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EFFECT OF CHINESE KNOTWEED EXTRACT ON

HSV-1 INFECTION OF VERO CELLS

By

Derek J. Prince

Submitted in partial fulfillment of the requirements for the
degree of Master of Science in Microbiology from the
Department of Biological Sciences of Seton Hall University

May 2015

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Abstract

HSV-1 is an opportunistic virus responsible for infecting the majority of the global population. The ability of this virus to strategically alternate between lytic and latent states has made the development of effective treatments challenging. *Polygonum multiflorum* (Chinese Knotweed, CK) is a perennial plant local to the south central regions of China. The thunb (root) constituents of this compound have been used in traditional Chinese medicines to treat a broad range of illnesses for centuries. In the present study, CK root extracts were evaluated for potential anti-HSV-1 activity. Cytopathic effect (CPE) monitoring studies indicate that Vero cells infected with HSV-1 pre-treated with 0.1% CK showed no signs of CPE after seven days. The potential cytotoxic effect of the compound was also assessed via cytotoxicity and cell proliferation assays. These results indicate that a 1% CK concentration displayed no signs of cytotoxicity. In order to quantify viral inhibition and determine the compounds potential mechanism of action (MOA), plaque reduction and binding assays were performed. These assays suggest that 0.1% CK concentrations are able to reduce viral infectivity by >99% by inhibiting viral binding. Similarly, viral tracking assays using flow cytometry and fluorescent microscopy suggest that as the CK concentrations are increased, HSV-1 infection is decreased. Furthermore, molecular analysis from real-time quantitative PCR (qPCR) assays suggest that 0.1% CKs concentrations are able to inhibit >98% of HSV-1 viral entry when compared to untreated HSV-1 positive controls. Overall, CKs ability to prevent *in vitro* HSV-1 infection may provide clinicians a potential natural alternative and/or synergistic agent to current HSV-1 therapies.

Introduction

Herpes simplex viruses (HSV), such as herpes simplex virus type-1 and type-2 (HSV-1 & HSV-2), are capable of infecting a broad range of animal hosts (Karasneh and Shukla, 2011). These viruses are well studied and it is accepted that up to 80% of the global population is infected with HSV-1 while close to 40% are infected with HSV-2 (Akhtar and Shukla, 2009). Symptoms of HSV-1 infection are primarily categorized as oral and ocular lesions while HSV-2 is generally responsible for genital lesions. However, either viral type can infect oral or genital sites (Lafferty et al., 2000). Although HSV-1 symptoms usually result in mild to moderate blistering of oral or genital tissues, severe complications such as corneal blindness or herpetic encephalitis can develop as a result of this infection (Wuest et al., 2011).

HSV-1 is an enveloped virus composed of linear double stranded DNA (dsDNA) approximately 152 kb in size (Karasneh and Shukla, 2011). Encoded within the genome are 74 viral genes responsible for mediating host cell infection and viral replication. HSV-1 infection begins with viral attachment to susceptible host cell receptors. HSV-1 viral attachment is a complex and cell specific process facilitated by at least seven HSV glycoproteins; however, four are primarily relied upon for proper virus/cell membrane adhesion (Karasneh and Shukla, 2011). These glycoproteins, located along the outer envelope of HSV-1 include glycoproteins gB, gD, gH, and gL. Following attachment to host cell receptors, the nucleocapsid containing viral DNA and the surrounding tegument proteins penetrate host cells through processes such as fusion or endocytosis (Akhtar and Shukla, 2009). Following penetration, the nucleocapsid will migrate from the cytoplasm

into the nucleus via microtubules where host cell machinery is utilized to facilitate viral replication. Eventually, newly synthesized HSV-1 virions burst out of the infected cell through a process known as exocytosis (Miranda-Saksena et al., 2009) (Figure 1). Depending upon environmental conditions, escaped virions can continue infecting susceptible epithelial cells or establish a latent infection within the trigeminal ganglion (Theil et al., 2003). The strategic ability of these viruses to alternate between latent and lytic life cycles and the lack of a true vaccine for HSV-1 makes it an important virus to research.

Figure 1. The lytic life cycle of HSV-1. Initial HSV-1 infection begins when HSV-1 virion(s) attach and enter host cells via fusion or endocytosis. Upon entry into the host cytoplasm, the virus's lipid envelope is dissolved and enables the tegument proteins to separate from the DNA containing nucleocapsid. Two of the main tegument proteins, virion host shut off (VHS) proteins and VP16, are then able to remain in the cytoplasm to inhibit host mRNAs (VHS) and/or travel to the nucleus to aid in viral transcription (VP16 and VHS). Concurrently, the nucleocapsid is transported to the nuclear membrane where it releases the DNA. Here, the DNA circularizes and is able to replicate via rolling circle replication. VP16, along with other viral proteins, utilizes host machinery to aid in the production of immediate early genes, early genes and late genes to aid in DNA synthesis as well as the complete reconstruction and packaging of progeny virions. The newly structured viruses are then exported out of the nucleus where they pass through the endoplasmic reticulum (ER), where some of the late envelope proteins are synthesized, and the golgi apparatus (GA) to attain their final envelope and tegument structures, ultimately exiting the infected cell through exocytosis. At this point, newly synthesized virions can repeat the lytic cycle by infecting neighboring host epithelial cells or alternatively establish a latent infection by traveling to trigeminal ganglion nerve endings.

Traditional herbal medicines (THMs) have been used as natural remedies throughout Asian cultures for centuries. Chinese herbal medicines (CHMs), for example, are well documented in their usage with regards to treating insomnia, increasing hair growth and other general illnesses or discomforts (Kim et al., 2008; Chen et al., 2011; Sun et al., 2013). Chinese knotweed, an herbaceous vine found throughout the south central regions of China, is an example of a CHM that has been used as a natural therapeutic and potent antioxidant for hundreds of years. In the past decade, researchers have provided detailed information regarding the chemical root constituents of this natural compound. High speed counter-current chromatography including high liquid performance chromatography (HPLC) techniques indicate that the root is composed of at least nine active compounds including emodin, chrysophanol, rhein, 6-OH-emodin, emodin-8-β-D-glucoside, polygonimitin B, 2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glucoside, gallic acid and an unknown glucoside (Yao et al., 2006). Recent *in vitro* and *in vivo* studies present data suggesting the components of Chinese Knotweed to have a broad range of therapeutic activity. For example, the 2,3,4′,5-tetrahydroxystilbene-2-O-β-D-glucoside root component extracted has shown anti-atherosclerotic activity while other studies suggest that entire root extracts may increase the activity of mitochondrial cytochrome oxidase (COX) in rats with Alzheimer's Disease (AD), thus offering a potential unique natural therapeutic to people suffering with AD (Hou et al., 2008 & Zhang et al., 2009). This may represent an important finding due to the controversial yet potential link between HSV-1 and AD (Piacentini et al., 2014). Other independent studies show similar cognitive improvements in mice supplemented with Chinese Knotweed

extracts (Chan et al., 2003). Although the root constituents are also believed to have antibacterial and antiviral activities (Kim et al., 2008; Lin et al., 2010), there is minimal research supporting these claims.

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A)

B)

Figure 2. Chinese Knotweed. Chinese Knotweed, native to the south central regions of China, produces a large, dense root packed with natural compounds. **A)** Chinese Knotweed chopped roots. **B)** Chinese Knotweed extract.

2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside

Emodin-8-β-D-glucoside

6-OH-emodin

Gallic acid

Chrysophanol

Polygonimitin B

Emodin

Rhein

Figure 3. Chinese Knotweed root major constituents. The 8 chemical structures of the major root constituents found in Chinese Knotweed root extracts (adapted from Yao et al., 2006).

With regards to potential antiviral activity, compounds of a stilbenoid nature make up a large amount of the root constituents. Stilbenes, such as resveratrol, are known to have impressive anti-herpesviral activity (Docherty et al., 1999). Although Chinese Knotweed does not contain resveratrol, it contains other stilbenes such as 2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glucoside that may present similar or improved activity. The current study will assess this stilbenoid in combination with the at least 8 other Chinese Knotweed root constituents for any possible anti-HSV-1 activity.

Recombinant DNA technology has provided researchers the ability to introduce exogenous DNA into susceptible host genomes. For example, the HSV-1 strain used in the present study is a recombinant strain (GHSV-UL46) that has been modified to carry the gene that encodes for the green fluorescent protein (GFP). This gene is fused to the viral tegument protein pUL46 and allows for real time monitoring of HSV-1 binding, penetration, and subsequent infection through the use of fluorescence-based techniques. Flow cytometry, for example, is a technology that is able to characterize particulates within liquid samples according to a variety of parameters such as size, granularity, and fluorescence. With regards to detecting fluorescence, flow cytometers are able to simultaneously shine multiple lasers at single cells or particles. The wavelength emitted from this excitation is captured by a series of optics and mirrors and converted to electronic signals, or fluorescent peaks. In the case of GFP, excitation occurs at 488 nm and emission is captured at 530 nm. With regards to the present study, cells infected with the recombinant strain of HSV-1 should produce a large fluorescent peak under the respective wavelength while cells that have not been infected should lack a signal.

Similarly, fluorescent microscopy techniques can be used to visually track viral binding, penetration, and/or infection by analyzing infected and non-infected cells at varying time points. The ability to track viral infection by following the GFP signal is a critical component of determining the mechanism of MOA of a potential therapeutic, such as Chinese Knotweed. In order to determine whether or not Chinese Knotweed is delaying HSV-1 viral infection or completely inhibiting it, further experimentation is required.

The daily monitoring of cells infected with treated and untreated HSV-1 provides critical information with regards to the ability of a potential therapeutic to either delay or completely inhibit viral infection. In order to study this, cytopathic effect (CPE) monitoring studies can be performed. This study, which may run over the course of 3-7 days, provides evidence as to whether or not the potential therapeutic of interest is completely inhibiting, partially inhibiting, or simply delaying viral infection. This study, however, fails to provide any concrete quantitative data with regards to Chinese Knotweeds potential ability to decrease HSV-1 infectivity.

Plaque assays are commonly used to quantitate a virus's infectivity. This infectivity is expressed as plaque forming units per milliliter (PFU/mL) and represents the titer of the specific viral pool tested. Only the virions that are capable of infecting and lysing host cells will be calculated into this titer, hence the phrase "plaque forming" units. Depending upon the dilution and volume of the viral titer used to infect the cells, the infectivity of the viral pool can be determined with a simple calculation after counting the amount of plaques. Similar studies, such as plaque reduction and binding assays are modeled after this assay and follow similar guidelines. For example, the plaque reduction

assay can be used to determine if treating viral stocks with a suspected antiviral causes any significant reduction in infectivity when compared to untreated stocks from the same pool. The percentage of reduction between the two samples can then be used as a preliminary way to assess any potential antiviral activity. Binding assays follow a similar protocol, however, the experimental design is setup to favor the attachment or binding process. For HSV-1, this is achieved by performing the assay at 4°C. At this temperature, HSV-1 virions will bind to host receptors and avoid penetration (Cantatore et al., 2013). Once the environment is altered to conditions favoring penetration, the procedure can be continued the same way as the plaque and plaque reduction assays. If treatments inhibited viral binding, no binding should take place when subjected to 4°C temperatures. Therefore, when temperatures are set back to 25-37°C and the cells are washed; there should be no infection in the treated samples. However, if the treatment did not affect viral binding and instead was working at a later stage of the viral life cycle, viral infection would occur as normal.

Prior to assessing a potential therapeutics antiviral activity, several tests need to be performed to determine whether or not the compound expresses any toxicity. Preceding *in vivo* experimentation, several tests have been designed in order to determine this *in vitro*. For example, the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay is a colorimetric experiment used to measure cell proliferation. Metabolically active cells reduce the MTS kit compound tetrazolium via mitochondrial reductase to produce an insoluble purple colored compound called formazan. A spectrophotometer can be used to detect the intensity of

the formazan product by measuring its absorbance at 490 nm. The intensity of formazan is thus directly proportional to the amount of living cells. Similar experiments such as the MTT and trypan blue exclusion assay can also be used to further assess potential cytotoxicity.

HSV-1 remains one of the most invasive viruses in the world. The strategic life cycles and defense mechanisms it has evolved have made it nearly impossible to cure and difficult to treat. Acyclovir (ACV), for example, is one of the most common and effective anti-herpesviral agents due to its ability to insert itself into the viruses elongating DNA chain, halting DNA synthesis (Bacon et al., 2003). However, HSV-1's strategic ability to evade the inhibitory DNA synthesis mechanism of this drug by developing resistance is well documented (Pottage and Kessler, 1995; Smith et al., 2001; Bacon et al., 2003). Without the threat of resistance, ACV is primarily effective at targeting virions undergoing their lytic cycle but shows no evidence of preventing the virus from establishing a latent infection (Smith et al., 2001). Furthermore, research also suggests that HSV-1 virions that develop ACV resistance and enter their latency stage are able to maintain this resistance when reactivated, thus nullifying any potential relief by using ACV as a treatment (Bacon et al., 2003). Due to these concerns, developing new anti-HSV-1 therapies to either work together with current drugs or to function as separate treatments is important. THM's such as Chinese Knotweed may offer researchers unique methods to combat the battle against resistance.

Materials and Methods

Preparation of solutions

Chinese Knotweed compound was extracted by Dr. Chih-Yu Lo (Department of Food Science, National Chiayi University, Chiayi, Taiwan). A 10% stock solution was prepared in dimethyl sulfate (DMSO).

Cell and Viral Maintenance

Green monkey kidney (Vero cells) were cultured in T-75 flasks containing Dulbecco Minimal Essential Media (DMEM) supplemented with 5% FBS and 1% gentamycin at 37ºC and 5% CO_2. A recombinant strain of HSV-1, GHSV-UL46, expressing the tegument protein pUL46 gene fused to the gene encoding for GFP was used for all viral assays (donated by Dr. Sandra Adams; Department of Biology and Molecular Biology, Montclair State University, Montclair, NJ 07043, USA). Viral propagation was performed in T-75 flasks and cells were allowed to reach complete CPE. The media was then collected, centrifuged and the supernatant containing virus was stored in cryogenic vials at -80°C.

Viral Infection

HSV-1 viral stocks were treated with varying concentrations of Chinese Knotweed. Vero cells were incubated with the various concentrations in triplicate at 37ºC and 5% CO_2 for 1 hour. Viral infection was monitored as follows:

Cytopathic effect monitoring

Unabsorbed virus (0.1 MOI) was removed and the wells were washed two times with 1X phosphate buffered saline (PBS). Fresh culture medium was added to each well and the cultures were incubated at 37ºC and 5% CO_2 for seven days. The cultures were examined daily with an inverted light microscope and CPE was expressed as a percentage of infected cells throughout the well.

Cytotoxicity

Vero cells were plated in 6-well cell culture dishes and varying concentrations of Chinese Knotweed were added to each well. Cells were studied for morphological and proliferation changes 24 and 48 hours later using an ACCU-Scope 3002 microscope.

MTS Assay

Cell proliferation kit (G5421, Promega Corp.) was used. This is a colorimetric method for determining cell proliferation. Metabolically active cells will reduce the kit reagent tetrazolium into a soluble formazan product. Therefore, the amount of formazan measured at an absorbance of 490 nm is directly proportional to the number of metabolically active cells. Vero cells were seeded into a 96 well plate and allowed to grow for 24 hours. Along with the experimental controls, varying concentrations of Chinese Knotweed and HSV-1 mixtures were added to the monolayers in triplicate for 1 hour. After 1 hour, the controls and treatments were removed, the wells were washed and fresh cell culture media was added. After 24 hours, the MTS reagent was added and a 4

hour incubation period at 37ºC and 5% CO_2 took place. Following incubation, the absorbance was read at 490 nm.

Plaque reduction assay

Unabsorbed virus was removed and 3mL of fresh culture medium supplemented with 0.9% bacto agar was added over each well. After five days of incubation at 37ºC and 5% CO_2, a 2 mL media topping of fresh culture medium was added. The cultures were incubated for an additional 24 hours. At the end of the experiment, on day 6, the media topping and agar overlay was removed; the cells were stained with 0.1% crystal violet and then washed with PBS. Plaques were then counted and expressed as PFU/mL.

Binding assay

Six-well plates were seeded with Vero cells and allowed to reach confluency**.** On the day of experimentation, the plates were removed from the incubator and left at room temperature for 20 minutes. Viral adsorption and infection occurred at 4ºC for 1 hour. The wells were washed three times with cold PBS and the plates were then allowed to equilibrate at room temperature. The plaque assay protocol was followed as previously described to determine if binding occurred.

Flow Cytometry

Vero cells were grown in 12-well plates and allowed to reach confluency. HSV-1 virions (0.5 MOI) treated with 0.1, 0.05, and 0.025% Chinese Knotweed concentrations for 1

hour were then added to their respective wells. PBS was used for viral dilutions and cell controls. The unabsorbed liquid was aspirated and the cells were washed with PBS. Fresh media was added to each well and the plates were incubated at 37ºC and 5% CO_2 for an additional 8 hours to promote infection. Cells were then trypsinized and resuspended in PBS for flow cytometry analysis. The flow cytometric antiviral assay was carried out in triplicate and the GFP signal was excited with a blue 488 nm laser and detected at 530 nm. Flow cytometry was carried out using a MACSQuant Analyzer, serial #MQ2492 (MACS Miltenyi Biotec).

Fluorescence Microscopy

Vero cells were grown on sterile glass cover slips in 6-well plates for 24 hours. HSV-1 virions (0.5 MOI) treated with 0.1, 0.05, and 0.025% Chinese Knotweed concentrations for 1 hour were then added to their respective wells. PBS was used for viral dilutions and cell controls. The unabsorbed liquid was aspirated and the cells were washed with PBS. Fresh media was added to each well and the plates were incubated at 37ºC and 5% CO_2 for an additional 8 hours to promote infection. The cells were then stained with 300 µL of 300 nM DAPI (4,6-diamidino- 2-phenylindole) for 5 minutes at room temperature in the dark. Next, cells were fixed with a 1:1 acetone/methanol solution for 20 minutes at 20°C. The glass cover slips were then placed on to a glass slide with 20 µL of glycerol as the mounting solution and a drop of nail polish to keep the cover slip in place. Cells were analyzed using an Olympus Fluoview FV-1000 confocal laser-scanning microscope (CLSM).

Real-time quantitative PCR

Vero cells seeded in 60 mm dishes were allowed to reach confluency. HSV-1 virions (0.5 MOI) without treatment and treated with Chinese Knotweed were added to the cell monolayers for 1 hour. Unabsorbed virus was removed and the cells were washed with PBS. Three (3) mL of fresh cell culture media was added to each dish and infection was allowed to develop for 8 hours. Following the 8-hour infection, cell monolayers were collected for PCR analysis. The forward and reverse primers used in this study are fwd: 5'-CAACCCTACAACCTGACCATC-3' and rev: 5'TTGTAGGAGCATTCGGTGTAC-3' and were designed to recognize the US6 gene of HSV-1 responsible for coding glycoprotein D. DNA isolation was followed as per manufacturer instructions (QIAGEN DNeasy Blood & Tissue Kit). Using a 96-well plate, 10 µL of Fast SYBR Green Master Mix, 1 µL of forward primer, 1 µL of reverse primer, 2 µL of respective genomic DNA (100 ng), and 6 µL of sterile diH_2O was added in triplicate to the appropriate wells. The DNA volume for the negative controls was replaced with 2 µL of sterile diH_2O. qPCR analysis was carried out using an ABI StepOnePlus Real-Time PCR System (Applied Biosystems; Carlsbad, CA) thermocycler with the following reaction settings: 2 minute hold @ 50°C, 2 minutes @ 95°C, 15 seconds @ 95°C, 1 minute @ 60°C, 15 seconds @ 95°C and 1 minute at 60°C for 40 cycles.

Results

Cytopathic effect monitoring studies indicate that Chinese Knotweed decreases HSV-1 infectivity over a seven-day period.

As the Chinese Knotweed concentration was increased from 0.05% to 0.1%, the CPE was decreased (Figure 4). Although the 0.05% Chinese Knotweed concentration did not completely inhibit CPE, a significant delay was observed. This can be seen when comparing the 0.05% treatment to the HSV-1 control. After only ~12 hours post adsorption, up to 50% of the HSV-1 control infected cells showed notable CPE characteristics. It took up to three days for the 0.05% treatment to show a similar effect. This suggests a significant delay of infection when HSV-1 is treated with 0.05% Chinese Knotweed. However, when the HSV-1 treatment concentration is increased to 0.1% Chinese Knotweed, no CPE is observed throughout the seven-day course of the experiment.

Chinese Knotweed concentrations up to 1% show no negative effect on Vero cell morphology or proliferation.

Vero cells were exposed to 0.1%, 0.5% and 1% Chinese Knotweed concentrations for 1 hour. The morphology was visually analyzed via microscopy 24 hours later and no morphological abnormalities or cytotoxicity characteristics were observed (data not shown).

The same treatments were set up and an MTS assay was performed in order to determine whether or not Chinese Knotweed was negatively effecting cell proliferation.

As seen in Figure 5, 0.1%, 0.5% and 1% Chinese Knotweed treatments showed no significant reduction on Vero cell proliferation compared to untreated controls (blank). In order to express proliferation as a percentage, the average OD_{490nm} values (n=3) of each treatment were normalized according to the average of the blank, which had an average OD_{490nm} of 2.68 +/- 0.03. After dividing this by itself and multiplying by 100, the blank can be normalized to 100% cell proliferation. Therefore, the proliferation percentages of the subsequent treatments could be assessed. The average OD_{490nm} for the 0.1%, 0.5% and 1% Chinese Knotweed concentrations were 2.69 +/- 0.07, 2.62 +/- 0.05 and 2.51 +/- 0.14, respectively. By dividing these numbers by the blank and multiplying by 100, each samples corresponding proliferation value can be expressed as a percentage. Each sample showed a proliferation value of >90% when compared to the blank. To determine the efficacy of this assay, a 0.1% bleach solution as used as a control. The average OD_{490nm} for this control was 0.34 +/- 0.01 and resulted in a cell proliferation of only 13% compared to the blank. These assays suggest that concentrations of Chinese Knotweed as high as 1% show no significant reduction in cell proliferation of Vero cells.

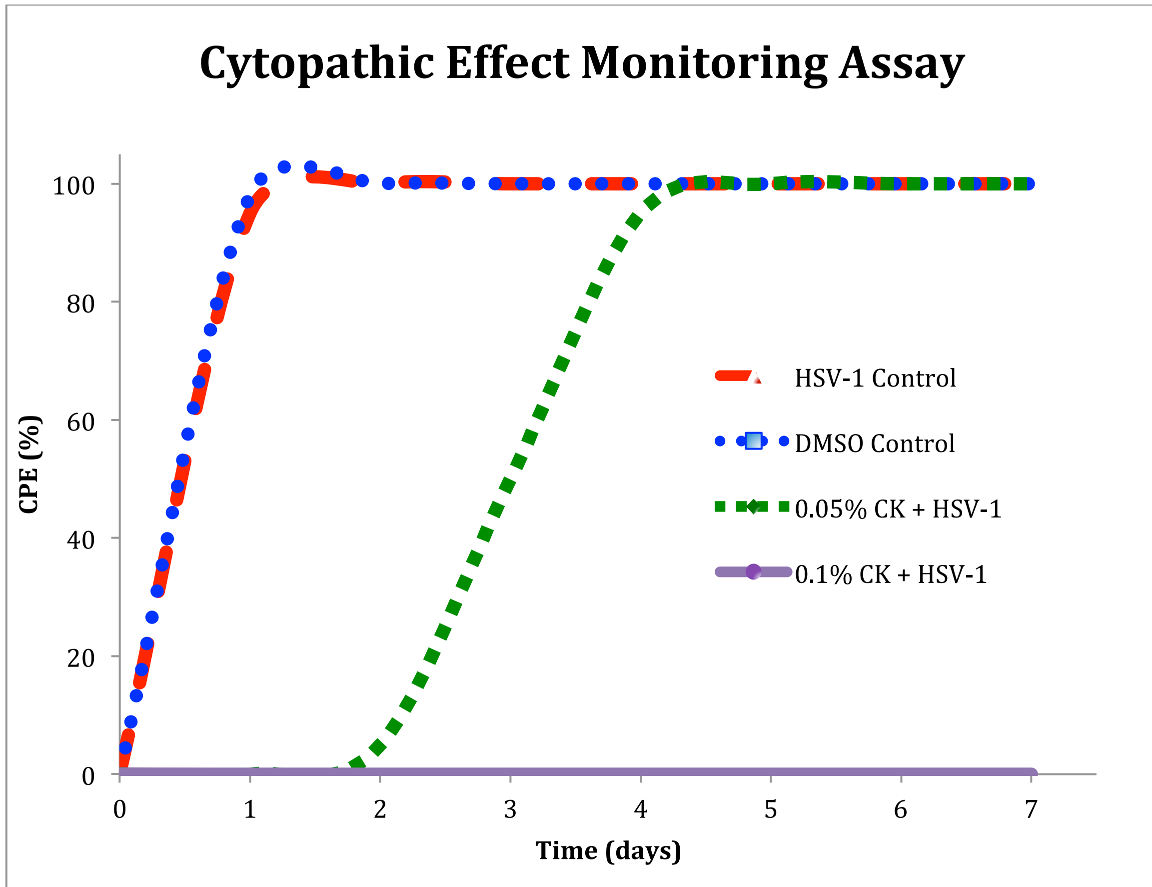


Figure 4. Cytopathic effect monitoring assay. Vero cells treated with 0.05% and 0.1% concentrations of Chinese Knotweed and HSV-1 mixtures. The plates were monitored via microscopy every day over a 7-day period and results are expressed as percentage of CPE. Percentage of CPE was based on the percent of the entire well that clearly expressed CPE. Red dashed line: HSV-1 positive control. Blue circled line: DMSO control. Green squared line: 0.05% Chinese Knotweed treatment. Purple solid line: 0.1% Chinese Knotweed treatment.

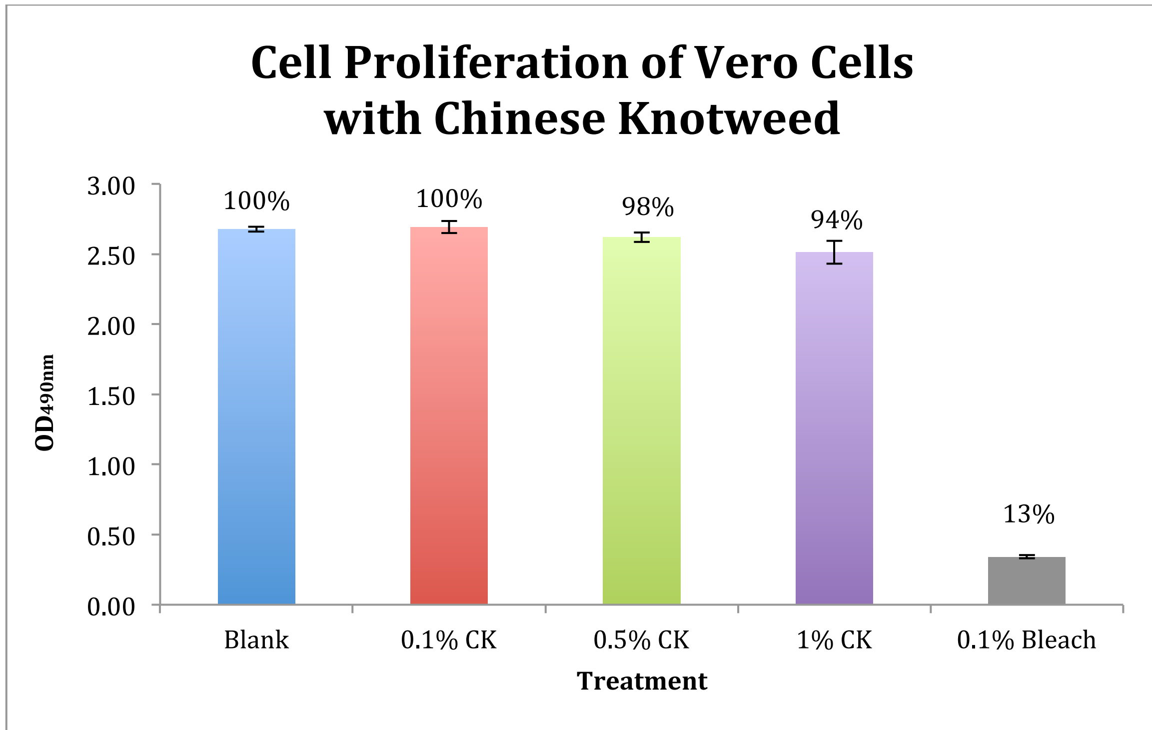


Figure 5. Effects of Chinese Knotweed on cell proliferation of Vero cells. MTS assays were performed to measure the cell proliferation of Vero cells treated with 0% (blank), 0.1%, 0.5% and 1% concentrations of Chinese Knotweed. As seen above, Chinese Knotweed concentrations as high as 1% showed no significant reduction in cell proliferation when compared to untreated cells (blank). Data are expressed by the mean of percent cell proliferation compared to the control (blank) after Chinese Knotweed exposure for 1 hour and a subsequent growth period of 24 hours +/- standard deviation (n=3).

Plaque reduction assays indicate that HSV-1 virions treated with 0.1% Chinese Knotweed extracts significantly reduce viral titers.

In order to assess Chinese Knotweeds potential ability to decrease HSV-1 titers, a plaque reduction assay was performed (Figure 6). In this experiment, Vero cells were infected with treated (0.1% of Chinese Knotweed) or untreated HSV-1 virions (PBS). It can be seen in Figure 6 that Vero cells infected with HSV-1 in the absence of Chinese Knotweed showed infection at all viral dilutions up to 10^{-5}. At the 10^{-4} dilution, countable plaques are observed and the infectivity of this virus can be calculated to ~2.2×10^{5} PFU/mL. However, when Vero cells are infected with the same virus concentration treated with 0.1% Chinese Knotweed, infection is inhibited even at the lowest dilution of 10^{-1}. Because no visible plaques are observed at the lowest dilution (10^{-1}), a complete or 100% reduction in infectivity can be expressed. These results indicate that 0.1% Chinese Knotweed concentrations are able to significantly reduce the number of viral plaques and therefore the infectivity of HSV-1.

Binding assays suggest that a Chinese Knotweed concentration of 0.1% is capable of inhibiting HSV-1 entry.

Binding assays were performed to examine if Chinese Knotweed had an inhibitory effect on the ability of HSV-1 to bind to Vero cells (Figure 7). This was done by performing the adsorption step of this assay at 4°C to promote viral binding while preventing penetration. When subjected back to favorable penetration conditions (37°C), any bound virions will enter and infect as normal. However, any virus left unbound after

the 1-hour adsorption period will be washed away, resulting in a decreased infection. As seen in the HSV-1 viral control plate with no Chinese Knotweed treatment, significant infection was observed in the 10^{-1} and 10^{-2} wells. This indicates that the HSV-1 virions were able to bind at 4°C and continue their life cycle when set back to 37°C. Alternatively, when the same viral concentration (10^{-1}) is treated with 0.1% Chinese Knotweed and undergoes the same viral adsorption and penetration conditions, no viral infection is seen. The lack of infection seen here suggests that Chinese Knotweed is preventing HSV-1 from entering susceptible host cells.

A)

B)

Figure 6. Comparison of HSV-1 titers with and without Chinese Knotweed treatment. Plaque assays were performed three times in duplicate to determine Chinese Knotweeds ability to decrease the titer of HSV-1. **A)** Virus control plate. From left to right: Cell control, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} viral dilutions. The viral titer was calculated by counting the plaques at 10^{-4}. **B)** HSV-1 treated with 0.1% of Chinese Knotweed. From left to right: Cell control, 10^{-1} + 0.1% Chinese Knotweed and 10^{-1} + 0.1% Chinese Knotweed.

A)

B)

Figure 7. Effects of Chinese Knotweed on HSV-1 binding. Binding assays were performed three times in duplicate to determine whether Chinese Knotweed was inhibiting HSV-1 binding. **A)** Viral control plate. From left to right: Cell control, 10^{-1} and 10^{-2}. **B)** Chinese Knotweed treatment plate. From left to right: Cell control, 10^{-1} + 0.1% Chinese Knotweed and 10^{-1} + 0.1% Chinese Knotweed.

Flow cytometric analysis indicates that Chinese Knotweed inhibits viral GFP expression in a concentration dependent fashion.

Flow cytometry was utilized to further assess Chinese Knotweeds anti-HSV-1 activity. The ability of HSV-1 to bind, enter, and infect Vero cells following treatments of 0.025%, 0.05%, and 0.1% Chinese Knotweed was evaluated by measuring the GFP signal associated with the virus. As the concentration of Chinese Knotweed is increased, the GFP signal is decreased. Thus, flow cytometry confirms that 0.1% Chinese Knotweed concentrations are capable of preventing HSV-1 infection. Previous studies indicated that DMSO controls mimic those of positive HSV-1 controls and do not inhibit HSV-1 infection (de Oliveira, et al., 2015).

A graphical representation of the flow cytometry results expressed percentage of GFP reduction.

The gating used in flow cytometry was set according to the GFP signal associated to Vero cells infected with untreated HSV-1. Normalizing the HSV-1 positive control to 100% GFP intensity, the percentage of GFP reduction associated with the remaining concentrations can be calculated. While 0.025% and 0.05% Chinese Knotweed concentrations show a reduction of 31.1% and 88%, respectively; the 0.1% concentration shows a GFP reduction of 97.2% compared to the HSV-1 controls.

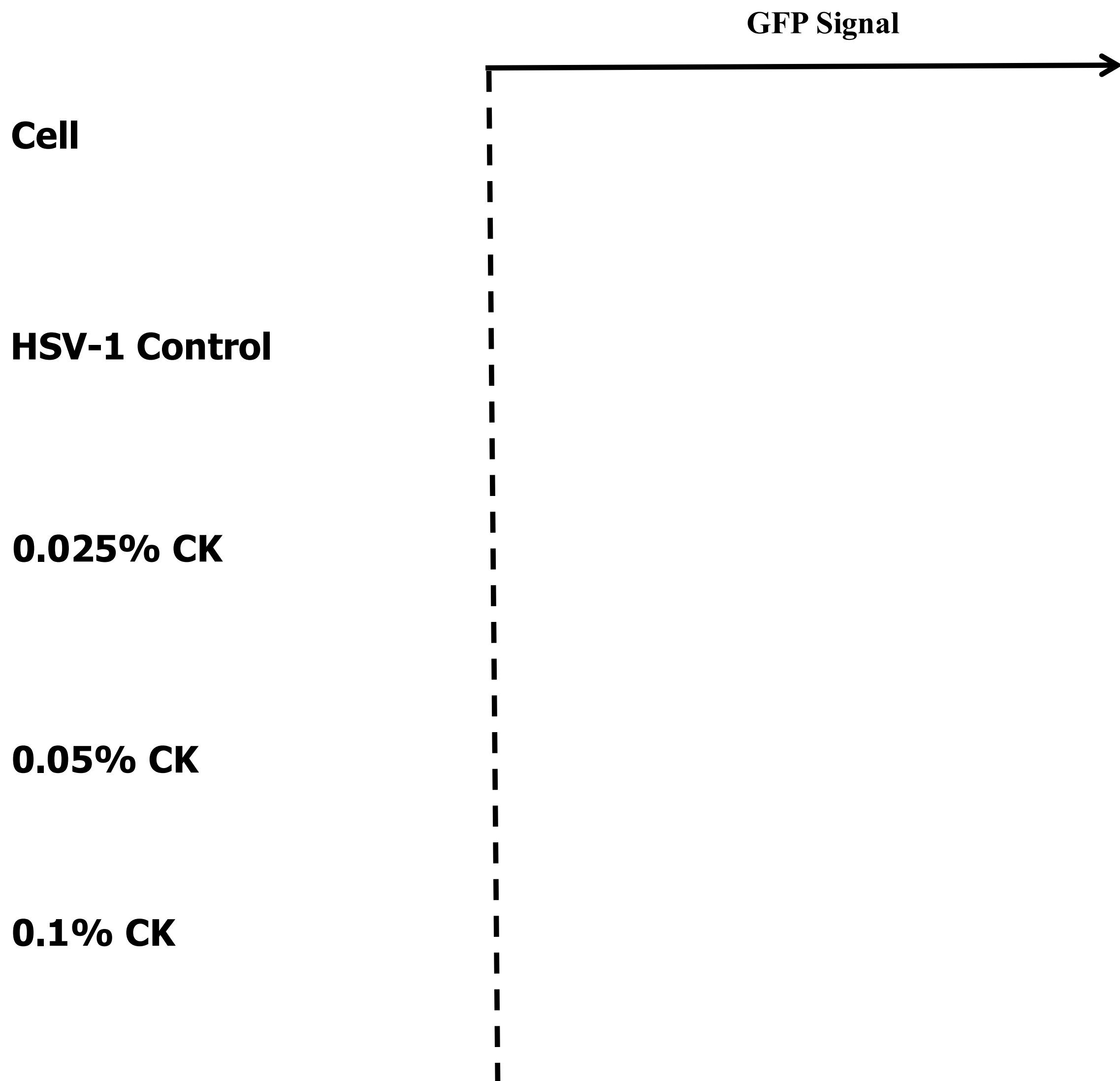


Figure 8. Histogram of HSV-1 GFP signal in Vero cells. Flow cytometry was utilized to further assess Chinese Knotweeds anti-HSV-1 activity. The ability of HSV-1 to bind, enter, and infect Vero cells following treatments of 0.025%, 0.05%, and 0.1% Chinese Knotweed was evaluated by measuring the GFP signal associated with the virus. As the concentration of Chinese Knotweed is increased, the GFP signal is decreased. Thus, flow cytometry confirms that 0.1% Chinese Knotweed concentrations are able to inhibit viral infection.

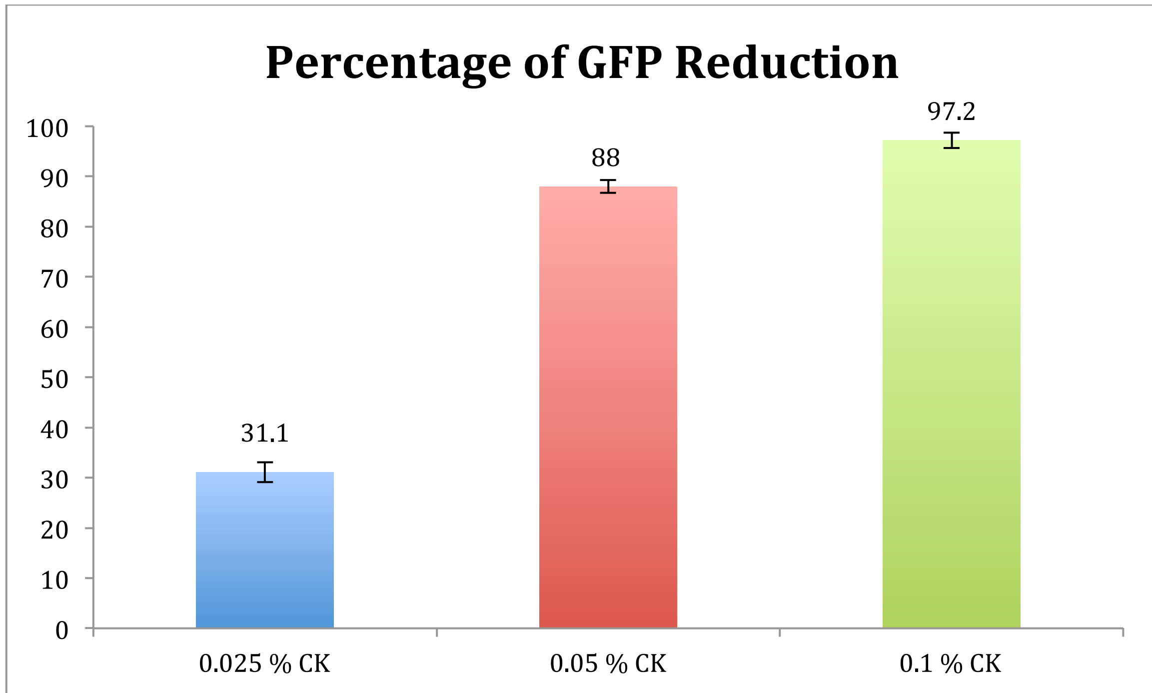


Figure 9. Flow cytometric analysis of GFP reduction. Flow cytometry gating is based on Vero cells infected with untreated HSV-1 and is expressed as a percentage of GFP. Normalizing the HSV-1 positive control to 100% GFP intensity, the percentage of GFP reduction associated with the remaining concentrations can be calculated. While 0.025% and 0.05% Chinese Knotweed concentrations show a reduction of 31.1% and 88%, respectively; the 0.1% concentration shows 97.2% GFP reduction compared to the HSV-1 controls.

Fluorescent microscopy results further confirm that the viral GFP signal is diminished according to a concentration dependent treatment.

In order to better visualize the GFP reduction seen in the flow cytometry data, fluorescence microscopy was employed. Vero cells were infected with 0% (HSV-1 positive control), 0.025%, 0.05% and 0.1% Chinese Knotweed treated virions and were observed 12 hours post adsorption utilizing a fluorescent microscope. The cells were stained with DAPI and the localization and fluorescent intensity of the GFP signal associated with the viral pUL46 tegument protein was monitored. Similar to the response seen using flow cytometry, as the concentration of Chinese Knotweed increased, the GFP signal intensity decreased. All images were taken 8 hours post adsorption at 600x magnification.

Figure 10. Healthy, uninfected Vero cells. The cells were stained with DAPI (blue fluorescence) which targets and binds to nuclear DNA.

Figure 11. HSV-1 positive control. Vero cells infected with HSV-1 show a strong green fluorescence signal throughout their cytoplasm and along their nuclear envelopes.

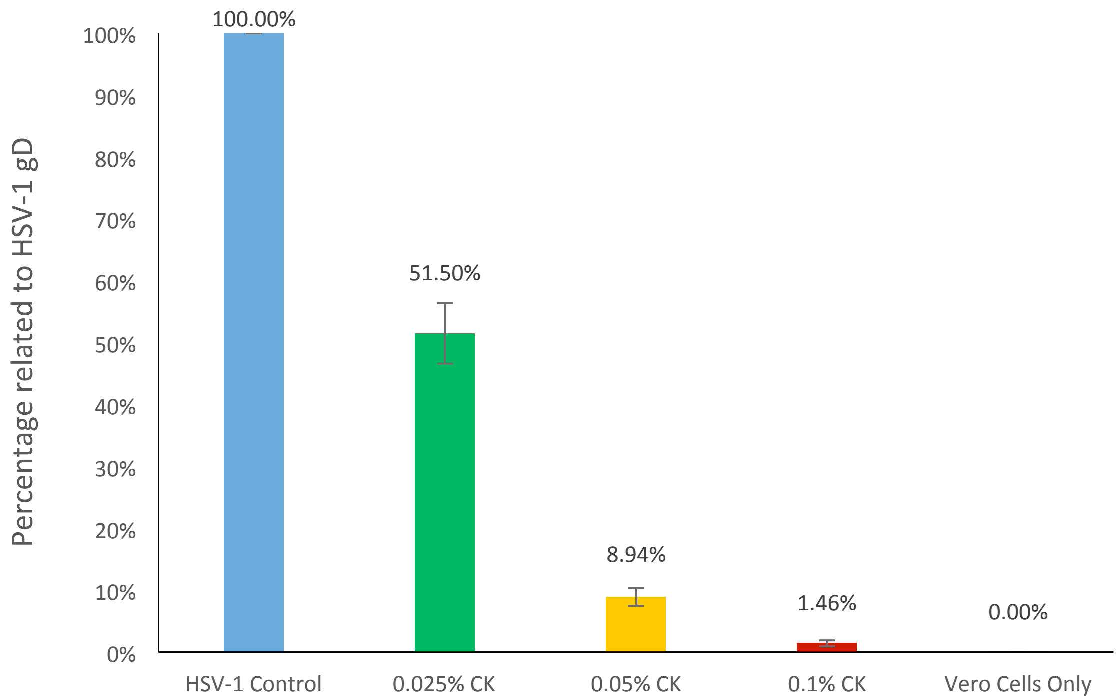
Figure 12. Vero cells infected with HSV-1 pretreated with 0.025% Chinese Knotweed. The majority of the cells show a similar GFP signal to the HSV-1 positive control.

Figure 13. Vero cells infected with HSV-1 pretreated with 0.05% Chinese Knotweed. GFP intensity has significantly decreased, however, at least 50% of the cells show some signal.

Figure 14. Vero cells infected with HSV-1 pretreated with 0.1% Chinese Knotweed. This treatment shows an almost complete reduction of the GFP signal, closely mimicking that of the untreated cell controls.

Real-time quantitative PCR (qPCR) analysis of gene expression in HSV-1 treated Vero cells.

Real-time quantitative PCR (qPCR) analysis was used to compare the expression of the HSV-1 envelope glycoprotein D gene, US6 in untreated and Chinese Knotweed treated samples. As concentrations of Chinese Knotweed were increased, the expression of the US6 gene decreased. Because the US6 gene codes for a glycoprotein that is crucial to viral binding, the ability of Chinese Knotweed to inhibit the production of this gene by >98% at a concentration of 0.1% suggests that it is preventing viral infection. Furthermore, 0.025% and 0.05% Chinese Knotweed concentrations further confirm that Chinese Knotweed is working against HSV-1 in a concentration dependent manner.



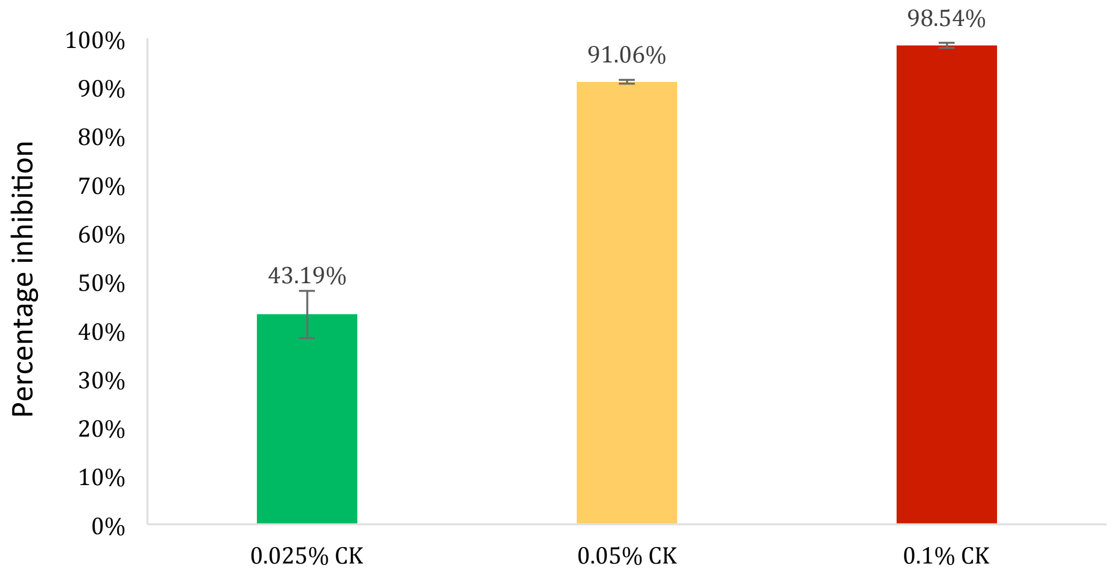


Figure 15. Real time PCR (qPCR) analysis of US6 gene expression. A) Percentage of glycoprotein D amplification. **B)** Percent inhibition of the glycoprotein D gene when virions are treated with 0.025%, 0.05% and 0.1% Chinese Knotweed concentrations.

Conclusion

In the present study, the anti-HSV-1 activity and the potential toxicity of Chinese Knotweed root extracts were assessed through a variety of *in vitro* experimental approaches. Toxicity of the compound was analyzed through both a morphological cytotoxicity analysis assay as well as a quantitative colorimetric cell proliferation assay. Both assays indicated that Chinese Knotweed has no toxic effect on Vero cells at concentrations as high as 1% (Figure 5). By using a recombinant viral stain (GHSV-UL46) with a GFP insert, both quantitative and qualitative based assays such as flow cytometry and fluorescence microscopy were used to track viral infection via GFP fluorescence. Both of these assays showed similar results in that HSV-1 virions treated with 0.1% Chinese Knotweed concentrations were able to inhibit HSV-1 infection by >95%. The plaque reduction assay, which was used to compare the infectivity of treated and untreated HSV-1 viral titers, showed similar results. As seen in Figure 6, HSV-1 titers treated with 0.1% Chinese Knotweed showed a >99% decrease in infectivity compared to viral controls. This indicates that Chinese Knotweed is able to significantly decrease HSV-1 infectivity by reducing the number of PFU/mL. Other antiviral assays showed similar results. The CPE monitoring assay, for example, showed that 0.05% Chinese Knotweed concentrations were able to delay an HSV-1 infection of 100% (the entire monolayer infected) by up to three days compared to HSV-1 controls. When increased to a concentration of 0.1% Chinese Knotweed, however, HSV-1 infection remained absent up to seven days post adsorption (Figure 4).

Due to Chinese Knotweeds (0.1%) ability to inhibit HSV-1 infection for up to seven days, it was critical to perform binding assays as a way to determine the compounds potential MOA. Binding assays are used as a way to promote HSV-1 binding while inhibiting premature viral penetration. Results from this assay indicate that Chinese Knotweed concentrations of 0.1% were able to completely inhibit viral binding and therefore subsequent viral infection (Figure 7). Furthermore, qPCR was used to monitor the expression of the glycoprotein D encoding gene US6 in treated and untreated cells. Similar to the reduction of the pUL46 gene seen in the flow cytometry and fluorescent microscopy based assays, a >98% reduction of the US6 gene was observed when HSV-1 virions were treated with 0.1% Chinese Knotweed concentrations, as seen in Figure 15. The reduction of the glycoprotein D gene, which is one of the four essential glycoproteins involved in host receptor recognition and binding, further points at Chinese Knotweeds ability to potentially inhibit HSV-1 binding. Overall, Chinese Knotweed appears to be an impressive inhibitor of HSV-1 infection.

Discussion

The potential development of antivirals through the isolation of natural components has been studied for years. In fact, several natural compounds from tea such as the modified polyphenol palmitoyl-epigallocatechin (pEGCG) from green tea and theaflavin digallate extracted from black tea have already demonstrated impressive anti-HSV-1 activity (de Oliveira et al., 2013; de Oliveira et al., 2015). Similarly, natural stilbenoids such as resveratrol, commonly found in red wine and grapes, and other dimeric and oligomeric stilbenoids have also been shown to display impressive anti-herpetic activity (Chen et al., 2012). The Chinese Knotweed root extract contains at least one main stilbene, 2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glucoside and several other polyphenolic compounds that appear to be the key components attributed to this compounds natural health benefits. In order to assess the potential toxicity associated with this compound, a MTS assay was performed to measure cell proliferation. Similarly, microscopic analysis revealed no morphological abnormalities or signs of cytotoxicity at the same concentrations (data not shown). These results suggest that Chinese Knotweed could potentially be used as a safe and natural therapeutic.

With regards to potential anti-HSV-1 activity, Chinese Knotweed appears to prevent HSV-1 infection of cultured Vero cells in a concentration dependent fashion. Based upon results gathered from multiple independent assays, the most effective Chinese Knotweed concentration is that of 0.1% (Figures 4, 6, 7, 8, 9, 14 and 15). Although both the 0.025% and 0.05% concentrations show notable HSV-1 inhibition, the 0.1% concentrations displayed truly impressive inhibition, preventing viral

infection/expression throughout all assays by >95%, including the inhibition of two critical genes involved during different stages of the viruses life cycle.

Due to the lack of a true cure or effective vaccine for HSV-1, it is a particularly important virus to study. Additionally, since the majority of the population is infected by HSV-1 and certain strains are beginning to show resistance to current drugs, finding alternative anti-HSV-1 agents are critical. In this study, Chinese Knotweed has shown promising evidence to be considered a natural anti-HSV-1 agent. Further *in vitro* experimentation should be performed in order to assess Chinese Knotweeds potential ability to work as a safe therapeutic or antiviral before proceeding to *in vivo* experimentation. Similarly, due to the diversity of compounds extracted from the root, it is important to identify which individual component may be attributing to the anti-HSV-1 activity seen throughout this study. By testing these individual compounds and comparing them to the results of the mixture, the compound which is specifically causing the anti-HSV-1 activity can be determined. Nonetheless, it appears Chinese Knotweed, as a mixture, appears to be inhibiting HSV-1 during the early stages of its life cycle. This can be observed throughout the fluorescent microscope data (Figures 10-14). The HSV-1 positive control as well as the 0.025%, and 0.05% treatment images all show a GFP signal localized within the host cells cytoplasm, indicating HSV-1 virions were able to bind and enter host cells (Figures 11, 12 and 13). However, in the treatment with 0.1%, virtually no GFP signal can be detected within the cells cytoplasm (Figure 14). This strongly suggests that HSV-1 virions are unable to bind to host cells when treated with 0.1% Chinese Knotweed concentrations. Although these images were obtained 8 hours

post adsorption, there is always the possibility that Chinese Knotweed is simply delaying the infection period, as opposed to completely preventing it. In order to assess this, the CPE monitoring assay was performed. Here it can be seen that the 0.1% Chinese Knotweed concentration was not just delaying infection but completely inhibiting infection even seven days post adsorption (Figure 4). Furthermore, binding assays agree with the previous results and further suggest that HSV-1 virions treated with 0.1% Chinese Knotweed are unable to bind to and therefore infect the host Vero cells (Figure 7). Additionally, the vehicle of the compound (DMSO) displayed no viral inhibition as seen in Figure 4. Supplementary DMSO controls, using the same viral strain and cell line, have been carried out and can be found in our recent publication (de Oliveira, et al., 2015).

Continuing to study the ability of natural compounds to prevent viral infections can potentially provide researchers the confidence to develop novel anti-HSV-1 treatments. The current drug market is flooded with synthetic agents that are not fully effective. The fact that the majority of people infected with HSV-1 rarely show symptoms implies that the virus relies on environmental cues such as immunocompromised situations to initiate an outbreak. Studies suggest that a healthy and natural diet is a critical factor with regards to promoting a healthy immune system, which in turn may drastically limit the number of HSV-1 outbreaks (Chew et al., 2009). Ingesting Chinese Knotweed extract as a dietary supplement such as a daily multi-health vitamin or concentrated pill could potentially help suppress the amount of outbreaks an infected individual may experience. Furthermore, due to the natural compounds low

toxicity, it could also be used as a topical ointment either individually or in combination with lip balms to treat an infected area or to prevent the severity of a potential outbreak. Based on Chinese Knotweeds broad range of therapeutic activity, the applications are endless and could be modified depending upon specific needs.

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