 µl
2.5 nm probe	1 µl
Water	3.375 µl
Standard sample	5 µl
Total	20 µl

After loading the plate into the PCR machine, use follow PCR conditions to run the assay: Hold for 30 minutes at 48°C. Hold for 10 minutes at 95°C. Run 40 cycles; during one cycle, denaturing of the RNA will occur at 95°C for 15 seconds and annealing will occur at 60°C for 1 minute.

Appendix I: P-values: p-values for significant differences between viral copies per cell rates in *P. niruri* extracts and DMSO vehicle control at 24 hours (a), 48 hours (b), and 96 hours (c).

Table 7a

Concentration	**Extract 1**	**Extract 2**	**Extract 3**	**Extract 4**	**Extract 5**
0.001 µg/mL	0.032515	0.129655	0.018673	0.054401	0.275389
0.01 µg/mL	0.049336	0.048443	0.059523	0.009211	0.441174
0.1 µg/mL	0.001672	0.492549	0.035099	0.001803	0.000477
1 µg/mL	0.014218	0.868622	0.610586	0.209952	0.012689
10 µg/mL	0.015148	0.019641	0.040052	0.001511	0.012548

Table 7b

	Extract 1	Extract 2	Extract 3	Extract 4	Extract 5
0.001 µg/mL	0.214168	0.050031	0.355779	0.625189	0.00144
0.01 µg/mL	0.48885	0.105866	0.349787	0.022063	0.025158
0.1 µg/mL	0.080976	0.595292	0.324903	0.025015	0.33275
1 µg/mL	0.013046	0.837104	0.34127	0.032028	0.033256
10 µg/mL	0.013235	0.000812	0.135046	0.058814	0.010656

Table 7c

	Extract 1	Extract 2	Extract 3	Extract 4	Extract 5
0.001 µg/mL	0.013329	0.004465	0.014136	0.211342	0.029411
0.01 µg/mL	0.045416	0.37786	0.039387	0.343234	0.814709
0.1 µg/mL	0.089056	0.006538	0.062638	0.337574	0.074147
1 µg/mL	0.488162	0.034517	0.202324	0.007149	0.003391
10 µg/mL	0.099404	0.248611	0.528244	0.001034	0.002403

Appendix J: Time-course treatment schedule: Time-course treatment schedule

The following is a schedule of the time scale treatment. Our cell samples will be treated as follows:

- Sample A: 10 µg/mL plant extract
- Sample B: 1 µg/mL
- Sample C: 0.1 µg/mL
- Sample D: 0.01 µg/mL
- Sample E: 0.001 µg/mL
- Sample F: Positive Control (IFN-alpha for HCV)
- Sample G: Negative Control (No treatment)

Table 8 a: Time Scale Treatment Schedule

Treatment Day/Sample	Day -1	Day 0	Day 4
Sample A-G	Seed the cells	Add plant extract treatment	End of treatment

Table 8 b: Time Scale Treatment and Plates Schedule

Treatment Day/Plate	Day 0	Day 1	Day 2	Day 4
Plate to be sampled on Day 1	Seed the cells	Measure cell viability and save the supernatant to extract viral RNA. Viral RNA levels will be determined by real-time PCR		
Plate to be sampled on Day 2	Seed the cells		Measure cell viability and save the supernatant to extract viral RNA. Viral RNA levels will be determined by real-time PCR	
Plate to be sampled on Day 4	Seed the cells			Measure cell viability and save the supernatant to extract viral RNA. Viral RNA levels will be determined by real-time PCR

Four identical plates will be running at the same time with the exact same conditions as indicated above. At Day 0, we will be seeding the cells, which means

we will be plating freshly split cells onto cell culture plates. After 24 hours, when the cells have had a chance to adhere to the cell culture plate and grow, treatment can begin. At different points in time, we will "sacrifice" a plate to get a measurement of the cell viability and the medium, or supernatant, will be saved and stored in -80°C freezer. That way, we will be able to extract viral RNA from it later and run a real-time PCR assay to determine viral load concentrations.

Appendix K: Set-up of Cell Studies Plates

Plates

Huh7.5 cells

24 hours	1	2	3	4	5	6	7	8	9	10	11	12
concentration of extract	100 ug/ml	10 ug/ml	1 ug/ml	100 ng/ml	10 ng/ml	1 ng/ml	100 ug/ml	10 ug/ml	1 ug/ml	100 ng/ml	10 ng/ml	1 ng/ml
A	Extract 1											
B							Extract 4					
C												
D	Extract 2											
E							Extract 5					
F												
G	Extract 3											
H							Blank					

J6JFH cells

24 hours	1	2	3	4	5	6	7	8	9	10	11	12
concentration of extract	100 ug/ml	10 ug/ml	1 ug/ml	100 ng/ml	10 ng/ml	1 ng/ml	100 ug/ml	10 ug/ml	1 ug/ml	100 ng/ml	10 ng/ml	1 ng/ml
A	1											
B							4					
C												
D	2											
E							5					
F												
G	3											
H							Blank					

Controls at 24 hours

	1	2	3	4	5	6	7	8
concentration of extract	100 ug/ml	10 ug/ml	10 ug/ml	100 ng/ml	10 ng/ml	1 ng/ml	Untreated control	10 IU/ml IFN on J6JFH cells
A	DMSO + **HUH** cells + medium at 96 hours						Huh	
B							Huh	
C							Huh	
D	DMSO+**J6JFH** cells+medium at 96 hours						J6JFH	IFN+J6
E							J6JFH	IFN+J6
F							J6JFH	IFN+J6
G	Blank	Blank	Blank					
H								

Standard Curve

Day 0	1	2	3	4	5	6
Concentration of cells	40 x 10^3 cells/well	20 x 10^3 cells/well	10 x 10^3 cells/well	5 x 10^3 cells/well	2.5 x 10^3 cells/well	1.25 x 10^3 cells/well
A	Huh 7.5 cells					
B						
C						
D	J6JFH					
E						
F						
G						
H	Blank	Blank	Blank			

Appendix L: HPLC Chromatograms

==== Shimadzu LCsolution Analysis Report ====

Acquired by	: Admin
Sample Name	: sample 1 0.5mg/mL, trial 1, day 2
Sample ID	: sample 1
Vail #	: 1
Injection Volume	: 20 uL
Data File Name	: Sample 1 25_75 Solvent Comp2.lcd
Method File Name	: hplc_isocratic.lcm
Batch File Name	:
Report File Name	:
Data Acquired	: 12/1/2011 1:55:35 PM
Data Processed	: 12/1/2011 2:05:30 PM

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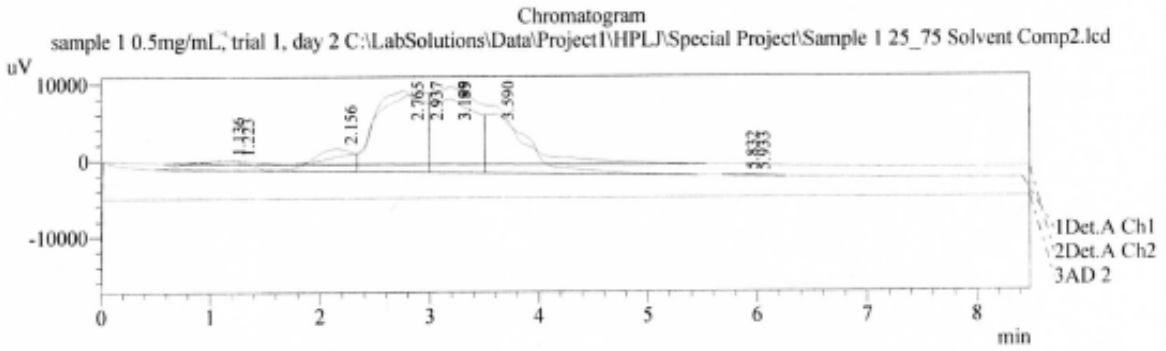

1 Det.A Ch1 / 281nm
2 Det.A Ch2 / 254nm
3 AD 2 /

<Results>

Quantitative Results

Detector A

ID#	Name	Ret. Time	Area	Height	Conc.
1	RT1.136	1.136	42837	1254	0.000
2	RT2.156	2.156	69826	2876	0.000
3	RT2.765	2.765	325108	10588	0.000
4	RT3.189	3.189	264974	9572	0.000
5	RT3.590	3.590	206447	7686	0.000

==== Shimadzu LCsolution Analysis Report ====

Acquired by	: Admin
Sample Name	: sample 2 0.5mg/mL, trial 1, day 2
Sample ID	: sample 2
Vail #	: 1
Injection Volume	: 20 uL
Data File Name	: Sample 2 25_75 Solvent Comp2.lcd
Method File Name	: hplc_isocratic.lcm
Batch File Name	:
Report File Name	:
Data Acquired	: 12/1/2011 2:07:48 PM
Data Processed	: 12/1/2011 2:23:29 PM

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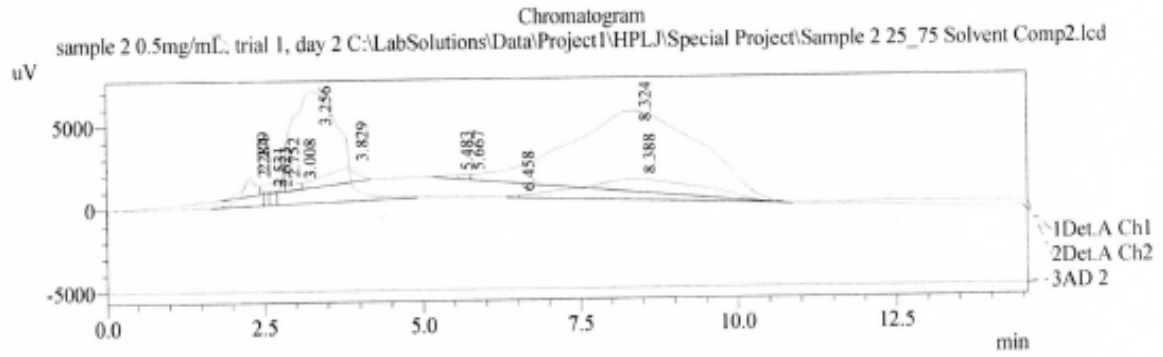

1 Det.A Ch1 / 281nm
2 Det.A Ch2 / 254nm
3 AD 2 /

<Results>

Quantitative Results

Detector A

ID#	Name	Ret. Time	Area	Height	Conc.
1	RT2.284	2.284	14494	856	0.000
2	RT2.752	2.752	13210	598	0.000
3	RT3.829	3.829	35023	972	0.000
4	RT8.324	8.324	714653	4848	0.000
5	RT2.279	2.279	30314	1765	0.000
6	RT3.256	3.256	353447	6666	0.000
7	RT8.388	8.388	165890	1245	0.000

==== Shimadzu LCsolution Analysis Report ====

Acquired by	: Admin
Sample Name	: sample 3 0.5mg/mL, trial 1, day 2
Sample ID	: sample 3
Vail #	: 1
Injection Volume	: 20 uL
Data File Name	: Sample 3 25_75 Solvent Comp2.lcd
Method File Name	: hplc_isocratic.lcm
Batch File Name	:
Report File Name	:
Data Acquired	: 12/1/2011 2:26:39 PM
Data Processed	: 12/1/2011 2:34:23 PM

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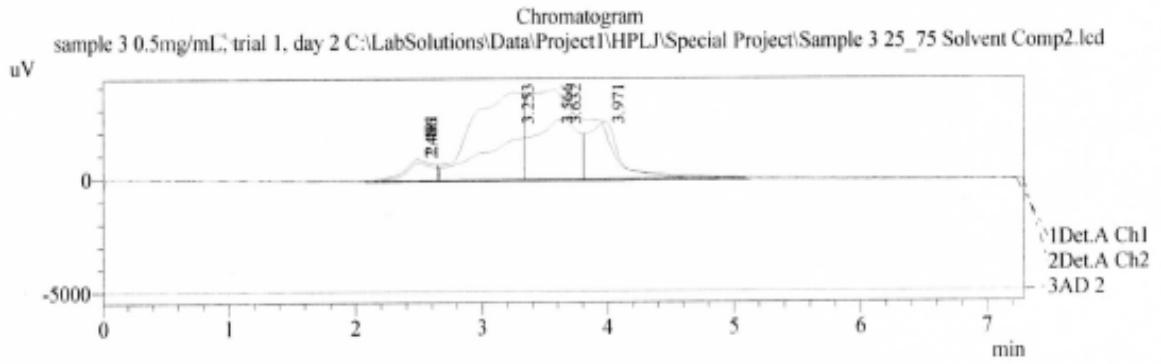

1 Det.A Ch1 / 281nm
2 Det.A Ch2 / 254nm
3 AD 2 /

<Results>

Quantitative Results

Detector A

ID#	Name	Ret. Time	Area	Height	Conc.
1	RT2.486	2.486	12929	799	0.000
2	RT3.632	3.632	113080	2676	0.000
3	RT3.971	3.971	46013	2587	0.000
4	RT2.481	2.481	14888	921	0.000
5	RT3.253	3.253	105624	3819	0.000
6	RT3.566	3.566	143199	3931	0.000

==== Shimadzu LCsolution Analysis Report ====

Acquired by	: Admin
Sample Name	: sample 4 0.5mg/mL, trial 1, day 2
Sample ID	: sample 4
Vail #	: 1
Injection Volume	: 20 uL
Data File Name	: Sample 4 25_75 Solvent Comp2.lcd
Method File Name	: hplc_isocratic.lcm
Batch File Name	:
Report File Name	:
Data Acquired	: 12/1/2011 2:36:56 PM
Data Processed	: 12/1/2011 2:45:31 PM

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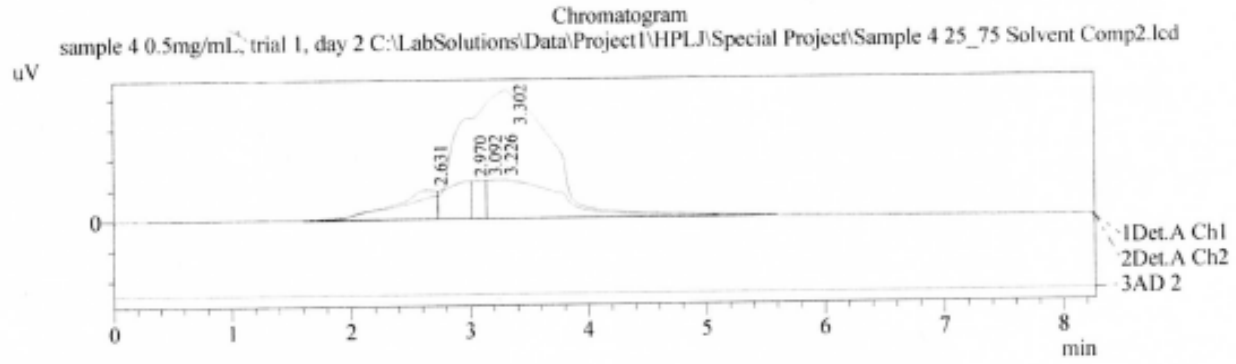

1 Det.A Ch1 / 281nm
2 Det.A Ch2 / 254nm
3 AD 2 /

<Results>

Quantitative Results

Detector A

ID#	Name	Ret. Time	Area	Height	Conc.
1	RT2.631	2.631	49069	1905	0.000
2	RT2.970	2.970	37647	2456	0.000
3	RT3.092	3.092	18730	2523	0.000
4	RT3.226	3.226	108684	2545	0.000
5	RT3.302	3.302	478741	8417	0.000

==== Shimadzu LCsolution Analysis Report ====

Acquired by	: Admin
Sample Name	: sample 5 0.5mg/mL, trial 1, day 2
Sample ID	: sample 5
Vail #	: 1
Injection Volume	: 20 uL
Data File Name	: Sample 5 25_75 Solvent Comp2.lcd
Method File Name	: hplc_isocratic.lcm
Batch File Name	:
Report File Name	:
Data Acquired	: 12/1/2011 2:47:20 PM
Data Processed	: 12/1/2011 2:56:05 PM

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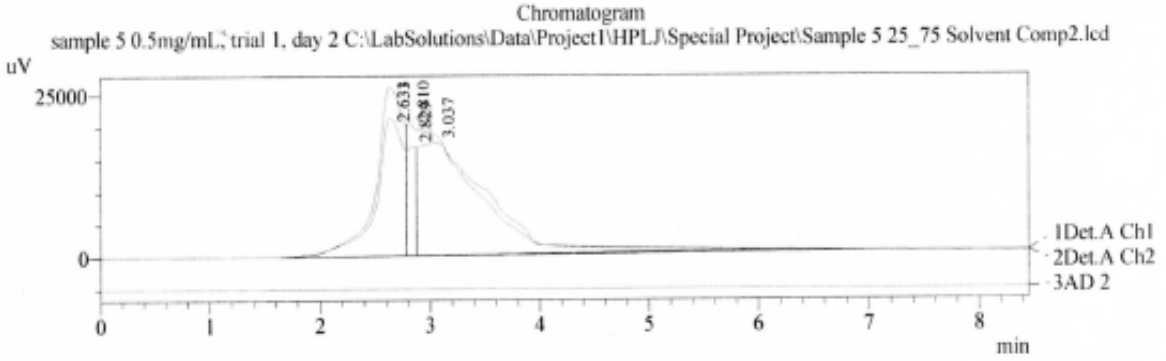


1 Det.A Ch1 / 281nm
2 Det.A Ch2 / 254nm
3 AD 2 /

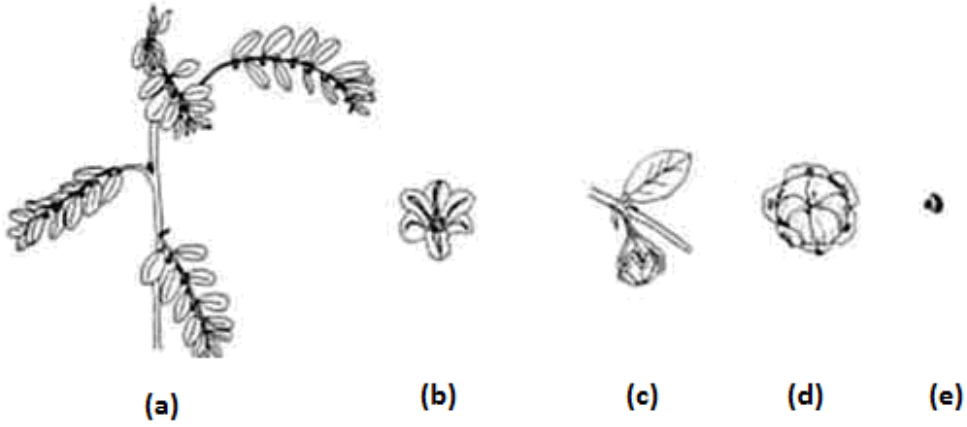
<Results>

Quantitative Results

Detector A

ID#	Name	Ret. Time	Area	Height	Conc.
1	RT2.631	2.631	455804	25949	0.000
2	RT2.810	2.810	862804	20346	0.000
3	RT2.633	2.633	374112	21261	0.000
4	RT2.829	2.829	91643	16847	0.000
5	RT3.037	3.037	802205	17351	0.000

Appendix M: Anatomy of *Phyllanthus Niruri* (Krishnamurthy, 2012)



Drawing of the anatomy of *Phyllanthus niruri* that includes the (a) the branch, (b) male flower structure, (c) female flower structure, (d) capsule, and (e) seed.

Glossary

Amplify: increase the number of copies of a specific gene using PCR

Analytical HPLC: High Pressure Liquid Chromatography. This type of chromatography follows the same principles as basic chromatography but allows the user far better control over the system. With HPLC you are able to control solvent flow, gradient, and specific run time. The columns utilized in this form of chromatography also are far more controlled.

C4/C8/C18 HPLC Column: HPLC column are filled with tiny glass beads coated with varying sizes of hydrocarbon chains. The length of the carbon chain determines the "stickiness of a column". A C4 column would mean that there is are many four carbon chains attached to the bead available to interact with what is injected into the column. A long carbon chain has a better capability to interact with an hold on longer to compounds flowing through the column. The more nonpolar the compound flowing through, the even strong the interaction where as a polar molecule will be able to flow through with less interactions. To have optimal separation, meaning compounds will come out separately from each other, selection of the proper column is essential. Since nonpolar molecules will be mostly found in the organic extracts, which utilize non polar solvents, you would want to use a column with a short carbon chain (C4 or possibly C8) otherwise the column may never eluted the compound. (Think about stick two sides of tape together). Where as aqueous extract which would contain polar compounds, thus you would want to use a "stickier" column to maximize the interactions thus get better separation.

Conventional drug therapy: standardized treatment currently used against HIV and HCV; T-20 is a potent synthetic protein that inhibits the replication of HIV-1. Interferon-alpha is a chemokine, or a white blood cell that attracts other immune system cells to a site of injury in the body, and is the standard treatment against HCV. This drug is often combined with ribavirin to stabilize the interferon in the body to prevent it from degradation by enzymes.

Chromatography: is a method of separating compounds based on physically properties, in our case polarity. All chromatography has three major elements. The mobile phase is the solvent you flow through the column. The stationary phase part that remains stationary in the column. Both of these interact with your the compound. The compound will want to flow through the column with the mobile phase, but will also interact with the stationary phase to prevent movement. The relative polarities of the compound compared to the mobile phase and the stationary phase determine how it will move through the column. If there are multiple compounds, each with different physical properties they will move through the column with different rates, thus allowing you to separate one from another.

DNeasy spin column: a type of microcentrifuge tube with a filter to catch DNA

Flow cytometry: technique for counting, examining, and sorting microscopic particles, such as cells and chromosomes. The particles are suspended in fluid and analyzed through electronic detection.

Flow rates: the actual amount of liquid moving through the HPLC column per a unit of time

Genetic barcode: due to the evolution of DNA over time, certain genes can be found ubiquitously in many living organisms, such as plants. For a plant genetic barcode, the gene must be common enough across all plant species, but distinct enough that the sequence of the gene is different in each species. If a gene fulfills these requirements, then the sequence of that gene will be associated with a specific species, and thus be used to identify a plant.

LC/MS Mass spectrometry: Liquid Chromatography/Mass Spectrometer. Molecules are separated through HPLC and then aerosol and hit with a beam of high speed electrons to cause fractionation. The fractions each have a positive charge in at least one place, and pulled across a magnetic field to a detector on the other side. More massive particles will be more difficult to move to the detector and will hit lower on the detector depended on their mass. the place where their hit the detector, combined with their know charge can be used to find the molecular weight of a molecule.

Lyse: break open cells for the purposes of extracting intracellular material, such as DNA, RNA, proteins, etc...

Lyophilize: freeze-dry

Lymphocyte: white blood cell of the immune system to help fight off infection or disease

Mist room: a room that maintains humidity and soil moisture at appropriate levels in a greenhouse using misting apparatuses

Molecular barcode: see genetic barcode

NMR: Nuclear Magnetic Resonance; the effect of a magnetic field on a molecule will result in a distinct graph of speaks, which can be used to identify the molecule's structure

Partitioned: separation of compounds through their solubility in two immiscible layers ie. two liquids that do not mix are water and oil

Polymerase chain reaction (PCR): a technique to amplify DNA by generating thousands of copies of a particular DNA sequence through a repeated cycle of heating and cooling.

Primer: a series of nucleotides that catalyze DNA replication.

QIA shredder: type of microcentrifuge tube that is used in the lysis of cells

Reflux: involves letting a reaction boil with a condenser above it. This serves to allow the liquid to completely enter the gaseous phases before recondensing and dripping back down into the reaction mixture. It is a technique often used in many organic chemistry processes.

Retention Time: this is a property of HPLC. Once we have specifically defined the parameters for our HPLC separation methods (flow rate, solvent gradient, column length and type, run length) the time at which a compound elutes is known as the retention time. It is specific to that compound and can be used for identification.

Sequencing: to sequence a fragment of DNA means to find the order of the DNA nucleotides (adenosine (A), cytosine (C), thymine (T), and guanine (G))

Soxhlet extraction: an extraction performed by boiling a solvent with the plant material and allowing the solvent, along with the soluble compounds, to vaporize and recondense into a separate collection flask.

Solvent gradient: this involves change the rate and percentage of solvent flow optimal peak shape in HPLC. HPLC utilizes two different solvents denoted by A and B. We will have solvent A as water and B as acetonitrile. The solvent in chromatography is known as the mobile phase and is what essentially pulls the compounds along the beads (known as the stationary phase). Starting with a solvent ratio of A 100: B 0 you are able to pull along compounds that are highly polar as it will have a stronger interaction with the mobile phase than with the stationary phase. Then by changing the solvent ration you can pull along other compounds with different polarities. The method through which you change the solvent ratios over time is known as the solvent gradient. Different gradients, combined with flow rates change how the compounds exit the column and are picked up by the UV detector.

Washing: using solvents and wash detergents to purify genetic material, such as RNA or DNA

UV Spectra: a graph of the ultraviolet absorbency of compounds at different ultraviolet wavelengths.

Vacuum dessicator: specialized container to protect moisture-sensitive reagents

Viral load: level of viral genetic material in the cell and serves as an indication for the severity of a advancement of in cases such as HIV and HCV

Bibliography

Aita, A. M., Matsuura, H. N., Machado, C. A., & Ritter, M. R. (2009). Medicinal species sold as "quebra-pedras" in Porto Alegre, Rio Grande do Sul, Brazil. *Revista Brasileira De Farmacognosia-Brazilian Journal of Pharmacognosy, 19*(2A), 471-477.

Applied Biosystems. (2010). TaqMan One-Step RT-PCR Master Mix Reagents Kit Protocol. pp. 1-44.

Bartenschlager, R. (2005). The hepatitis C virus replicon system: From basic research to clinical application. *Journal of Hepatology, 43*(2), 210-216.

Bartenschlager, R., & Lohmann, V. (2000). Replication of hepatitis C virus. *Journal of General Virology*, *81*, 1631-1648.

Bartenschlager, R., & Sparacio, S. (2007). Hepatitis C virus molecular clones and their replication capacity in vivo and in cell culture. *Virus Research, 127*(2), 195-207.

Bhattacharjee, R., & Sil, P.C. (2006). The protein fraction of *Phyllanthus niruri* plays a protective role against acetaminophen induced hepatic disorder via its antioxidant properties. *Phytotherapy Research, 20*(7), 595-601.

Bhattacharyya, S., & Bhattacharya, R. (2003). Establishment of *Phyllanthus amarus* root clone with significant activity against bovine viral diarrhoea virus. *Current Science*, *84*(4), 529-533.

Bjelakovic, G., Gluud, L.L., Nikolova, D., Bjelakovic, M., Nagorni, A., & Gluud, C. (2011). Antioxidant supplements for liver diseases. *Cochrane Database of Systematic Reviews*, 3.

Brok, J., Gluud, L.L., Gluud, C. (2010). Meta-analysis: ribavirin plus interferon vs. interferon monotherapy for chronic hepatitic C – an updated Cochrane review. *Alimentary Pharmacology and Therapeutics, 32*(7), 840-850.

Buckwold, V.E., Beer, B.E., & Donis, R.O. (2003). Bovine viral diarrhea virus as a surrogate model of hepatitis C virus for the evaluation of antiviral agents. *Antiviral Research, 60*(1), 1-15.

Calixto, J.B. (2000). Efficacy, safety, quality control, marketing and regulatory guidelines for herbal medicines (phytotherapeutic agents). *Brazilian Journal of Medical and Biological Research, 33*(2), 179-189.

Calixto, J.B. (2005). Twenty-five years of research on medicinal plants in Latin America: A personal view. *Journal of Ethnopharmacology, 100*, 131-134.

Calixto, J.B., Santos, A., Cechinel Filho, V., & Yunes, R. (1998). A review of the plants of the genus Phyllanthus: their chemistry, pharmacology, and therapeutic potential. *Medicinal Research Reviews, 18*(4), 225-258.

Canavan, N. (2012, March 30). Boceprevir, interferon alfa-2a, ribavirin effective in hcv. *Medscape*. Retrieved from http://www.medscape.com/viewarticle/761264

Cartaxo, S. L., Souza, M. M. D., & de Albuquerque, U. P. (2010). Medicinal plants with bioprospecting potential used in semi-arid northeastern Brazil. [Article]. *Journal of Ethnopharmacology, 131*(2), 326-342.

CDC. (2009, June 9). *Hepatitis C faqs for the public*. Retrieved from http://www.cdc.gov/hepatitis/C/cFAQ.htm

Chatterjee, M., Sarkar, K., & Sil, P. (2006). Herbal (*Phyllanthus niruri*) protein isolate protects liver from nimesulide induced oxidative stress. *Pathophysiology, 13*(2), 95-102.

Chevallier, A. (1996). *The Encyclopedia of Medicinal Plants: A Practical Reference Guide to over 550 Key Herbs and Their Medicinal Uses*. University of Michigan, Michigan: DK Pub.

Chirdchupunseree, H., & Pramyothin, P. (2010). Protective activity of phyllanthin in ethanol-treated primary culture of rat hepatocytes. *Journal of Ethnopharmacology*, *128*(1), 172-176.

Chitturi, S., & Farrell, G.C. (2000). Herbal hepatotoxicity: An expanding but poorly defined problem. *Journal of Gastroenterology and Hepatology, 15*, 1093-1099.

Colombo, R., de L Batista, A., Teles, H., Silva, G., Bomfim, G., Burgos, R., . . . Heimberg, M. (2009). Validated HPLC method for the standardization of *Phyllanthus niruri* (herb and commercial extracts) using corilagin as a phytochemical marker. *Biomedical Chromatography, 23*(6), 573-580.

Das, B., Bepary, S., Datta, B., Chowdhury, A., Ali, M., & Rouf, A. (2008). Hepatoprotective activity of Phyllanthus reticulatus. *Pakistan Journal of Pharmaceutical Sciences, 21*(4), 333-337.

de Mochel, N. S. R., Seronello, S., Wang, S. H., Ito, C., Zheng, J. X., Liang, T. J., …Choi, J. (2010). Hepatocyte NAD(P)H oxidases as an endogenous source of reactive oxygen species during hepatitis C virus infection. *Hepatology, 52*. 47–59. doi: 10.1002/hep.23671

Dugdale, D. C., Longstreth, G. F., & Zieve, D. (2010, November 23). *Hepatitis C*. Retrieved from http://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0001329/

Duke, J. A. (2009). Dr. Duke's Phytochemical and Ethnobotanical Databases. Retrieved February 11, 2012, from http://sun.ars-grin.gov:8080/npgspub/xsql/duke/plantdisp.xsql?taxon=725

Duke, J. A. (2002). Your instructor: Jim Duke (James A. Duke), from http://www.ars-grin.gov/duke/syllabus/instructor.htm

Duverlie, G & Wychowski, C. (2007). Cell culture systems for the hepatitis C virus. *World Journal of Gastroenterology*. *13*(17): 2442-2445.

Elvin-Lewis, M. (2000). Should we be concerned about herbal remedies. *Journal of Ethnopharmacology, 75*, 141-164.

FDA. (2011, May 25). *Viral hepatitis therapies*. Retrieved from http://www.fda.gov/ForConsumers/ByAudience/ForPatientAdvocates/ucm151494.htm

Fried, M.W. (2002). Side effects of Therapy of Hepatitis C and Their Management. *Hepatology, 36*, 237-244.

Gane, E. J., Weilert, F., Orr, D. W., Keogh, G. F., Gibson, M., Lockhart, M. M., Frampton, C. M.,…Murphy, M. P. (2010). The mitochondria-targeted anti-oxidant mitoquinone decreases liver damage in a phase II study of hepatitis C

patients. *Liver International*, *30*, 1019–1026, doi: 10.1111/j.1478-3231.2010.02250.x

Gosser, Y., Hermann, T., Majumdar, A., Hu, W., Frederick, R., Jiang, F.,…Patel, D.J. (2001). Peptide-triggered conformational switch in HIV-1 RRE RNA complexes. *Nature Structural Biology, 8,* 146-150.

Harish, R., & Shivanandappa, T. (2006). Antioxidant activity and hepatoprotective potential of *Phyllanthus niruri*. *Food Chemstry, 95*(2), 180-185.

Harmon, K., (2012, February 20). Hepatitis C now killing more Americans than HIV. Retrieved from http://blogs.scientificamerican.com/observations/2012/02/20/hepatitis-c-now-killing-more-americans-than-hiv/

Hollingsworth, P. M. (2008). DNA barcoding plants in biodiversity hot spots: Progress and outstanding questions. *Heredity, 101*(1), 1-2.

Hollingsworth, P. M., Forrest, L. L., Spouge, J. L., Hajibabaei, M., Ratnasingham, S., van der Bank, M., ...Little, D.P. (2009). A DNA barcode for land plants. *Proceedings of the National Academy of Sciences of the United States of America, 106*(31), 12794-12797.

Kapoor, L. D. (2001). *Handbook of Ayurvedic Medicine*. CRC Press LLC.

Khanna, A.K., Rizvi, F., & Chander, R. (2002). Lipid lowering activity of *Phyllanthus niruri* in hyperlipemic rats. *Journal of Ethnopharmacology, 82*(1), 19-22.

Khatoon , S., Rai, V., Rawat, A. K., & Mehrota, S. (2006). Comparative pharmacognostic studies of three phyllanthus species. *Journal of Ethnopharmacology*, *104*, 79-86.

Kottilil, S., Jackson, J., & Polis, M. (2005). Hepatitis B & hepatitis C in HIV-infection. *Indian Journal of Medical Research, 121*(4), 424-450.

Kress, W., & Erickson, D. (2007). A two-locus global DNA barcode for land plants: the coding rbcL gene complements the non-coding trnH-psbA spacer region. *Public Library of Science, 2*(6), e508.

Kress, W. J., & Erickson, D. L. (2008a). DNA barcodes: Genes, genomics, and bioinformatics. *Proceedings of the National Academy of Sciences of the United States of America, 105*(8), 2761-2762.

Kress, W. J., & Erickson, D. L. (2008b). DNA barcoding - a windfall for tropical biology? *Biotropica, 40*(4), 405-408.

Kress, W., Wurdack, K., Zimmer, E., Weigt, L., & Janzen, D. (2005). Use of DNA barcodes to identify flowering plants. *Proceedings of the National Academy of Sciences of the United States of America,* 102(23), 8369-8374.

Krishnamurthy, K. (2012). Bhūmī āmalakī, Phyllanthus. *Journal of New Approaches to Medicine and Health*, 19(4).

Lambert, J. B., Shurvell, H. F., Lightner, D. A., & Cooks, R. G. (Eds.). (1998). *Organic Structural Spectroscopy* (1st ed.). Upper Saddle River, New Jersey: Prentice-Hall Inc.

Lee, S. H., Jaganath, I. B., Wang, S. M., & Sekaran, S. D. (2011). Antimetastatic effects of *Phyllanthus* on human lung (A549) and breast (MCF-7) cancer cell Lines. [Article]. *Public Library of Science ONE, 6*(6), 14.

Lindenbach, B., Evans, M., Syder, A., Wölk, B., Tellinghuisen, T., Liu, C.,...Rice, C. (2005). Complete replication of hepatitis C virus in cell culture. *Science*, *309*(5734), 623-626.

Linneaus, C. (1738). *Hortus cliffortianus*. (p. 439).

Manjrekar, A. P., Jisha , V., Bag, P. P., Adhikary, B., Pai, M. M., Hegde, A., & Nandini, M. (2008). Effect of phyllanthus niruri linn. treatment on liver, kidney and testes in ccl4 induced hepatotoxic rats. *Indian Journal of Experimental Biology*, *46*, 514-520.

Markom, M., Hasan, M., Daud, W. R. W., Singh, H., & Jahim, J. M. (2007). Extraction of hydrolysable tannins from Phyllanthus niruri Linn.: Effects of solvents and extraction methods. *Separation and Purification Technology, 52*(3), 487-496.

Martin, G.J., (1995). *Ethnobotany: A People and Plants Conservation Manual*. London: Chapman & Hall.

Martins, L.R., Pereira-Filho, E.R., & Cass, Q.B. (2011). Chromatographic profiles of *phyllanthus* aqueous extracts samples: a proposition of classification using chemometric models . *Analytical and bioanalytical chemistry*, *400*, 469-481.

Mitra, R. L., & Jain, S. K. (1985). Concept of *phyllanhus niruri* (euphorbiaciae) in Indian floras. *Bulletin of the botanical survey of india*, *27*(1-4), 161-176.

Moradpour, D., Penin, F., & Rice, C. (2007). Replication of hepatitis C virus. *Nature Reviews Microbiology*, *5*, 453-463.

Moriya, K., Miyoshi, H., Shinzawa, S., Tsutsumi, T., Fujie, H., Goto, K., …Koike, K. (2010). Hepatitis C virus core protein compromises iron-induced activation of antioxidants in mice and HepG2 cells. *Journal of Medical Virology, 82*, 776–792.

Murugaiyah, V., & Chan, K. L. (2007a). Analysis of lignans from *Phyllanthus niruri L.* in plasma using a simple HPLC method with fluorescence detection and its application in a pharmacokinetic study. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences, 852*(1-2), 138-144.

Murugaiyah, V., & Chan, K. L. (2007b). Determination of four lignans in *Phyllanthus niruri L.* by a simple high-performance liquid chromatography method with fluorescence detection. *Journal of Chromatography A, 1154*(1-2), 198-204.

Naik, A.D., & Juvekar, A.R. (2003). Effects of alkaloidal extract of *Phyllanthus niruri* on HIV replication. *Indian Journal of Medical Sciences, 57*(9), 387-393.

Nakamura, M., Saito, H., Hokari, R., Kato, N., Hibi, T., & Miura, S. (2010). An antioxidant resveratrol significantly enhanced replication of hepatitis C virus. *World Journal of Gastroenterology*, *16*(2), 184-192.

NIAID. (2009, October 1). *Hepatitis C*. Retrieved from http://www.niaid.nih.gov/topics/hepatitis/hepatitisc/Pages/Default.aspx

Nikam, M.S., Nikam, S.V., Sontakke, A.V., Khanwelkar, A.A. (2011). Role of *Phyllanthus amarus* treatment in Hepatitis-C. *Biomedical Research, 22*(30), 319-322.

Ogata, T., Higuchi, H., Mochida, S., Matsumoto, H., Kato, A., Endo, T., . . . Kaji, H. (1992). HIV-1 reverse-transcriptase inhibitor from *Phyllanthus-niruri*. *Aids Research and Human Retroviruses, 8*(11), 1937-1944.

Ottesen, A. (2009). [Personal Communication].

Patel, J. R., Tripathi, P., Sharm, V., Chauhan, N. S., & Dixit, V. K. (2011). *Phyllanthus amarus*: Ethnomedicinal uses, phytochemistry and pharmacology. *Journal of ethnopharmocology*, *138*, 286-313.

Promega (2009). CellTiter 96 AQueous One Solution Cell Proliferation Assay. pp. 1-11.

Qian-Cutrone, J., Huang, S., Trimble, J., Li, H., Lin, P., Alam, M., Klor, S. E., & Kadow, K. F. (1996). Niruriside, a new hiv rev/rre binding inhibitor from *Phyllanthus niruri. Journal of Natural Products*, *59*, 196-199.

Qiagen. (2006). DNeasy Plant Handbook. pp. 1-58.

Qiagen. (2010). QIAmp Viral RNA Mini Handbook. pp. 1-44.

Ravikumar, Y.S., Ray, U., Nandhitha, M., Perween, A., Naika, H.R., Khanna, N., Das, S. (2011). Inhibition of hepatitis C virus replication by herbal extract: *Phyllanthus amarus* as potent natural source. *Virus Research, 158*, 87-97.

Russo, M.W., & Fried, M.W. (2003). Side effects of therapy for chronic hepatitis C. *Gastroenterology, 124*(6), 1711-1719.

Sabir, S.M., & Rocha, J.B.T. (2008). Water-extractable phytochemicals from *Phyllanthus niruri* exhibit distinct in vitro antioxidant and in vivo hepatoprotective activity against paracetamol-induced liver damage in mice. *Food Chemistry, 111*(4), 845-851.

Sahoo, N., Manchikanti, P., & Dey, S. (2010). Herbal drugs: Standards and regulation. *Fitoterapia, 81*(6), 462-471.

Saitou, N., & Nei, M. (1987). The neighbor-joing method-a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution, 4*(4), 406-425.

Sarkar, M., & Sil, P. (2007). Hepatocytes are protected by herb *Phyllanthus niruri* protein isolate against thioacetamide toxicity. *Pathophysiology, 14*(2), 113-120.

Singh, A. K., Raghubanshi, A. S., & Singh, J. S. (2002). Medical ethnobotany of the tribals of Sonaghati of Sonbhadra district, Uttar Pradesh, India. *Journal of Ethnopharmacology, 81*(1), 31-41.

Srirama, R., Senthilkumar, U., Sreejayan, N., Ravikanth, G., Gurumurthy, B. R., Shivanna,...Shaanker, R.U. (2010). Assessing species admixtures in raw drug trade of *Phyllanthus*, a hepato-protective plant using molecular tools. *Journal of Ethnopharmacology*, *130*, 208-215.

Unander, D. W., Bryan, H. H., Lance, C. J., & McMillan, R. T. (1993). Cultivation of *Phyllanthus-amarus* and evaluation of variables potentially affecting yield and the inhibition of viral-DNA polymerase. *Economic Botany, 47*(1), 79-88.

U.S. Department of Veterans Affairs, (2011). Hepatitis C treatment: Entire lesson. Retrieved from website: http://www.hepatitis.va.gov/patient/treat/single-page.asp

Venkateswaran, P.S., Millman, I., & Blumberg, B.S. (1987). Effects of an extract from *Phyllanthus niruri* on hepatitis B and woodchuck hepatitis viruses: in vitro and in vivo studies. *Proceedings of the National Academy of Sciences, 84*(1), 274-278.

Venturini D., Simão, A.N., Barbosa, D.S., Lavado, E.L., Narciso, V. E., Dichi, I.,… Dichi, J. B. (2010). Increased oxidative stress, decreased total antioxidant capacity, and iron overload in untreated patients with chronic hepatitis C. *Digestive Diseases and Sciences*, *55*(4), 1120-1127.

Vijendra, N., Khatri, P.K. (2010). Traditional knowledge on ethno-medicinal uses prevailing in tribal pockets of Chhindwara and Betul Districts, Madhya Pradesh, India. *African Journal of Pharmacy and Pharmacology, 4*(9), 662-670.

Vieira, R. (1999). Perspectives on new crops and new uses. In J. Janick (Ed.), *Perspectives on new crops and new uses* (pp. 152-159). Alexandria, VA: ASHS Press.

What is dna barcoding? - identifying species with dna barcoding. (2012). Retrieved from http://www.barcodeoflife.org/content/about/what-dna-barcoding

White, C.P, Hirsch, G., Patel, S., Adams, F., & Peltekian, K.M. (2007). Complementary and alternative medicine use by patients chronically infected with hepatitis C virus. *Canadian Journal of Gastroenterology, 21*(9), 589-595.

Webster, G. L. (1957). A monographic study of the west indianspecies of *Phyllanthus*. *Journal of the Arnold Arboretum*, *38*, 51-80, 170-178, 295-373 .

Whiting, P.W., Clouston, A., & Kerlin, P. (2002). Black cohosh and other herbal remedies associated with acute hepatitis. *Medical Journal of Australia,* 177(8), 440-443.

Wiart, C. (2006a). *Ethnopharmacology of medicinal plants: Asia and the pacific*. (pp. xxi-228). Totowa, NJ: Humana Press.

Wiart, C. (2006b). *Medicinal plants of the Asia-Pacific: Drugs for the future?* (pp. 1-756). Toh Tuck Link, Singapore: World Scientific Co. Pte. Ltd.

World Health Organization, (2000). General guidelines for methodologies on research and evaluation of traditional medicine. Retrieved from website: http://whqlibdoc.who.int/hq/2000/WHO_EDM_TRM_2000.1.pdf

World Health Organization. (2011, June). *Hepatitis C fact sheet*. Retrieved from http://www.who.int/mediacentre/factsheets/fs164/en/index.html.

Yang, C., Cheng, H., Lin, T., Chiang, L., & Lin, C. (2005). Acetone, ethanol and methanol extracts of *Phyllanthus urinaria* inhibit HSV-2 infection in vitro. *Antiviral Research, 67*(1), 24-30.

Zhang, X. (2009). [Personal Communication].