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## **Inhibition of Herpes Simplex Virus-1 by Black Tea Extract**

Sadé Diahann Randall

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**Abstract.** Tea is a highly consumed beverage with numerous health benefits, such as antioxidant and antiviral properties. The compounds within tea that are believed to be associated with these benefits are polyphenols. Black tea polyphenols are known as theaflavins, and in previous studies have exhibited inhibitory effects on influenza and human immunodeficiency virus 1. Herpes simplex virus -1 (HSV-1) is an extremely common virus and has the ability to cause recurrent infections. A vast majority of adults have been exposed to the virus and could be asymptomatic. The purpose of this study was to determine the lowest inhibitory concentration of black tea extracts on the herpes simplex virus -1. Utilizing plaque assays, DNA extraction, PCR, and gel electrophoresis, it was determined that viral inhibition occurs at a concentration of 0.1 $\mu$ g/mL BTE.

Montclair State University

Inhibition of Herpes Simplex Virus-1 by Black Tea Extract

by

Sadé Diahann Randall

A Master's Thesis Submitted to the Faculty of

Montclair State University

In Partial Fulfillment of the Requirements

For the Degree of

Master of Science

May 2012

College of Science and Mathematics

Department of Biology & Molecular Biology



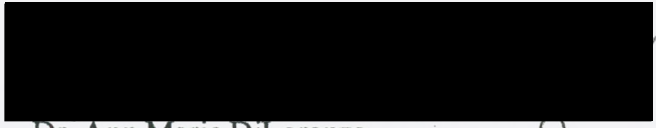
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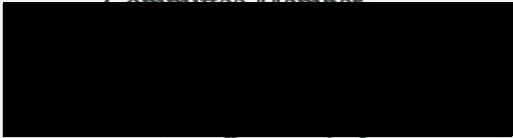
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INHIBITION OF HERPES SIMPLEX VIRUS – 1 BY BLACK TEA EXTRACT

A THESIS

Submitted in partial fulfillment of the requirements

For the degree of Master of Science

by

SADÉ DIAHANN RANDALL

Montclair State University

Montclair, NJ

2012



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## **Herpes Simplex Virus – 1**

**Classification.** Herpes simplex virus (HSV) belongs to the *Herpesviridae* family, and is a member of the *Alphaherpesvirinae* subfamily. There are eight human herpes viruses, which include Herpes simplex virus 1 and 2. Biological properties such as growth cycle, cytopathology, and location of latent infection determine the classification of the herpes viruses into their respective subfamilies. HSV-1 is a double-stranded DNA virus composed of four parts: an icosahedral capsid, a core containing viral DNA, a tegument, and an envelope accented with glycoprotein spikes. Its diameter can range from 125nm – approximately 200nm based on whether the virion is enveloped or naked (Brooks et al., 2010). HSV-1 contains about 150,000 base pairs, which allows it to produce 80 gene products. The genome is separated into segments called Long or Short as indicated by the respective lengths of each segment (Taylor et al., 2002). Each HSV type encodes at least 70 distinctive polypeptides, of which many of their functions remain unknown. HSV-1 exhibits a short growth cycle and establishes latent infections within neurons (Brooks et al., 2010).

**Viral Entry/Latency.** HSV-1 is often transmitted via oral contact, while HSV-2 transmission occurs most often through genital contact. The transmission locations are not exclusive to each type and may be used as a route of entry by either type of HSV. Viruses gain entry to host cells via different mechanisms; herpes virus type 1 exhibits multiple entry routes. One entry mechanism is virus attachment to filopodia; filopodial bridges were also observed between cells and assisted in the migration of extracellular HSV-1 virions from an infected to uninfected cell (Akhtar and Shukla 2009). Carfi et al (2001) indicated that viral fusion at membranes was triggered by glycoprotein conformational

changes. Also, the use of paired immunoglobulin-like receptor (PILR) as a co-receptor may be utilized as a route of entry by HSV-1 (Sato et al., 2008). HSV-1 utilizes viral envelope proteins such as glycoprotein B, C, and D in steps to gain entry. Initially, HSV-1 begins to attach to the host cell using glycoprotein C (Harold et al., 1991). Next, the glycoprotein D and cellular receptor interaction stabilize the attachment to the host cell (Spear et al, 2000). Subsequently, the host cellular membrane and viral envelope fuse via an unknown mechanism (Ligas and Johnson, 1988).

Previous research indicated HSV-1 entry occurred through pH-independent viral envelope and plasma membrane fusion (Spear, 1993). One strategy was to utilize bioengineering to remove the recognition of the natural receptors and replace them with a new target receptor (Menotti et al., 2009; Zhou and Roizman, 2006). A second mechanism was to develop an attachment bridge to transport the virus to its target. Adaptors may function to target one specific mode of entry for HSV-1 (van Beusechem et al., 2003; Verheije et al., 2006). O'Donnell et al (2010) stated that 3-O sulfated heparin sulfate's (3-OS HS) precursors are readily available leading to the possibility of increased amounts of 3-OS HS. The results also suggest that HeLa, Vero, and retinal pigment epithelia (RPE) cell lines express a unique group of 3-O sulfotransferases (3-OST), which suggests 3-OS HS may be an important receptor in HSV-1 entry. A reduction in viral entry was observed in all cell types when either exposed to 2-OST siRNA or when cell lines expressed decreased amounts of 3-OS HS. This further supports that 3-OS HS plays a significant role in viral entry. 3-OS HS may be a coreceptor for nectin-1 or may have an uninvestigated role in post-entry processes. Studies with 3-OS HS knockout mice indicate that 3-OS HS functions in other signal pathways by the inability of the knockout mice to



develop normally. The inhibition of 3-OS HS precursors may result in attachment, penetration, and cell-cell fusion inhibition. Further research is required to determine why down-regulation of 3-OS HSs precursors inhibits viral entry and cell-cell fusion as well as the specific role of 3-OS HS in HSV-1 (O'Donnell et al., 2010). Future research should also identify HSV protein-protein interactions during the process of infection host cells. Identification of the mechanisms will allow for the development of novel anti-viral medications and treatments (Kelly et al., 2009).

Upon entry, herpes virus establishes latent infections within humans and produces lesions at the entry location upon reactivation. HSV incubation can last approximately 2 – 12 days, may excrete virus for about 7-10 days and can recur more frequently in individuals with underlying immune deficiencies (Taylor et al., 2002; Whitley and Roizman 2001). During latent infection, viruses are virtually undetectable in the host. Once the virus enters the host, the infection either proceeds as lytic or latent. Latent infections are unable to be reproduced in vitro. There are numerous regulatory mechanisms that facilitate latency and subsequent reactivation (Bloom et al., 2010).

**Replication/Assembly.** The HSV-1 genome consists of a minimum of 79 genes, with approximately four being diploid. Within the genome are three origins of replication; all of which can function independently of the other origins. Viral replication occurs within specific areas of the host cell nucleus. Most proteins utilized for replication are virus encoded. HSV-1 exhibits highly recombinant characteristics (Bataille and Epstein 1995). Changes within the pattern of viral gene expression results in a different replication pattern of HSV-1, which can range from explosive replication to a complete arrest. With increased early gene activity comes faster replication speed. Mutations in the

promoter have the ability to increase or decrease binding affinity. The replication speed of a virus is more important than replication efficiency due to the limited time before the infected cells dies (Nakabayashi and Sasaki 2009). After replication, the HSV virus must assemble prior to lysing the host cell. Herpesvirus capsids form within the nucleus. Specialized structures house capsid maturation. The nucleocapsids then migrate and make contact with the inner nuclear membrane; subsequently budding occurs. Cellular kinases enable this contact by phosphorylation of the nuclear lamins. The nucleocapsids obtain proteins and acquire an additional envelope as they lyse the host cell. Further research on the assembly mechanisms will allow for the development of drugs/treatments that can target replication and assembly mechanisms individually or in a number of combinations (Mettenleiter et al., 2009).

**Detection/Treatment.** HSV-1 infection rates are significantly higher among young children, ranging in age from birth up until about three years old in comparison to adults. However, 70-90% of adults will develop HSV-1 antibodies within their lifetime (Brooks et al., 2010). Ohana et al (2000) have developed an extremely sensitive and specific HSV-1 and HSV-2 antibody screening tool. The test detects total antibodies present and is a better indicator of actual prevalence rates. The testing is intended to determine if the infection is caused by HSV-1 or HSV-2 however, is not engineered for mass screenings. The main goal of the screening is to achieve an accurate account of HSV prevalence (Ohana et al., 2000).

HSV may infect the body by a number of routes, including the eye. A feature of all herpes viruses is the ability to reactivate the infection. Recurring infections in the eye could lead to permanent damage up to and including blindness. Peptide-conjugated



phosphorodiamidate morpholino oligomers (PPMO) targeting HSV-1 genes may prevent viral replication in the eyes of rats. This method of treatment was found to be more effective against Acyclovir sensitive and/or resistant HSV-1 strains. Results indicate that topical treatment utilizing PPMOs are most effective on recurrent HSV-1 as the viral load is lower in reactivation (Moerdyk-Schauwecker et al., 2009).

Bacteriophages are used as the delivery mechanism for some DNA vaccines. Mice receiving injections containing recombinant phage are able to induce antibody production and elicited immune responses that were significantly higher than the control groups. Bacteriophages are cost-effective and safe method to administer vaccinations against infections and cancer. However, further research is required to indicate the potential usage of bacteriophage vaccines for HSV-1 protection and the degree of immunity provided (Hashemi et al., 2010; Parsania et al., 2010). Some suggested ways to improve DNA vaccinations were to include a nontoxic adjuvant that would heighten the immune response, and administer the vaccine with an antigen that would prolong the life cycle of dendritic cells. Co-injection of proapoptotic box gene with a HSV-1 plasmid exhibited increased immune response. A moderate amount of Bax plasmid is necessary for enhanced immune response. Expression vectors, doses of vaccination, regimens of vaccine and route of administration require further evaluation (Parsania et al., 2010). Currently, there are antiviral drugs available as a treatment to reduce the number of recurrences, shorten healing time, and suppress the infection. However, there are not any preventative methods to combat HSV-1 infections.

## Tea and Tea Extracts

**Background and Uses of Polyphenols.** *Camellia sinensis* is a plant whose leaves when processed will produce tea in all its available forms. The fermentation level determines tea categories such as, green (unfermented), black (fermented), and oolong (partially fermented) (Cheng 2006). Theaflavins are a natural antioxidant, derived from tea, which have been shown to have a greater antioxidant effect than vitamin E (Shiraki et al., 1994). The inhibition stems from decreased superoxide production. Experiments have shown that theaflavins target cell-signaling pathways in tumors and as a result are able to suppress cancer (Park and Dong, 2003). Theaflavins are also able to inhibit the initiation of tumor formation thereby preventing carcinogenesis (Mari et al., 1999; Maniti et al., 2002). Inhibition of inflammation occurs after theaflavin ingestion. Theaflavins can inhibit viral adsorption into the cell but seem to lack an effect on viral replication in cells infected with influenza, rotavirus, or enteroviruses (Mikio et al., 1990; Mukoyama et al., 1991). A number of diseases can benefit from regular theaflavin intake such as: hyperglycemia, atherosclerosis, heart disease, cancer, and aging. Black tea theaflavins may be beneficial in future pharmacological developments (Wang and Li 2006). Bonnely et al. (2003) created a new model set up to effectively oxidize tea leaves while maintaining high polyphenol levels and reducing or removing the majority of impurities. The oxidation of tea leaves over two hours was found to be most effective. Polyvinylpyrrolidone (PVPP) removed additional polyphenol substances to increase accuracy of the activity measurement. The model oxidation system required no buffer, and indicated that agitation of tea leaves in water does not affect the tea's enzymatic activity (Bonnely et al., 2003).



The antioxidant activity of the *Camellia sinensis* plant has been suggested to protect against atherosclerosis. Polyphenols have exhibited positive effects on hypertension cases. The reduced incidences of atherosclerosis have the ability to reduce diabetic mortality. It is unknown if tea drinking should be recommended to diabetics or those at risk. However, recent studies have indicated green tea may be beneficial in metabolic syndromes (Campbell 2004). Green tea has a higher antioxidant level than black tea. Both green and black tea cause a similar degree of microbial degradation (Cheng 2006). Regular consumption of green tea has been found to reduce oxidative damage within the body (Erba et al., 2005). Green tea is often more effective than black tea as an antioxidant. The effects of the green or black teas' antioxidant functions are short lived and best results are experienced with repeated tea intake (approximately 6 cups daily) for several days (Klaunig et al., 1999). Tea consumption has an inverse relationship with developing gastric cancer as well as some other cancers. Tea polyphenols have also been used as a topical treatment. Additionally, tea consumption is beneficial in cardiovascular disease, diabetes and obesity (Khan and Mukhtar 2007). Animal models were created to mimic diabetes and tea catechins, such as epigallocatechin gallate (EGCG), that exhibited antidiabetic and antiobesity effects (Kao et al., 2006). Green tea catechins' biological activity has been well studied and documented. However black tea requires further inquiry (Vinson 1998). Investigations found increased catechin levels in the blood after black tea ingestion (Leenen 2000). Black tea was determined to not have diminished antioxidation properties; rather, theaflavins contain more hydroxyl groups than catechins, which are key to scavenging free radicals (Leung et al., 2001). There are additional antioxidative features, such as

black tea polyphenols binding to transition metal ions. EGCG affected free radicals via reduction of reductase activity (Rice-Evans et al., 1997). Previous research has indicated that epigallocatechin gallate (EGCG) is able to inhibit influenza when there is direct contact. EGCG and epicatechin gallate (ECG) were determined to inhibit influenza growth in all strains of influenza. A strong inhibitory effect was evident after analyzing plaque assays of influenza infected Madin-Darby canine kidney (MDCK) cells, which led to the belief that the catechins were combating multiple steps of the viral cycle (Song et al., 2005). Black tea also protects against oxidation of phospholipids in the membrane. Some previous black tea studies have indicated theaflavins may cause some oxidative damage occasionally resulting in apoptosis (Oikawa et al., 2003). Although further research is required to understand the conditions to which theaflavins are best used for health benefits (Luczaj and Skrzydlewska 2005), research indicates that black tea extract can prolong the average lifespan and increase survival of fruit flies. Black tea extract has contributed to upregulation of superoxide dismutase (SOD) and catalase (CAT) resulting in increased fruit fly lifespan. SOD and CAT expression decreases with aging so there could be a correlation between BTE intake and extended life (Peng et al., 2007). Scavenging of free radicals and upregulation of antioxidant enzymes as a result of black tea intake may be the mechanisms by which the fruit fly life is extended (Gutteridge and Halliwell, 2000). Additionally, Kitani et al. (2007) reported that the inclusion of BTE in water consumed by male mice increased their life span by 6.4%. There is no evidence that this same result will occur in humans, however the ingestion of black tea may prevent premature death (Nakachi et al., 2003).



**Antiviral Studies.** Black tea theaflavins were found to have antimutagenic activities in microbial systems. Tea functions through inhibition of reductase and enzymes, induction of detoxification pathways and removal of free radicals and electrophiles (Apostolides et al., 1996). Previously, researchers hypothesized that the mechanism by which tea exhibited anti-mutagenic properties was through enzyme inhibition (Bu-Abbas et al., 1994) Black tea blocks the production of free radicals; it also protects DNA and may reverse many mutagenic changes (Hasaniya et al., 1997).

Khan et al. (2005) reviewed various natural forms of anti-HSV extracts and compounds. Researchers described 18 different plants that were tested to determine their antiviral effects on HSV infected Vero cells. *Plantago major L* extract exhibited minimal anti-HSV effects. High concentrations of *Melia dubia*, and *Cryptostegia grandiflora* displayed partial antiviral activity. Approximately 11 medicinal plant extracts derived from Thailand exhibited a reduction in HSV plaque formation. Some plants, *Hemidesmus indicus*, *Paederia foetida*, and *Shorea robusta* were shown to exhibit antiviral resistance against herpes strains resistant to Acyclovir (Khan et al., 2005). Plant extract, derived from *Rhododendron ferrugineum*, was used to prevent viral absorption and penetration. These results imply the use of polyphenols from other plant types as possible antiviral substances. Further research is required to determine which surface locations on the viruses are affected by polyphenols (Khan et al., 2005).

Attachment of virus to host cells was affected by extracts of *Rhododendron ferrugineum* and increased concentrations of the substance inhibited penetration. Attacking the viral envelope produced the most anti-adhesive effect on HSV infections (Gescher et al., 2011). Concentrated tea extract was more effective than diluted or

mixtures of polyphenols at inhibiting influenza virus replication. Antiviral activity is observed throughout the infection cycle leading to the conclusion that the primary target is the membrane. Catechins may also affect conformation and virus-host cell interaction but more investigation is required (Song et al., 2005).

Black tea, derived from *Camellia sinensis*, has approximately 1/10 of the catechins in green tea. The black tea theaflavins inhibited growth and triggered apoptosis in cancerous cells. Immortalized cells were more affected by theaflavin cytotoxicity than normal cells. Theaflavins were found to inhibit growth of abnormal cells and may have a protective role against human cancers. Toxicity of theaflavins was dependent on length of exposure time, and thought to occur as a result of antioxidant action. Cytotoxic effects of black tea theaflavins are thought to cause cell cycle arrest and induce apoptosis (Babich et al., 2006).

Black tea theaflavins were found to be more effective against HIV activity than green tea catechin derivatives. Four theaflavins inhibited HIV-1 replication, while maintaining minimal cytotoxicity. Approximately seven theaflavins inhibited cell fusion in HIV cells. Results indicated that tea polyphenols were unable to prevent coreceptor binding in HIV however, black tea polyphenols inhibited HIV entry by disrupting formation of the glycoprotein 41 (gp41) six helix bundle, subunit of the envelope. EGCG combated HIV infection by three mechanisms: inhibition of HIV-1 reverse transcriptase activity, binding to cellular glycoprotein CD4 and preventing interaction with glycoprotein 120 (gp120), and destroyed viral particles. EGCG blocked viral entry of influenza and may inhibit entry of HIV-1 (Liu et al., 2005).

The antiviral effects that black tea extracts (BTE) have exhibited on HIV and influenza (Song et al., 2005; Liu et al., 2005) coupled with green tea's catechin effectiveness has led to the proposed use of BTE to inhibit Herpes simplex virus1 replication in cultured cells (Khan et al., 2005). Tea polyphenols have inhibited HIV entry and replication of influenza when in contact with the respective viruses. It is reasonable to presume that HSV-1 may demonstrate similar outcomes when exposed to BTE. Therefore the goal of this project is to determine the lowest inhibitory concentration of black tea extract that would affect Herpes simplex virus 1 infection of cultured cells. Plaque assays will be used to quantify the viral inhibition at various concentrations of BTE. Subsequently, spectrophotometry, DNA extraction and gel electrophoresis will be utilized to determine the effect on viral replication.



## Materials and Methods

### Cells

**Cell line.** African green monkey kidney (Vero) cells [American Type Culture Collection (ATCC), Manassas, VA, USA] were grown in ATTC-formulated Dulbecco's Modified Eagle's Medium (Catalog No. 30-2002), which is composed of 1mM sodium pyruvate, 4mM L-glutamine, 1500 mg/L sodium bicarbonate and then supplemented to contain fetal bovine serum (FBS) (Biowest, Miami, FL, USA) to a final concentration of 5%.

**Cell cultures.** Vero cells were kept in T25 flasks with 5 mL 5% FBS-DMEM in an incubator at 37°C with an atmosphere of 5-7.5% CO<sub>2</sub>. To passage the cells, the media was removed and the cells were washed twice with 1X phosphate buffered saline (PBS) (Fisher Scientific, Fair Lawn, NJ, USA). Cells were trypsinized with 0.5 mL of 1X trypsin-EDTA (Mediatech Incorporated, Manassas, VA, USA) at 37°C with an atmosphere of 5-7.5% CO<sub>2</sub> for approximately 5 minutes to detach cells. Cells are maintained at a 1:3 to 1:6 ratio and subcultured one to two times per week. □

### Virus

A recombinant strain of HSV-1, GHSV-UL46, which contains the sequence for green fluorescent protein (GFP) fused to the tegument protein pUL46, was used for all experiments (Willard, 2002) (ATCC, Manassas, VA, USA). □

### Black Tea Extract (BTE)

Black tea extract: 80% theaflavins (BTE)(10 mg/mL) (Sigma-Aldrich, Saint Louis,

MO, USA) was dissolved in 1 mL of 10% FBS-DMEM (Mediatech Incorporated, Manassas, VA, USA) to produce a stock concentration of 10 mg/mL of BTE solution. Ten-fold dilutions of stock were stored in Eppendorf tubes at 4°C.

### **Viral Inhibition**

**Virus-treated cells.** Vero cells were plated in 6-well plates with 2.5 mL of cell suspension added to each well and incubated at 37°C with an atmosphere of 5% CO<sub>2</sub> until confluent, about 24 – 48 hours. The media was aspirated and cells were treated with 100 µL of each of the four concentrations of BTE solution; as controls, 100 µL of 5% FBS-DMEM was added to two separate wells. Plates were rocked to ensure complete coverage and kept in an incubator at 37°C with an atmosphere of 5% CO<sub>2</sub> for 30 minutes. Any unabsorbed solution was aspirated and 100 µL of stock virus was added to each well, with the exception of one of the two wells to which 100 µL of 5% FBS-DMEM was added, which served as a control (mock-infected). The cells were returned to an incubator at 37°C with an atmosphere of 5% CO<sub>2</sub> for 1 hour and rocked every 15 minutes to ensure complete coverage. After 1 hour, any unabsorbed virus was aspirated from each well and 2.5 mL 5% FBS-DMEM was added to each well. The plates were returned to the incubator and kept at 37°C with an atmosphere of 5% CO<sub>2</sub>. The media from each well was harvested after 48 hours and transferred to cryogenic vials, which were stored at -80°C.

**Plaque assay.** To determine viral titers, ten-fold serial dilutions of virus-treated cells were performed by taking 100 µL of the desired treated media and combining it with 900 µL of 1% FBS-PBS, which was made by mixing 0.5 mL of FBS with 49.5 mL of



PBS, to produce the first,  $10^{-1}$ , dilution. Further dilutions proceeded in the same fashion, in which the starting solution was the previously made diluted solution. To the wells of a 6-well plate that contained a confluent monolayer of Vero cells, from which media had been aspirated, 500  $\mu$ L of 1% FBS-PBS was added as a control to the first well, and 500  $\mu$ L of the third through seventh ( $10^{-3}$ - $10^{-7}$ ) dilutions were separately added to each of five remaining wells. This process was replicated for each desired concentration of BTE extract that was studied. The plates were returned to an incubator at 37°C with an atmosphere of 5% CO<sub>2</sub> for 1 hour and rocked every 15 minutes to ensure thorough coverage; after 1 hour, any unabsorbed virus was aspirated from each well. Following this, a nutrient agar was poured over each well. The nutrient agar consisted of a mixture of two components, Solutions A and B, that were kept in separate flasks in a 41°C water bath prior to use. Solution A contained the following in an autoclaved Erlenmeyer flask: 17 mL of 3X Eagle (Gibco Invitrogen Corporation, Grand Island, NY, USA), 1.0 mL of 5% sodium bicarbonate (Gibco Invitrogen Corporation, Carlsbad, CA, USA), 0.5 mL FBS, 0.1 mL DEAE-dextran (ICN Biomedicals Incorporated, Aurora, OH, USA), 0.1 mL penicillin/streptomycin (Cambrex, Walkersville, MD, USA), and 0.05 mL gentamicin. Solution B consisted of mixing 30 mL MQ water and 0.2 g of agar bacteriological (Oxoid Limited, Basingstoke, Hampshire, England) and autoclaved. After 1 hour incubation, combine Solutions A and B and overlay cells with 4 mL of plaque media. Once solidified, store plates in an incubator at 37°C with an atmosphere of 5% CO<sub>2</sub> for 72 hours. After 72 hours incubation, remove agar and stain cells with crystal violet. The number of plaques formed in each well was counted to determine the titer in plaque forming units (PFU) per milliliter.

**DNA extraction.** DNA was extracted from infected Vero cells that contained 100  $\mu$ L HSV-1 virus, 100  $\mu$ L HSV-1/BTE lysate (0.1 mg/mL), 100  $\mu$ L HSV-1/BTE lysate (0.01 mg/mL), 100  $\mu$ L HSV-1/BTE lysate (1.0  $\mu$ g/mL), and 100  $\mu$ L HSV-1/BTE lysate (0.1  $\mu$ g/mL), respectively. Cells were incubated for 24 hours at 37°C with an atmosphere of 5% CO<sub>2</sub>. The DNA from each of the five groups of cells was extracted with the Qiagen DNeasy® Blood & Tissue Kit (Qiagen Sciences, Germantown, MD, USA), following the protocol provided by the manufacturer for cultured cells.

**Polymerase chain reaction (PCR).** Viral DNA was amplified by PCR. The primers were for sequences specific for the strain of HSV-1 used in all experiments, which coded for glycoprotein D (gD) and GFP. The following virus-specific primers were used: Glycoprotein D: □

gD2F. 5'-TTGTTTGTCTCATAGTGGGCCTC-3'

gD2R. 5'-TGGATCGACGGTATGTGCCAGTTT-3'

Green Fluorescent Protein (Willard, 2002):

GFP1F. 5'-GTCAAAGCTTAAGATGGTGAGCAAGG-3'

GFP1R. 5'-CTTGAAGCTTCTTGTACAGCTCGTCC-3'

**Gel electrophoresis.** PCR products were visualized on a 1% agarose gel.

**DNA quantification.** To quantify the total amount of DNA in both the extracted DNA and PCR products, a NanoDrop ND-1000 Spectrophotometer with accompanying computer software (NanoDrop Technologies Incorporated, Wilmington, DE, USA) was utilized, following the protocol provided by the manufacturer.

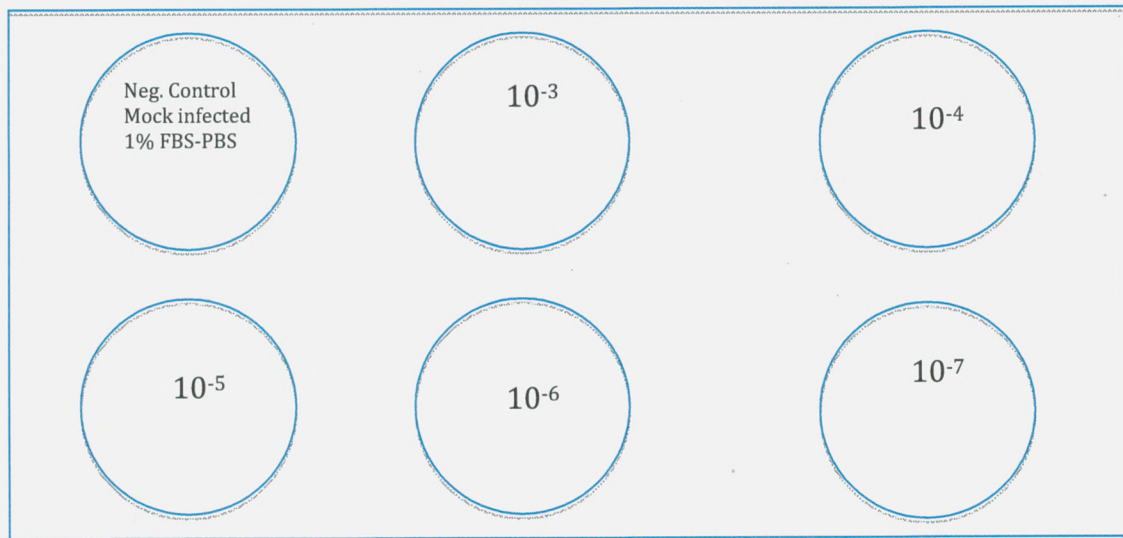
## Results

## **Viral Inhibition**

**Virus treated cells.** In order to visualize the cytopathic effect (CPE) of HSV-1 on Vero cells, treated and untreated Vero cells were observed with a light microscope. Differences could be observed between the cytopathic effects of cells treated with the highest concentration BTE (0.1 mg/mL) and the lowest concentration of BTE (0.1  $\mu$ g/mL). Cells treated with 0.1  $\mu$ g/mL BTE exhibited CPE, rounded and detached cells, close to that of untreated HSV-1. The cells treated with higher concentrations of BTE (0.1 mg/mL, 0.01 mg/mL), exhibited few cells displaying CPE. However, the control group treated with 5% FBS-DMEM had few dying cells.



**Plaque Assay.** In order to investigate the effect of BTE on HSV-1 infection, plaque assays (Figure 1) were conducted on untreated HSV-1 for use as a comparison to treated plates. Plaque assays were also conducted by infecting the monolayers of Vero cells with viral media harvested from infected cells previously treated: 0.1 mg/mL, 0.01 mg/mL, 1.0  $\mu$ g/mL, or 0.1  $\mu$ g/mL (Figure 2).

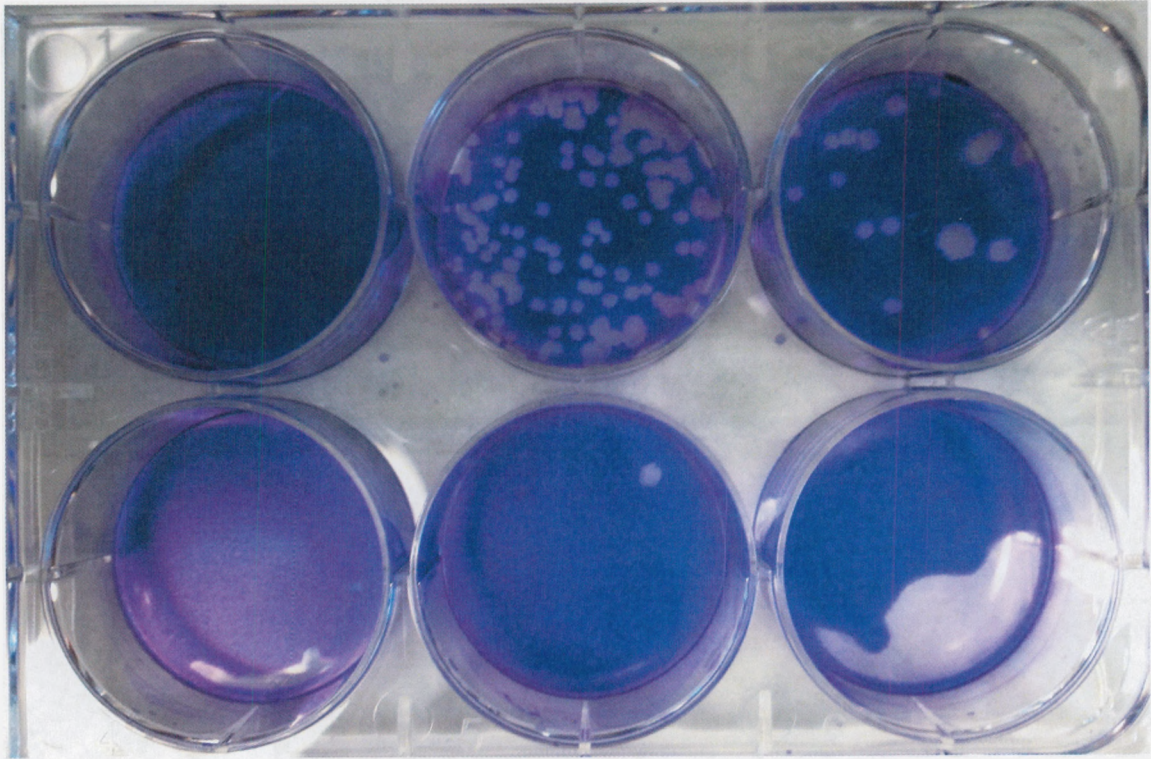


*Figure 1.* Schematic diagram of the set-up for plaque assays. Each of the six wells, confluent with Vero cells, were treated with a different dilution of either untreated HSV-1 or HSV-1 treated with BTE. The 1<sup>st</sup> well contained no virus and served as a control.

Neg. Control  
Mock infected  
1% FBS-PBS

$10^{-3}$

$10^{-4}$



$10^{-5}$

$10^{-6}$

$10^{-7}$

*Figure 2.* Plaque assay for HSV-1 lysate harvested from 0.1  $\mu\text{g}/\text{mL}$  concentration of BTE in cultured Vero cells. At the  $10^{-4}$  viral dilution, the viral titer is calculated to be  $3.4 \times 10^5$  PFU/mL.

The viral titer produced by HSV-1 alone and when treated with various concentrations of BTE was determined by plaque assays (Figure 1). The viral titer for HSV-1, 1.0 µg/mL and 0.1 µg/mL were determined (Table 1). The untreated HSV-1 viral titer is  $8.0 \times 10^6$  PFU/mL (plaque forming units per milliliter). The viral media harvested from infected cells treated with 1.0 µg/mL BTE produced 15 plaques for 0.5mL/well of viral solution at the  $10^{-4}$  dilution resulting in a viral titer of  $3.0 \times 10^5$  PFU/mL. However, the infected cells treated with 0.1 µg/mL BTE produced similar results as infected cells treated with 1.0 µg/mL BTE, 17 plaques for 0.5 mL/well of viral solution at the  $10^{-4}$  dilution, thus calculating a viral titer of  $3.4 \times 10^5$  PFU/mL.

Table 1  
*Plaque assay results*

Vero Sample	Viral titer (PFU/mL)
Untreated HSV-1	$8.0 \times 10^6$
BTE Treated HSV-1 (1.0 µg/mL)	$3.0 \times 10^5$
BTE Treated HSV-1 (0.1 µg/mL)	$3.4 \times 10^5$



**DNA extraction and Spectrophotometric Analysis.** To compare the amount of DNA present in Vero infected cells exposed to either untreated or BTE treated HSV-1, Vero cells were either exposed to 5% FBS-DMEM (mock-infected), untreated HSV-1 (HSV-1 suspended in 5% FBS-DMEM) or HSV-1 treated with four serial dilutions of lysate 4 (0.1  $\mu\text{g}/\text{mL}$  BTE treated HSV-1). The DNA from these cell samples was extracted and compared using a spectrophotometer (Table 2). The cells infected with untreated HSV-1 were shown to have relatively the same amount of DNA present (50.6  $\text{ng}/\mu\text{L}$ ) as compared to infected cells exposed to BTE treated HSV-1 (53 $\text{ng}/\mu\text{L}$  – [0.01 $\mu\text{g}/\text{mL}$  BTE treated HSV-1], 45.2  $\text{ng}/\mu\text{L}$  – [1.0  $\text{ng}/\text{mL}$  BTE treated HSV-1], 66.1 $\text{ng}/\mu\text{L}$  – [0.01  $\text{ng}/\text{mL}$  BTE treated HSV-1]). However, 0.1  $\text{ng}/\text{mL}$  of BTE treated HSV-1 resulted in a lower concentration DNA (38.5  $\text{ng}/\mu\text{L}$ ), which may indicate a reduction in the replication of viral DNA and thus, viral inhibition.

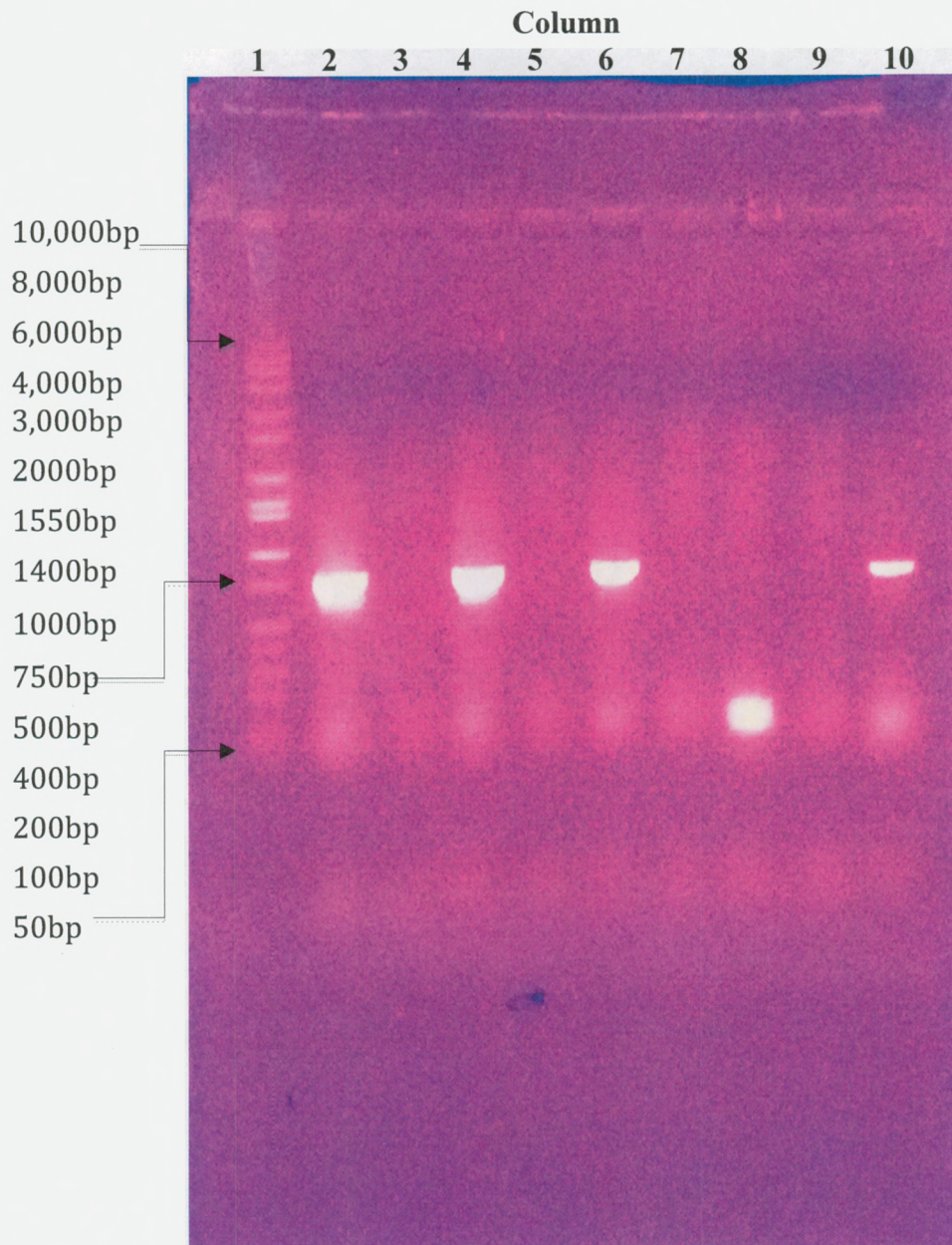


Table 2  
*Comparison of DNA from infected Vero Cells*

Vero Sample	Total DNA Present (ng/ $\mu$ L)
Untreated HSV-1	50.6
BTE Treated HSV-1 (.1 mg/mL)	53
BTE Treated HSV-1 (0.01mg/mL)	45.2
BTE Treated HSV-1 (1.0 $\mu$ g/mL)	38.5
BTE Treated HSV-1 (0.1 $\mu$ g/mL)	66.1

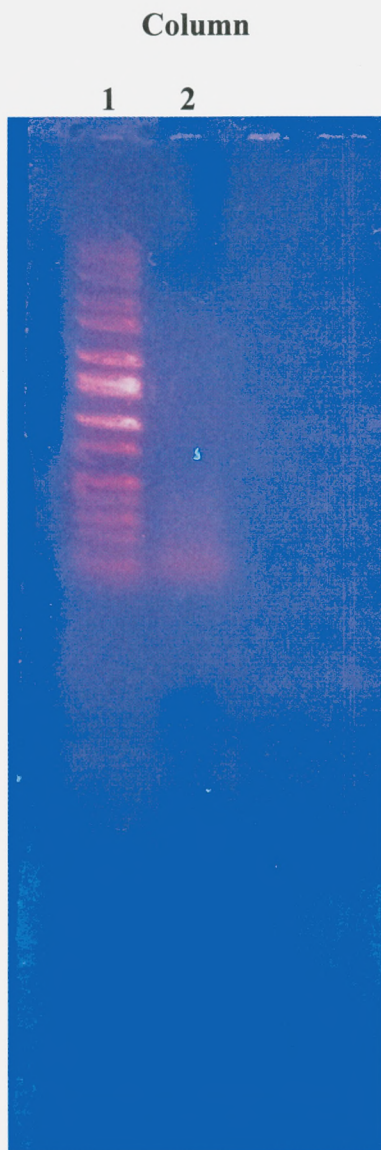
*Note.* For each sample, the total DNA present denotes a combination of cellular and any viral DNA. Vero cells were infected with HSV-1 that was either untreated or treated with four serial dilutions of the 0.1  $\mu$ g/mL sample (0.01  $\mu$ g/mL - 0.01 ng/mL BTE treated HSV-1). The spectrophotometer determined the amount of DNA present by analyzing 2  $\mu$ L of each sample. As a blank, 2  $\mu$ L of the elution buffer was utilized and registered as 0.0 ng/ $\mu$ L (data not shown).

**PCR and gel electrophoresis.** To determine if replication of HSV-1 DNA was affected by the treatment with BTE, two sets of viral primers were used in PCR to amplify any viral DNA extracted from infected cells. Sequence-specific primers were used to amplify the DNA encoding gD and GFP. Gel electrophoresis of the PCR products resulted in no visible bands on the gel corresponding to gD, however bands on the gel corresponding to GFP were apparent for all untreated and treated HSV-1 (0.01  $\mu\text{g/mL}$ , 1.0  $\text{ng/mL}$ , 0.1  $\text{ng/mL}$ , 0.01  $\text{ng/mL}$ ). These results indicate a direct correlation between the decrease of BTE concentration and the intensity of samples containing GFP. The intensity of samples 0.01  $\mu\text{g/mL}$  (column 2) and 1.0  $\text{ng/mL}$  (column 4) indicate that larger quantities of DNA fragments are present as opposed to the lower intensity observed in samples 0.1  $\text{ng/mL}$  (column 6) and 0.01  $\text{ng/mL}$  (column 10).



*Figure 3.* Gel electrophoresis of PCR products extracted from HSV-1 infected Vero cells either treated with BTE (columns 4 - 10) or untreated (columns 2 - 3). Column 1 contains the DNA ladder, with visible bands identified to the left in base pairs (bp). Columns 2, 4, 6, 8, and 10, and 3, 5, 7, and 9 contain DNA amplified with primers for the HSV-1 GFP and gD genes, respectively.





*Figure 4.* Gel electrophoresis of PCR products extracted from HSV-1 infected Vero cells treated with BTE (Column 2). Column 1 contains the same DNA ladder seen in the previous gel (Figure 3). Column 2 contains DNA amplified with primers for the HSV-1 gD gene.

## Discussion

The leading consumed beverage worldwide, after water, is tea. For over 50 centuries, tea has been recognized for its medicinal uses as an herbal treatment of multiple ailments from simple indigestion to atherosclerosis (Cheng, 2006; Khan and Mukhtar, 2007). Tea is available in multiple forms including, green, black and oolong, of which all contain polyphenols. Theaflavins, black tea polyphenols, are a natural antioxidant and inhibits viral adsorption into cells. As such, theaflavins may be useful in future pharmaceutical developments (Wang and Li 2006).

Herpes simplex virus is found all over the world and 70 – 90% of adults have HSV-1 antibodies. Once contracted, the primary infection lasts approximately 10 days with an average of 2-6 recurrences per year. There are about 300,000 HSV eye infections, as well as cases of HSV encephalitis which if left on treated results in death for 7 out of every 10 incidences. HSV-1 infection is not restricted to adults, as neonatal herpes occurs approximately 1 in 5000 deliveries each year (Whitley and Roizman, 2001; Brooks et al., 2010). Maternal infections are transferred to 30-50% of vaginally delivered neonates. To combat HSV-1 infection, Acyclovir is the drug of choice as it inhibits viral DNA synthesis, shortens healing time and can reduce the number of HSV recurrences. Nevertheless, HSV maintains the ability to establish latent infections within the neurons and also develop a resistance to Acyclovir and other related drugs (Brooks et al., 2010).

The purpose of this study was to assess the antiviral qualities of black tea extracts and determine the lowest inhibitory concentration against HSV-1. Black tea extract has previously been found to block the production of free radicals, inhibited growth of cancerous cells, as well as exhibited cytotoxic effects against immortalized cells (Babich



et al., 2006). BTE also inhibited viral activity of both HIV and influenza. If the lowest inhibitory concentration could be determined, future research could then be done to determine its effectiveness in conjunction with Acyclovir or as an alternative for Acyclovir resistant strains of HSV-1.

Previous research conducted in this lab determined the cytotoxicity of BTE as well as the highest concentration of BTE that can be used without causing toxic effects. Vero cells were treated with concentrations of BTE (0.1 mg/mL, 0.01 mg/mL, 1.0 µg/mL, 0.01 µg/mL, respectively), infected with HSV-1, and then the lysate was harvested. Inhibition of viral activity can be due to various mechanisms such as inhibition of viral entry, DNA replication, or any other step in the process of creating viral progeny. HSV-1 inhibition was observed through conducting plaque assays utilizing the previously obtained lysates. Data from each plaque assay indicated that inhibition occurred when compared to the HSV-1 assay.

DNA extracted from cells infected with each dilution of the lysate resulting from treatment of Vero cells with 0.01 µg/mL of BTE were analyzed by spectrophotometer. All the extracts had relatively similar concentrations of DNA; meanwhile, the DNA extracted from Vero cells treated with 0.1 ng/mL of BTE resulted in a lower DNA concentration than was present in any of the other samples including untreated HSV-1 (Table 2). This result indicates that there may be some viral inhibition taking place since there is less DNA found in the sample. To determine if replication of HSV-1 DNA was affected by treatment with BTE, PCR was used to amplify the DNA extracted from infected Vero cells and PCR products were visualized using agarose gel electrophoresis. Genome specific primers for GFP amplified the DNA extracted from each infection. The

gel electrophoresis also confirmed the spectrophotometer results as all PCR samples had a band at 750 bp, the approximate size of the GFP (727bp). If there is inhibition, the mechanism is not known. Oikawa et al. (2003) state that theaflavins may cause some oxidative damage. However, prior research in this lab found that various concentrations of BTE ( $10 - 10^{-8}$  mg/mL) exhibited no cytotoxic effects. The concentration of BTE in the 0.1 ng/mL sample may be low enough to perform viral inhibition without inflicting additional cytopathic effects against the Vero cells. Furthermore, the concentration of BTE in the 0.01 ng/mL sample may be below the required threshold to exhibit inhibition and thus the viral DNA levels increase back to the concentrations found within untreated HSV-1 and the other dilutions of lysate 0.1  $\mu$ g/mL.

It appears that 0.1 ng/mL is the lowest concentration that exhibits inhibitory effects on HSV-1. It remains to be determined the mechanism by which inhibition is occurring. Current antiviral drugs, such as Acyclovir, inhibit viral DNA synthesis (Brooks et al., 2010). There are also other means of inhibition that BTE could be acting upon such as attachment, penetration, or BTE could be slowing the replication cycle.

In conclusion, the 0.1 ng/mL sample was found to contain the lowest concentration of BTE that exhibits inhibitory effects on HSV-1. Previous research in this lab showed reduction due to binding to human epithelial A549 cells. This is also a possible mechanism for inhibition in Vero cells. The determination of which mechanism BTE is exploiting to reduce viral activity remains to be found. Further studies will be beneficial as BTE is a readily available substance and has a history of improving health with regular usage.



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