Effect of Black Tea Extract on Herpes Simplex Virus Type 1 Propagation

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EFFECT OF BLACK TEA EXTRACT ON HERPES SIMPLEX VIRUS TYPE 1 PROPAGATION

by

Anthony Cantatore

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

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Abstract

The purpose of this investigation was to determine if flavanol compounds, called theaflavins, found only in black tea and black tea extract (BTE) could inhibit herpes simplex virus type 1 (HSV-1) infection in cultured A549 human epithelial cells. The effect of BTE both on A549 cultured cells and HSV-1 was assessed by using phase contrast and fluorescent microscopy, as well as trypan blue and WST-1 assays and gel electrophoresis; the effect of infectivity was quantified by plaque assays and compared using a spectrophotometer to examine the extracted DNA and PCR products. Results indicated that HSV-1 did not cause cytopathic effects in A549 cells when exposed to BTE at a concentration of 1 mg/mL. Furthermore, HSV-1 treated with BTE resulted in no visible plaques at $10^3$ viral dilution and a reduction in viral DNA, indicating that BTE is capable of inhibiting HSV-1 in A549 cell cultures. The results of this study are promising for the future development of BTE into a treatment for HSV-1 infections.
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ANTHONY CANTATORE

Montclair State University
Montclair, NJ
2011
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Herpes Simplex Virus Type 1

Classification. Human herpesvirus 1, more commonly known as herpes simplex virus type 1 (HSV-1), is only one of eight viruses belonging to the Herpesviridae family that is known to infect humans. The Herpesviridae family contains over 100 different herpesviruses that infect a multitude of host organisms, including fish, birds, horses and humans. While some herpesviruses can infect multiple types of host organisms, most herpesviruses, including HSV-1, are restricted to just one host type. Aside from host range, other differences among the herpesviruses include genetic content, reproductive cycle duration and mechanism of latency. Yet, all herpesviruses are united by some basic properties: DNA genome, virion structure and latent infection cycle (Mettenleiter, Klupp, & Granzow, 2009; Roizman & Baines, 1991).

Herpes virus particles can range from 120 - 300 nm in diameter, though many are approximately 200 nm in diameter. All herpesviruses, however, contain several distinct morphological features, including: core, capsid, tegument and envelope. The core of a herpesvirion consists of a linear double-stranded deoxyribonucleic acid (dsDNA) genome arranged in toroid form, ranging from 120 - 230 kilo-base pairs (kbp) in length; about 30 - 35 different proteins reside with the genome in the core. The core is protected by an icosahedral capsid composed of 150 hexons and 12 pentons, which has a diameter ranging from 100 - 110 nm. A tegument separates the inner capsid from the outer envelope; the tegument contains several proteins, some of which are present at up to 2,000 copies. The viral envelope is composed of a lipid bilayer and contains various
glycoprotein spikes (Fatahzadeh & Schwartz, 2007; Kelly, Fraefel, Cunningham, & Diefenbach, 2009; Roizman & Baines, 1991).

The HSV-1 linear dsDNA genome is 152 kbp in length and consists of two main protein coding components, the unique long (UL) and unique short (US) sequences, which can be inverted to produce four isomers. Each of the two sequences are flanked by a pair of inverted repeat regions, TRl-IRl and TRs-IRs, respectively, which have functions in gene regulation and genome replication. Residing between two of the inverted repeat regions, which connects the UL and US sequences, and at the ends of these repeat regions, which serve as the terminal ends of the genome, are "a" sequences that contains packaging signals. The UL sequence contains 59 genes, while the US sequence contains 14 genes, for a total of 73 genes encoded by HSV-1, which are activated at different times during its 18 hour life cycle. Yet, it has been shown that HSV-1 can produce over 80 different proteins, some of which have been found to be the same protein, but with various post-translational modifications. While many of the proteins serve a structural purpose, others are solely for viral DNA replication, while other proteins have multiple functions (Bataille & Epstein, 1995; Garner, 2003; Kelly et al., 2009; Mettenleiter et al., 2009; Watanabe, 2010).

Based on biological characteristics, the Herpesviridae family is further classified into three subfamilies: alphaherpesvirinae, betaherpesvirinae and gammaherpesvirinae. HSV-1, a member of the simplexvirus genera that contains the eight human herpesviruses, belongs to the alphaherpesvirinae subfamily. Other members of the alphaherpesvirinae subfamily that infect humans include herpes simplex virus type 2 and
varicella zoster virus. This subfamily is distinguished by its short reproductive cycle, rapid spread, destruction of host cells and location in establishing its latent cycle (Mettenleiter et al., 2009; Roizman & Baines, 1991).

**Infection cycle.** The infection cycle of HSV-1 is typical of that for other viruses: attachment and fusion of virion to host cell, transcription - and subsequent mRNA translation (protein synthesis) - and replication of the viral genome, and assembly and release of new virus particles. However, HSV-1, as with the other members of the *Herpesviridae* family, is unique in that it is also capable of establishing a latent infection cycle. The lytic infection cycle of HSV-1 begins with adsorption: when the virion first attaches to then fuses with a host cell, typically a neuron or epithelial cell. Both attachment and fusion take place when glycoproteins on the virus particle bind to suitable receptors on the plasma membrane of the host cell. Important HSV-1 glycoproteins extruding from the viral envelope include glycoprotein (g)B, gC, gD, gH and gL, as well as gE, gI and gK; cell receptors to which these glycoproteins bind comprise of the following: heparan sulfate (HS), nectin-1, nectin-2 and herpesvirus entry mediator (HVEM). While HS is found on many types of cells, nectin-1 and nectin-2 are found primarily at junctions of epithelial cells, as well as the synaptic junctions of neurons, and HVEM is located on T lymphocytes and trabecular meshwork cells. The location of the receptors influences the tropism of HSV-1 and places a limit on the types of cells to which it is capable of attaching, and thus, infecting (Akhtar & Shukla, 2009; Garner, 2003; O'Donnell, Kovacs, Akhtar, Valyi-Nagy, & Shukla, 2010).
As with other viruses, HSV-1 adsorption into host cells requires the successful attachment of viral glycoproteins to host cell receptors, followed by a fusion of the viral envelope to the host cell plasma membrane. Initial attachment of HSV-1 to its host cell occurs when gB with or without gC on the viral envelope binds to HS on the host cell's membrane. HS is a glycosaminoglycan, in which the polysaccharides, composed of repeating disaccharide units of uronic (either glucuronic or iduronic) acid and (either N-acetylated or N-sulfo-) glucosamine, are covalently linked to a protein core, which is located on the plasma membrane of most cells in humans. Binding of the glycoprotein to HS is only facilitated by gC; gB alone can successfully bind to the HS cell receptor to allow HSV-1 attachment to a host cell. While attachment of HSV-1 gB to its HS receptor on a host cell can occur anywhere along the plasma membrane of the host cell, it has been found to often occur along filopodia or protrusions of the plasma membrane that contain an abundant actin network. Aside from occurring on the edges of various tissue layers, filopodia are also prevalent during wound healing, which allows for easier access of HSV-1 to suitable attachment sites. Viral particles attached to HS on filopodia have been observed to be transported to the cell body for subsequent fusion in a process called viral surfing. Viral surfing is a likely result of the reorganization of the host cell's actin cytoskeleton that occurs when HSV-1 gB binds to HS, which functions as a signaling pathway for the aforementioned process. In addition to HS, HSV-1 gB has also recently been found to bind to paired immunoglobulin-like type 2 receptor-alpha (PILR-α), in order to achieve attachment to monocytes, macrophages and dendritic cells. Binding of gB to PILR-α has also been implicated in the subsequent process of viral fusion to the
host cell, although this is still poorly understood (Akhtar & Shukla, 2009; O'Donnell et al., 2010; Watanabe, 2010).

Once the viral particle has attached itself to a suitable host cell, the viral envelope must fuse with the plasma membrane of the host cell; this process too, like that of viral attachment, is dependent upon viral glycoproteins and host cell receptors. Fusion begins when gD of HSV-1 binds to a cellular receptor such as nectin-1, nectin-2, HVEM or a modified HS molecule called 3-O-sulfated HS (3-OS HS), which is found on corneal cells; binding of gD to any of these receptors results in a conformational change in the gD molecule that recruits gH and gL, which form a heterodimer, as well as gB to form a multiprotein complex. Fusion then proceeds in a two step process that begins by bringing the HSV-1 envelope close to the host cell's plasma membrane; close contact is achieved by the gD/gH/gL portion of the complex and receptor binding, and results in the mixing of lipids between the viral envelope and the host cell's plasma membrane, which creates a fusion intermediate. The presence of gB in the glycoprotein complex allows for the subsequent fusion pore to form, which allows for mixing of the host cell's contents with that of the virus particle; specifically, the viral core, surrounded by its capsid and tegument, are released into the host cell's cytoplasm (Akhtar & Shukla, 2009; O'Donnell et al., 2010; Nakano, Kobayashi, Nakamura, Nakanishi, Asano, Kumagai, Tahara, Kuwano, Cohen, & Glorioso, 2011; Watanabe, 2010).

Viral entry can also be achieved by cell-to-cell spread, in which an infected cell transmits viral particles to an adjacent non-infected cell. This process still requires the interaction of HSV-1 gD with a suitable cell receptor; however, for cell-to-cell spread,
gD complexes with a heterodimer formed by gE and gI, which is transported to cell junctions via the trans-Golgi network (TGN) of cells infected with HSV-1. Fusion then proceeds in a manner similar to that previously described for viral adsorption. In addition, gK has recently been implicated in the cell-to-cell spread of specific cell types, including corneal cells and trigeminal ganglia cells. Regardless of how HSV-1 enters a host cell, viral adsorption is followed by transport of the capsid to the nucleus (Akhtar & Shukla, 2009; Watanabe, 2010).

The HSV-1 genome can undergo replication and transcription only when in the nucleus of the host cell. Thus, once a virion has gained entry into a host cell, transport of the viral particle to the nucleus must ensue. Upon entry into a cell, many viral proteins that make up the tegument dissociate from the viral capsid, while some tegument proteins still remain bound; disassociation of tegument proteins is aided by pUS3 and pUL13 (the "p" designates a protein, while "US" and "UL" signify that the protein is coded for by Us and UL segments in the viral genome, respectively), which are protein kinases that are part of the outer tegument of the viral particle. Viral transport to the nucleus soon follows when the capsid protein, pUL35, interacts with motor protein, dynein. Thus, HSV-1 uses the available microtubule network in host cells to engage in dynein-dependent transport in order to move to the nucleus. As transport occurs, the dissociated tegument proteins begin to alter cellular function to favor viral propagation; two of these proteins include the previously mentioned pUS3, which hinders apoptosis by inactivating members of the Bcl-2 pro-apoptotic family through phosphorylation, and pUL41, which is responsible for both the downregulation MHCII cell receptors, thereby avoiding an
immune response, and degradation of host mRNA, allowing for efficient viral protein synthesis (Kelly et al., 2009; Watanabe, 2010).

Once at the nucleus, HSV-1 will employ pUL36 to transport the viral genome and associated viral proteins from the capsid into the host nucleus by interacting with the nuclear pore complex. In the nucleus, HSV-1 DNA is transcribed in a regulated process that proceeds in three main stages, which is separated temporally into the following during an active or lytic infection: Immediate early (IE), early (E) and late (L). IE genes generally contain promoters with many binding motifs, and thus, are initially activated by a tegument protein, pUL48, which is a transcriptional activator and recruits host cell transcription factors such as HCF-1 and Oct-1. The five IE genes code for transcription factors, which serve to regulate HSV-1 gene expression. Specifically, infected cell protein (ICP)4 has been found to form a transcriptional complex on the promoter of viral E genes, thereby transitioning viral transcription to the second stage; activation of E genes proceeds via the blocking interferon by ICP0, thereby preventing E gene silencing. The 12 E genes mainly function to replicate the viral genome; some protein products of E genes include pUL23, thymidine kinase, which is involved in nucleic acid metabolism, pUL5, DNA helicase, and pUL30 and pUL42, which are subunits of DNA polymerase. The 56 L genes function mainly to produce structural proteins for the formation of new virions, including capsid proteins such as pUL19 and pUL26.5, structural and scaffolding proteins, respectively, tegument proteins such as pUL46 and pUL36, involved in viral envelopment and transport, respectively, and envelope proteins, such as pUL27 and pUS6, gB and gD, respectively. The HSV-1 genome in its latent state, which resides in
neuronal cells, is markedly different than in its lytic stage. Not only does the HSV-1 genome circularize in its latent stage, but it only produces one major transcript, Latency-Associated Transcript (LAT); LAT is usually spliced to produce a stable 2 kbp RNA intron, which is thought to repress lytic genes by recruiting histone modification enzymes that interact with the viral DNA genome. When exposed to certain stresses, however, this repression can be reversed in order to reactivate lytic genes, thereby causing viral propagation to resume (Bloom, Giordani, & Kwiatkowski, 2010; Kelly et al., 2009; Nakabayashi & Sasaki, 2009; Watanabe, 2010).

A lytic HSV-1 infection will result in the production of new viral DNA and proteins, which must then assemble to produce new, infectious virions. Viral assembly is a complex process, and different hypotheses have been proposed including the lumenal and nuclear pore pathways; however, one model, the envelopment-deenvelopment-reenvelopment model, is the most supported by a variety of studies and thus, will be the exclusive focus here. HSV-1 assembly begins with capsid formation in the infected host cell's nucleus, where the capsid proteins, pUL19, pUL18 and pUL38 assemble around pUL26.5, a scaffolding protein. Two other proteins, pUL17 and pUL32, are involved in cleaving the newly synthesized viral DNA and transporting it into the newly formed capsid. Tegument proteins may also begin to associate with the capsid, though this still remains unclear, and three separate models have been proposed, in which some tegument proteins like pUL16 attach to the capsid in the nucleus (nuclear loading model), cytoplasm (capsid loading model) or at the TGN (TGN loading model); most recent studies support the capsid loading model, where tegument proteins may be added while
the capsid is traveling through the host cell's cytoplasm. Regardless, the viral capsid must first exit the nucleus in a process called primary envelopment. In order for the capsid to attach to the inner nuclear membrane and bud into the perinuclear space, the nuclear lamin network must be disassembled; this occurs when pUL31 and pUL34 form a complex to recruit pUS3, a protein kinase, which causes changes in the nuclear lamin by phosphorylating lamins A and C, which are involved in maintaining nuclear integrity. Once the newly enveloped capsid buds into the perinuclear space, the virus soon looses this primary envelope, as it fuses with the outer nuclear membrane, in which pUS3 is again thought to play a significant role; this deenvelopment of the virus releases it into the cytoplasm of the host cell (Kelly et al., 2009; Meckes, Marsh, & Wills, 2010; Mettenleiter et al., 2009; Watanabe, 2010).

In the cytoplasm, several viral proteins associate with the capsid, including pUL36, pUL37 and pUS3, which constitute an inner tegument. Exit of the virus from the host cell proceeds as the viral particle moves along the microtubule network via kinesin-independent transport with the aid of pUL36 and pUL37. While in transport, additional viral proteins are recruited by pUL48, which interacts with pUL41, pUL46, pUL47 and pUL49 to form an outer tegument; pUL16 also associates with the outer tegument. Secondary envelopment or reenvelopment occurs at the TGN, where glycoproteins are also added. Glycoproteins such as gB and gD are found at the TGN, bound to pUL11, which in turn, binds to the tegument of the virus via interactions with pUL16. Similar interactions occur between pUL48 on the tegument and gB, gD and gH at the TGN. At this stage, the viral capsid with its tegument become enveloped and then proceed to exit
the cell via exocytosis; though this process is not well understood, pUL20 and gK have been implicated in viral egress or exit from the cell. Once released, these new virions are capable of infecting new host cells and starting the lytic cycle once again (Kelly et al., 2009; Meckes et al., 2010; Mettenleiter et al., 2009; Watanabe, 2010).

**Transmission.** HSV-1 infections in humans occur when an individual's mucous membranes or abraded skin is exposed either to lesions or mucosal secretions of a person who has an active HSV-1 infection, either primary or recurrent. The most common way for HSV-1 to be transmitted is by the oral secretions of an infected individual, whether symptomatic or not. An individual with an active infection can shed the virus onto various surfaces (e.g., skin, utensils, clothing), on which HSV-1 can remain intact for a short period of time; a non-infected person can then be exposed to one such surface and become infected. Thus, HSV-1 is often transmitted by kissing or sharing utensils. HSV-1 can also be transmitted from mother to child either in utero or during childbirth (Fatahzadeh & Schwartz, 2007).

Initial exposure to HSV-1 most commonly occurs to epithelial cells. After this primary infection, the virus travels to the cell bodies of sensory neurons via retrograde flow along the axons that innervate the site of initial infection. HSV-1 often travels to the trigeminal ganglia, where it will persist for the lifetime of the host in a latent state. However, the virus can become reactivated in neurons either spontaneously or due to a trigger, which may include one or more of the following: Physical or psychological stress, fever, fatigue, exposure to heat, cold or sunlight, tissue or nerve damage, immunosuppression, surgery or menstruation. Once reactivated, HSV-1 can either travel
in the anterograde direction and reinfect the original epithelial tissue, causing a recurrent infection, or it can move in the retrograde direction, to infect other, synaptically linked neurons. The difference in the direction that the reactivated virus travels produces the different symptoms found in various individuals infected with HSV-1 (Fatahzadeh & Schwartz, 2007; Garner, 2003; Whitley & Roizman, 2001).

**Symptoms and prevalence.** Since HSV-1 primarily affects skin, mucous membranes and neurons, this herpesvirus often results in the following infections: oral, facial, pharyngeal, ocular and central nervous system. Primary and recurrent infections generally produce the same symptoms with the exception that recurrent infections are often milder and persist for a shorter amount of time as compared to primary infections. HSV-1 infections can result in the following: Primary herpetic gingivostomatitis (PHGS), herpes simplex labialis (HSL), recurrent intraoral herpes (RIH), genital herpes, Kaposi's varicelliform eruption (KVE), herpes gladitorum, herpetic whitlow, ocular herpes, encephalitis and neonatal herpes. An individual's symptoms of these various HSV-1 manifestations may vary according to one's genetic makeup, immune status, site of infection and dose of inoculum (Fatahzadeh & Schwartz, 2007; Jordão, Ferreira, Souza, de Souza Faria, Machado, Abrantes, de Souza, & Cunha, 2011; Moerdyk-Schauwecker, Stein, Eide, Blouch, Bildfell, Iversen, & Jin, 2009).

PHGS, HSL, which is also known as fever blisters or cold sores, and RIH are all types of orofacial herpes, which are most common types of HSV-1 infections. Each is characterized by lesions that develop in and/or around the oral cavity. These lesions are often preceded or accompanied by a burning sensation, pain, discomfort, etc.; lesions
form when vesicles at the site of infection rupture to form erosions that coalesce into ulcerations. In each case, these symptoms persist for approximately 2 weeks after infection, while viral shedding can continue for up to several weeks after resolution of the symptoms. PHGS generally affects the oral mucosa, and can also cause gingivitis. HSL lesions, most commonly associated with HSV-1 infections, are located primarily on the outer vermilion border (the border marking the transition from one's lip to skin), and generally crust over. RIH typically affects the hard palate, surrounding gingiva and other keratinized tissue of the oral cavity. HSV-1 can also cause genital herpes, which produces symptoms similar to those of orofacial herpes; however, genital herpes is localized to the labia minora and urethra meatus in women and the shaft and glans of the penis in men (Fatahzadeh & Schwartz, 2007; Ohana, Lipson, Vered, Srugo, Ahdut, & Morag, 2000).

It is estimated that HSV-1 persists in approximately 45% - 98% of the world population and about 40% - 63% of the people in the United States. Of these infected individuals, about 15% - 40% experience symptomatic recurrent infections. While there is no seasonal variation to the spread of this herpesvirus, demographic factors seem to affect HSV-1 infection. For example, the infection rate in less developed/industrialized countries is higher than that in more developed/industrialized nations, 70% - 80% versus 40% - 60%, respectively. Race is another factor, with infection rates ranging from 35% in African American children under 5 years old, as compared to only 18% of their white counterparts in the United States. Socioeconomic conditions also influence HSV-1 infection, with 70% - 80% of adults belonging to a lower socioeconomic condition being
infected, as compared to only 40% - 60% of adults in an improved socioeconomic condition (Fatahzadeh & Schwartz, 2007; Whitley & Roizman, 2001).

**Detection and treatment.** In order to confirm HSV-1 infection, several methods of detection are employed, including: cytological smear, viral isolation, direct fluorescent antibody (DFA) testing, biopsy, polymerase chain reaction (PCR) and serological assays. Most of the detection methods rely on obtaining a sample from a lesion of a person with an active HSV-1 infection. Both a cytological smear and tissue biopsy require microscopic observation of a biopsied lesion to identify characteristic cytopathic or degenerative effects, with the former employing the use of a stain and the latter not doing so. Viral isolation requires culturing a sample transferred from a lesion and observing characteristic cytopathological features. DFA testing is a type of immunohistochemistry that detects HSV-1 antigens in a collected sample. Similarly, serological assays test for HSV-1 antibodies in a patient's blood. Lastly, PCR can detect the presence of HSV-1 DNA in lesions. Generally, viral isolation, sometimes coupled with DFA testing, is used to identify HSV-1, since it is fast, inexpensive, sensitive and specific, whereas serological assays may be utilized when none of the other tests are practical due to a lack of lesions; PCR is often employed to confirm herpes encephalitis (Fatahzadeh & Schwartz, 2007).

There is no cure that can eradicate HSV-1 from an infected individual. Thus, antiviral treatments, whether intravenous, oral or topical, focus on limiting the symptoms caused by HSV-1 usually by inhibiting viral replication. Generally, accessible lesions are treated by a topical antiviral agent, while inaccessible lesions are treated with systemic antiviral agents, whether oral or intravenous. The most common form of treatment is
with acyclovir by any of the three aforementioned methods. Acyclovir is a guanosine analogue that is modified by thymidine kinase (TK), a HSV-1 protein, and subsequently incorporated into viral DNA, where it terminates replication. Similar drugs include valacyclovir, penciclovir, and famciclovir. Other drugs such as foscarnet and cidofovir, which inhibit viral DNA polymerase, are often used on HSV-1 strains resistant to acyclovir and other TK-dependent drugs. While some of these drugs can have devastating side effects, the most widely used, acyclovir, has only a rare side effect of causing irreversible nephropathy. In addition, acyclovir has a low bioavailability and short half life in blood, requiring frequent doses to remain effective. To compound this problem, acyclovir is expensive, and patients who suffer from frequent HSV-1 reinfections may not be able to afford this medication (Fatahzadeh & Schwartz, 2007; Khan, Ather, Thompson, & Gambari, 2005; Thompson, 2006; Whitley & Roizman, 2001).

While acyclovir remains the current standard treatment for HSV-1 infections, new treatments are being developed to help combat resistant strains. Some of these treatments still focus on viral replication, though inhibit a different process. Once such class of compounds are called helicase-primase inhibitors (HPIs), which inhibit either pUL5 (helicase) or pUL52 (primase), both of which are vital to viral replication. However, mutant HSV-1 strains resistant to some of these drugs have already been detected, though a strategy of employing a combination of drugs that inhibit both the helicase and primase have proved successful thus far (Field & Biswas, 2011; Sukla, Biswas, Birkmann, Lischka, Ruebsamen-Schaeff, Zimmermann, & Field, 2010). Another strategy to inhibit
HSV-1 propagation is to inhibit protein synthesis; trichosanthin (TCS) is a compound extracted from the root of a plant that is capable of inactivating the 60s subunit of ribosomes, thereby preventing protein synthesis. The action of TCS is believed to induce cellular apoptosis, thereby inhibiting further HSV-1 propagation (He & Tam, 2010). Still another way of stopping HSV-1 is to vaccinate an individual against the virus. Two vaccines are being tested that allow the immune system to identify and aid in the eradication of HSV-1. One method employed plasmids that coded for Bax and gB; Bax is capable of inducing apoptosis, and thus, when these transfected cells died, they released large amounts of gB, which antigen presenting cells were able to present to T cells, which lead to an increase in the immune response (Parsania, Bamdad, Hassan, Kheirandish, Pouriayevali, Sari, & Jamali, 2010). Another vaccination method employed a phage particle expressing another HSV-1 glycoprotein, gD; these phages were injected into mice, which induced both a cellular and humoral response (Hashemi, Bamdad, Jamali, Pouyanfard, & Mohammadi, 2010). Still, other options for HSV-1 infections are being explored.

In an effort to find low-cost treatments for HSV-1 that can be taken more conveniently, with fewer side effects and a lower chance of developing resistant strains, a variety of compounds have been tested by researchers. Many of these treatments are focusing on compounds derived from plants, as many of them have been used throughout history to treat various human diseases (Khan et al., 2005). One study found that herbal extracts and compounds from a variety of medicinal plants, such as *Geum japonicum* and *Syzygium aromaticum*, were quite effective as anti-HSV agents; compounds identified
from these extracts had a wide range of structures, including: polyphenols, polysaccharides, tannins, and flavinoids (Thompson, 2006). Another study confirmed that tannins inhibit HSV-1 and revealed that these compounds block virus adsorption into cells (Fukuchi, Sakagami, Okuda, Hatano, Tanuma, Kitajima, Inoue, Inoue, Ichikawa, Nonoyama, & Konno, 1989), while similarly structured polyphenolic compounds inhibited attachment and penetration of HSV-1 into cells (Khan et al., 2005). The effectiveness of flavonoids, especially when used together instead of separately, was also confirmed to inhibit HSV-1, though the mechanism still remained unclear (Amoros, Simoes, & Girre, 1992). Finally, a specific compound found in green tea extract, (-)-epigallocatechin gallate (EGCG), was shown to effectively inhibit HSV-1 by binding to various glycoprotein receptors and preventing viral entry into the cell (Isaacs, Wen, Xu, Jia, Rohan, Corbo, DiMaggio, Jenkins, & Hillier, 2008).

EGCG and other catechins found in green tea have been shown to be effective antiviral agents against other viruses, capable of inhibiting the hepatitis B virus (Xu, Wang, Deng, Hu, & Wang, 2008) and the influenza virus (Song, Lee, & Seong, 2005). While the benefits of drinking green tea and the antiviral capability of its compounds have been studied and well documented (Cheng, 2006; Dufresne & Farnworth, 2001), those of black tea have comparatively remained unstudied. This is surprising given the fact that black tea is the most consumed beverage in the world, second only to water. Further still, black tea contains many of the same compounds and similar types of compounds that are found in green tea. Thus, while the health benefits of black tea
compounds may be just as effective as those of green tea, the former has gone largely unnoticed and remained unstudied until recently (Gupta, Saha, & Giri, 2002).

**Tea and Tea Extract**

**Background.** There are three main types of tea: Green, oolong and black. All three tea types are produced from the same plant, *Camellia sinensis*. Cultivation of tea from this plant can occur in many places with the following conditions: acidic soil, high humidity and fair temperature, although the majority is grown in subtropical and tropical zones (Dufresne & Farnworth, 2001; Gupta et al., 2002). Consumption of tea averages to about 120 mL per person each day, making tea, in terms of how much is consumed, the second most popular drink in the world, overshadowed only by water. Of the 2.5 million metric tons of dried tea that is produced annually, approximately 78% is black tea, 20% is green tea and only 2% is oolong tea. These different types of tea are favored in various parts of the world. Whereas green tea is mainly consumed in Asia, North Africa and the Middle East, oolong tea is confined mainly to China and black tea is preferred mainly in Western Europe and the United States (Cheng, 2006; Gupta et al., 2002; Khan & Mukhtar, 2007; Luczaj & Skrzydlewska, 2005).

The three types of tea arise due to differences in part of the manufacturing process often termed fermentation. Fermentation in the tea industry often refers to exposing the tea leaves to air in order to dry, which results in oxidation. The amount of fermentation that tea leaves undergo determine which tea type is produced; fermentation of the tea leaves is often halted by heating and dehydration, which inactivates certain enzymes in the tea leaves. Generally, green tea does not undergo fermentation, while black tea
undergoes a complete fermentation or 100% oxidation; oolong tea undergoes a partial fermentation and is thus only partially oxidized (Cheng, 2006; Luczaj & Skrzydlewska, 2005). The amount of fermentation that tea undergoes to produce the three different varieties is responsible for the characteristics of the tea, including color, aroma and taste, owing to the different compounds that are present (Babich, Pinsky, Muskin, & Zuckerbraun, 2006; Wang, Provan, & Helliwell, 2000).

**Compounds.** On average, tea leaves contain about 36% polyphenolic compounds, 25% carbohydrates, 15% proteins, 6.5% lignin, 5% ash, 4% amino acids, 2% lipids, 1.5% organic acids, 0.5% carotenoids and about 0.1% volatile substances, though the age of the tea leaves can affect their composition (Luczaj & Skrzydlewska, 2005). Since tea is often consumed as a hot water extract, as the tea leaves are steeped in hot water, it is often considered an extract of a plant (Gupta et al., 2002). Dry tea extracts or tea extract powders are made by first creating an infusion by soaking tea leaves in water and/or alcohol, then spray drying the infusion after it has been concentrated (Wang, Provan, & Helliwell, 2000). While the different tea extracts vary in the amount and nature of their compounds, they all contain polyphenolic compounds that fall into the class of flavonoids and are, more specifically, flavanols. However, it is the type and amount of flavanols originally produced during the fermentation process that gives the characteristics to each type of tea extract (Dufresne & Farnworth, 2001; Wang, Provan, & Helliwell, 2000).

The main type of flavanol in green tea extract are the catechins, which include the following compounds: (-)-epigallocatechin gallate (EGCG), (-)-epigallocatechin (EGC),
(-)-epicatechin gallate (ECG) and (-)-epicatechin (EC) (Dufresne & Farnworth, 2001; Wang, Provan, & Helliwell, 2000). Since green tea is not fermented, it contains a relatively high amount of catechins as compared to black tea; fermentation causes the catechins to polymerize and create theaflavins and thearubigens found in black tea, but not present in green tea. The four theaflavins are as follows: theaflavin (TFi), theaflavin-3-monogallate (TF2A), theaflavin-3'-monogallate (TF2B) and theaflavin-3,3'-digallate (TF3), which are formed by the combination of the following catechins: EC with EGC, EC with ECG, EGC with EGCG and ECG with EGCG, respectively (Bonnely, Davis, Lewis, & Astill, 2003). Thearubigens are even more complex and involve the combination of various theaflavins and catechin dimer products called theasinensins.

Combination of the catechin monomers occurs via an enzyme, polyphenol oxidase, in the tea leaves with the addition of oxygen (O2), which happens during the fermentation process to create black tea (Ferruzzi, 2010). As a result, in terms of dry weight, black tea contains a lower percentage of catechins, only 10% - 12%, as compared to 30% - 42% in green tea; however, black tea contains 3% - 6% theaflavins and 12% - 18% thearubigens, which are responsible for many of the distinctive properties of black tea (Babich et al., 2006; Dufresne & Farnworth, 2001; Khan & Mukhtar, 2007; Luczaj & Skrzydlewska, 2005).

Uses. While the applications of green tea have been explored and previously explained, that of black tea has only recently been investigated. The benefits of tea are often attributed to its antioxidant properties, which in turn has been ascribed to catechins, since green tea extract, with a higher concentration of catechins, has been found to have
more antioxidant activity than black tea extract (Cheng, 2006). However, recent studies have shown that certain extracts, specifically concentrated theaflavin extracts made from black tea, can be just as effective as catechins in terms of scavenging for and reacting with free radicals, which are indicative of antioxidant properties. It is believed that the high number of hydroxyl (OH) groups of theaflavins is responsible for its antioxidative properties (Luczaj & Skrzydlewska, 2005). Indeed, theaflavins have been shown to protect cells against oxidative damage, thus confirming its antioxidant capabilities (Yang, Tu, Xia, Jie, Chen, & He, 2007). Consequently, black tea extracts and theaflavins have been more vigorously studied and researched. As a result, it has been found that theaflavins are capable of inhibiting certain types of cancer (Kundu, Dey, Roy, Siddiqi, & Bhattacharya, 2005; Wang & Li, 2006), as well as inhibiting viruses, including bovine rotavirus and bovine coronavirus (Clark, Grant, Sarr, Belakere, Swaggerty, Phillips, & Woode, 1998) and HIV-1 (Liu, Lu, Zhao, He, Niu, Debnath, Wu, & Jiang, 2005).

While it has been shown that HSV-1 can be inhibited by compounds in green tea extract and a variety of other polyphenolic compounds, the purpose of this study is to determine if black tea extract with a concentrated amount of theaflavins (≥ 80% theaflavins) (BTE) can also inhibit HSV-1. Since theaflavins in BTE are composed of a dimer structure formed from catechin monomers found in green tea, which have been found to inhibit HSV-1, it is reasonable to infer that theaflavins in BTE may also produce similar results based on structural similarities. Despite the fact that theaflavin molecules are larger than catechins, larger polyphenolic compounds such as tannins have been shown to inhibit other viruses, which indicates that the size of the molecule may not
necessarily be a factor required for viral inhibition. Rather, the large amount of hydroxyl groups on these polyphenolic compounds seem to be the one common structural component among these various natural viral inhibitors; thus, theaflavins in BTE may be an effective inhibitor of HSV-1. In order to test this hypothesis, the cytotoxicity of BTE to cells that are similar to natural host cells for HSV-1, specifically, A549 or human epithelial cells, must first be tested; to this effect, trypan blue and WST-1 assays will be employed, as well as phase contrast microscopy. Once a safe concentration of BTE for the cell line has been established, the inhibitory effect that BTE has on HSV-1 propagation will be investigated through the use of phase contrast and fluorescent microscopy and plaque assays, as well as gel electrophoresis and spectrophotometry of viral PCR products.
Materials and Methods

Cells

**Cell line.** Human epithelial (A549) cells [American Type Culture Collection (ATCC), Manassas, VA, USA] were grown in 1X Ham's F-12K nutrient mixture, Kaighn's modification with 2 mM L-glutamine (Mediatech Incorporated, Manassas, VA, USA), supplemented to contain 10% fetal bovine serum (FBS) (Biowest, Miami, FL, USA) and 0.2% gentamicin (Lonza, Walkersville, MD, USA) (10% FBS-media).

**Cell cultures.** A549 cells were kept in T25 flasks with 5 mL 10% FBS-media in an incubator at 37°C with an atmosphere of 5% CO₂. To passage the cells, the media was removed and the cells were washed once 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% CO₂ for approximately 5 minutes to detach cells. Cells are maintained at a 1:3 to 1:8 ratio and subcultured two times per week.

Virus

A recombinant strain of HSV-1, GHSV-UL46, which contains the sequence for green fluorescent protein 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-media (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.

**Cytotoxic Activity of BTE**

**Phase contrast microscopy.** A549 cells were plated in 6-well plates (USA Scientific Incorporated, Ocala, FL, USA) with 2.5 mL of cell suspension added to each well and incubated at 37°C with an atmosphere of 5% CO₂ until confluent, about 48-72 hours. Pictures were then taken with a Motic AE31 microscope with a camera attached. The media was aspirated out of each well and 100 µL of 10 different concentrations of BTE solution ranging from 10⁻⁸ to 10 mg/mL was added to cells in each of 10 wells; as controls, 100 µL of 10% FBS-media and 100 µL of methanol (Fisher Scientific, Fair Lawn, NJ, USA) were each added to separate wells. Plates were kept in an incubator at 37°C with an atmosphere of 5% CO₂ for 1 hour and rocked every 15 minutes to ensure complete coverage. After 1 hour, any unabsorbed solution was aspirated from each well and 3 mL 10% FBS-media was added to each well. The cells were returned to the incubator and kept at 37°C with an atmosphere of 5% CO₂ for 48 hours, after which images were recorded and visualized with SPOT Advanced© software version 4.7 (Diagnostic Instruments Incorporated, Sterling Heights, MI, USA).

**Trypan blue assay.** A549 cells were plated in 6-well plates (USA Scientific Incorporated, Ocala, FL, USA) with 2.5 mL of cell suspension added to each well and incubated at 37°C with an atmosphere of 5% CO₂ until confluent, about 48-72 hours. The media was aspirated out of each well and 100 µL of each of the 10 concentrations of BTE solution was added to a separate well; as controls, 100 µL of 10% FBS-media and 100 µL of methanol were each added to separate wells. Plates were kept in an incubator
at 37°C with an atmosphere of 5% CO₂ for 1 hour and rocked every 15 minutes to ensure complete coverage. After 1 hour, any unabsorbed solution was aspirated from cells and 3 mL of 10% FBS-media was added to each well. The plates were returned to the incubator and kept at 37°C with an atmosphere of 5% CO₂ for 48 hours. Afterwards, 2 mL of 10% FBS-media was removed from each well, so that only 1 mL of 10% FBS-media remained. The cell monolayer of each well was then scraped with a sterile cell scraper and 100 µL of the cell suspension for each well was transferred to a separate Eppendorf tube. To each Eppendorf tube, 100 µL of trypan blue solution (Mediatech, Inc., Manassas, VA, USA) was added and then mixed with the cell suspension. After waiting 1-2 minutes, a portion of the mixture was placed in a hemacytometer (Hausser Scientific, Horsham, PA, USA) and observed under a Leica ATC 200 microscope at 400X magnification. Live cells (clear) and dead cells (dark blue) were counted in each of the four primary squares to obtain totals for each mixture.

**WST-1 reagent.** From the 0.9 mL of A549 cell suspension that remained for each well from the trypan blue assay, 300 µL of the cell suspension from each well was separately transferred to wells on a 96-well plate (Corning Incorporated, Corning, NY, USA), such that for each sample, there was 100 µL of the cell suspension in each of three wells. To each well that contained a sample, 10 µL of cell proliferation reagent WST-1 (Roche Diagnostics, Indianapolis, IN, USA) was added; controls included triplicates of 100 µL of 10% FBS-media both with and without the 10 µL of WST-1 reagent. The plate was gently rocked to mix the WST-1 reagent with each sample, then it was placed in an incubator at 37°C with an atmosphere of 5% CO₂ for 30 minutes. Afterwards, the
absorbance level for each well of the plate was measured at 450 nm in a microplate reader; for each sample that was repeated in triplicate, the absorbance levels were averaged.

**Viral Inhibition**

**Cell-treated virions.** A549 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% CO₂ until confluent, about 48-72 hours. The media was aspirated and cells were treated with 100 µL of each of the 10 concentrations of BTE solution; as controls, 100 µL of 10% FBS-media 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% CO₂ for 15 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 10% FBS-media was added, which served as a control (mock-infected). The cells were returned to an incubator at 37°C with an atmosphere of 5% CO₂ 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 10% FBS-media was added to each well. The plates were returned to the incubator and kept at 37°C with an atmosphere of 5% CO₂. The media from each well was harvested after 48 hours and transferred to cryogenic vials, which were stored at -80°C.

**Virus-treated virions.** To obtain media with virions from A549 cells that had been infected with virus particles treated with BTE solutions, 100 µL of stock virus was mixed with 100 µL of BTE solution in an Eppendorf tube; this was repeated in a separate
Eppendorf tube for each of the 10 concentrations of BTE solution, as well as a control, which contained 100 μL of 10% FBS-media instead of a BTE solution. Another control contained 200 μL of 10% FBS-media and was not mixed with the stock virus. The mixtures remained at room temperature (about 20°C-25°C) for 15 minutes. Then, 200 μL each mixture was added to a separate well on a 6-well plate containing A549 cells from which the media had been aspirated. The plates were returned to an incubator at 37°C with an atmosphere of 5% CO₂ 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 10% FBS-media was added to each well. The plates were returned to the incubator and kept at 37°C with an atmosphere of 5% CO₂. 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 cell-treated and virus-treated media 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 A549 cells, from which media had been aspirated, 500 μL of the fourth through seventh (10⁻⁴-10⁻⁷) dilutions were separately added to each of 5 wells; to the 6th well, 500 μL of 1% FBS-PBS was added as a control. This process was replicated for each desired treated media that was studied. The plates were returned to an incubator at 37°C with an
atmosphere of 5% CO₂ for 1 hour and rocked every 15 minutes to ensure complete 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 until needed. Solution A comprised of mixing the following in an autoclaved Erlenmeyer flask: 17 mL of 3X Eagle (Gibco Invitrogen Corporation, Grand Island, NY, USA), 1.5 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. Both solutions were placed in a 41°C water bath. After 1 hour incubation, combine Solutions A and B and overlay cells with 3 mL of plaque media. Store in plates in an incubator at 37°C with an atmosphere of 5% CO₂ for 72 hours. After 72 hours incubation, remove agar and stain cells with crystal violet. The number of plaques formed in each well were counted to determine the titer in plaque forming units (PFU) per milliliter.

**Fluorescent microscopy.** To visualize the effect that the 10⁻¹ diluted BTE solution had on viral propagation, A549 cells were plated in a 6-well plate. First, 100 μL of stock virus was mixed with 100 μL of 10⁻¹ diluted BTE solution in an Eppendorf tube. The controls contained 200 μL of 10% FBS-media and was not mixed with the stock virus, while a separate control consisted of 100 μL of stock virus mixed with 100 μL of
10% FBS-media. The mixtures remained at room temperature for 15 minutes. Then, 200 µL of each mixture was added to a separate well on a 6-well plate that contained confluent A549 cells from which the media had been aspirated. The cells were returned to an incubator at 37°C with an atmosphere of 5% CO₂ for 1 hour and rocked every 15 minutes to ensure complete coverage. After 1 hour, any unabsorbed solution was aspirated from the cells and 2.5 mL 10% FBS-media was added to each well. The plates were returned to the incubator and kept at 37°C with an atmosphere of 5% CO₂. Cells were observed with a fluorescent microscope, at 400X magnifications every 6 hours post infection for 24 hours.

**DNA extraction.** DNA was extracted from infected A549 cells that contained 200 µL HSV-1 virus treated with 200 µL of 1 mg/mL of BTE solution in an Eppendorf tube, 200 µL of 10% FBS-media, and 400 µL of 10% FBS-media alone, respectively. Cells were incubated for 12 hours at 37°C with an atmosphere of 5% CO₂. The DNA from each of the three 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 gB, gD, GFP and pUL46. The following virus-specific primers were used:

Glycoprotein B:

gB1F. 5'-AGATTCTGCGGTACTGCGATC-3'
gB1R. 5'-ACGGAACACAAACAAGCAGCGATG-3'

Glycoprotein D:

gD1F. 5'-AGACGTCCGGAACACCAACCCTACAA-3'
gD1R. 5'-ACACAAATTCCGCAAATGACCAGGG-3'

Green Fluorescent Protein (Willard, 2002):

GFP1F. 5'-GTAAGCTTTAGATGAGCAAGG-3'
GFP1R. 5'-CTTGAAGCTCTTTGTACAGCTCGTCC-3'

UL46 gene:

VP1F. 5'-ACCAAGCCTTGATGCTCAA-3'
VP1R. 5'-ACACACCGTTCCTCCGAGAGTTGA-3'

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

**DNA Purification.** PCR products were purified using QIAquick® PCR Purification Kit (Qiagen Sciences, Germantown, MD, USA), following the protocol provided by the manufacturer.

**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

Cytotoxicity

**Phase contrast microscopy.** Before BTE could be used to determine if it was capable of inhibiting HSV-1, its cytotoxicity to cells was assessed. This was to prevent a false reading that the BTE was lowering the viral titer due to cell death rather than viral inhibition. To this end, the cells were viewed with an inverted microscope both before and after treatment to observe any cytopathic effects, which include rounding and lifting of the cells.
Figure 1. Microscopy (200X magnification) of A549 cells treated with either (a) 10% FBS-media (mock-treated) or (b) $10^0$ mg/mL of BTE, to determine the cytotoxicity of BTE on A549 cells.
**Trypan blue assay.** In addition to microscopic observation, live and dead cells for each concentration of BTE, as well as positive and negative controls, were counted using the trypan blue assay. In this assay, live cells are able to exclude the blue dye and thus, appear clear, while the dead cells take up the dye and appear dark blue in a microscopic examination. For each group, a percentage of living cells was calculated by taking the number of live or viable cells and dividing it by the total number of (live and dead) cells (Table 1). The methanol group, which served as a negative control, had no living cells, whereas the positive control group treated with 10% FBS-media had 17% viable cells by the end of the experiment. Most of the BTE samples also had a percentage of viable cells close to that of the positive control group, and thus, the tested concentrations of BTE, from $10^{-8}$ to 10 mg/mL, did not appear to be cytotoxic to A549 cells. The only exception was the $10^1$ mg/mL of BTE, which had a significantly higher percentage of live cells compared to any other group (Figure 2).
Table 1

*BTE Cytotoxicity in Cultured A549 Cells: Trypan Blue Assay*

<table>
<thead>
<tr>
<th>BTE (mg/mL)</th>
<th>Viable Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Methanol)</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>0 (Media)</td>
<td>17 ± 6</td>
</tr>
<tr>
<td>$10^{-8}$</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>18 ± 4</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>16 ± 3</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>23 ± 3</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>$10^{-1}$</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>1</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>10</td>
<td>89 ± 7</td>
</tr>
</tbody>
</table>

*Note.* For each sample, the percentage of viable A549 cells was checked microscopically by first counting the number of viable or live (clear) and non-viable or dead (dark blue) A549 cells in each of four quadrants on the hemacytometer. The number of viable cells were then divided by the total (live plus dead) number of A549 cells for each sample to obtain the percentages. Values represent an average of three samples ± SD.
Figure 2. Trypan blue assay results for BTE cytotoxicity in A549 cultures, indicating the percentage of live or viable cells as compared to the total number of cells for each treated sample. Values represent the average of three samples ± SD.
WST-1 assay. To confirm the findings established by the trypan blue assay, an assay using WST-1 reagent was conducted. In this assay, only live cells can reduce WST-1, which is light red, to formazan, which is dark red; thus, when placed in a plate reader, the higher absorbance level is indicated by a darker color, which correlates to the number of living cells, such that a higher absorbance indicates more living cells. Overall, the findings with the WST-1 assay paralleled those found for the trypan blue assay, in that the sample treated with methanol had the lowest absorbance levels, while most of the BTE treated samples had approximately the same absorbance, 0.400, as the positive control group that was treated only with 10% FBS-media (Table 2). Thus, while methanol was cytotoxic to the A549 cells, the various concentrations of BTE did not seem to cause cell death. Again, cells treated with $10^1$ mg/mL BTE had a significantly higher absorbance level as compared to any other group (Figure 3). Once it was determined that BTE was not cytotoxic to A549 cells, experiments to study the inhibitory effects of BTE on HSV-1 were conducted.
### Table 2

**BTE Cytotoxicity in Cultured A549 Cells: WST-1 Assay**

<table>
<thead>
<tr>
<th>BTE (mg/mL)</th>
<th>Absorbance (450 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Methanol)</td>
<td>0.316 ± 0.033</td>
</tr>
<tr>
<td>0 (Media)</td>
<td>0.400 ± 0.005</td>
</tr>
<tr>
<td>10⁻⁸</td>
<td>0.372 ± 0.014</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>0.464 ± 0.033</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>0.406 ± 0.015</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>0.394 ± 0.022</td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>0.425 ± 0.017</td>
</tr>
<tr>
<td>10⁻³</td>
<td>0.490 ± 0.019</td>
</tr>
<tr>
<td>10⁻²</td>
<td>0.352 ± 0.018</td>
</tr>
<tr>
<td>10⁻¹</td>
<td>0.570 ± 0.036</td>
</tr>
<tr>
<td>1</td>
<td>0.556 ± 0.021</td>
</tr>
<tr>
<td>10</td>
<td>1.125 ± 0.033</td>
</tr>
</tbody>
</table>

*Note.* For each sample, the absorbance level is the average of three individual samples from the same plate. A higher absorbance level indicates a relatively higher number of living A549 cells, as compared to a lower absorbance level. As a reference, media containing no A549 cells with WST-1 reagent produced an absorbance level of 0.211 (an average of three separate samples on the same plate) (data not shown). Values represent the average absorbance level of three samples ± SD.
Figure 3. WST-1 assay results for BTE cytotoxicity in A549 cells, indicating the absorbance level (an average of three separate samples on the same plate), which relates to the amount of live or viable cells for each treated sample. A higher absorbance level indicates more living or viable cells than a lower absorbance level. As a reference, media containing no live cells with WST-1 produced an absorbance level of 0.211 (an average of three separate samples on the same plate) (data not shown). Values represent the average absorbance level of three samples ± SD.
Viral Inhibition

**Phase contrast microscopy.** To visually observe the cytopathic effect that HSV-1 had on A549 cells and to determine whether BTE could inhibit HSV-1, thereby reducing or preventing the observable cytopathic effect, treated and untreated A549 cells were observed with the Motic AE31 microscope at 400X magnification using phase contrast. Clear differences between each group were seen 12 hours and 24 hours post-infection (Figure 4). While the control group was treated only with 10% FBS-media and had relatively few dying cells at 12 and 24 hours, the cells infected with HSV-1 showed rounded and detached cells at 12 hours, characteristic of the cytopathic effect, in which the number increased after an additional 12 hours. The cells that were infected with a mixture of BTE and HSV-1 showed very few cells that displayed the cytopathic effect, which paralleled the control group.
Figure 4. Phase contrast microscopy (400X magnification) of A549 cells taken at (a) 12 hours and (b) 24 hours post-infection. Cells were observed for the cytopathic effect, which includes rounding and detachment of the cells from the substrate.
**Plaque Assay.** Once it was shown that BTE could reduce the number of cells infected by HSV-1 through a decrease in cells displaying the cytopathic effect, the viral titer produced by HSV-1 alone and when exposed to BTE was determined by plaque assays (Figure 5). The untreated virus produced 40 plaques for 0.5 mL of viral solution at the $10^5$ dilution, which is equivalent to a viral titer of $8.0 \times 10^6$ PFU/mL (plaque forming units per milliliter) (Figure 6). The virus media harvested from infected cells treated with 10% FBS-media resulted in a similar result, with 41 plaques produced at the $10^5$ dilution when cells were exposed to 0.5 mL of the solution, resulting in a viral titer of $8.2 \times 10^6$ PFU/mL (Figure 7).

Plaque assays were conducted to test the effect of BTE on HSV-1 infection by infecting fresh monolayers of A549 cells with viral media harvested from: (1) infected cells previously treated with either 10 mg/mL or 1 mg/mL of BTE solution and (2) cells infected with HSV-1 treated with either 10 mg/mL or 1 mg/mL of BTE solution prior to infection; the former assays are termed cell treated, whereas the latter are virus treated. In both the cell treated (Figure 8) and virus treated (Figure 9) plaque assays, no plaques were produced at the dilutions ranging from $10^{-7}$ to $10^{-3}$, as well as the mock-infected controls. Thus, treated cells and HSV-1 treated with 10 mg/mL or 1 mg/mL BTE resulted in a viral titer of 0 PFU/mL.
Figure 5. Schematic representation of the experimental set-up for plaque assays. As indicated, each of the six wells, confluent with A549 cells, was treated with a different dilution of the HSV-1, except for the well with no virus, which served as a control.
Figure 6. Plaque assay for HSV-1 in cultured A549 cells. At the 10^{-5} viral dilution, 40 plaques formed. Since 0.5 mL of HSV-1 was used, the viral titer is calculated to be $8.0 \times 10^6$ PFU/mL.
Figure 7. Plaque assay for infected A549 cells treated with 10% FBS-media. At the $10^{-5}$ viral dilution, 41 plaques formed. Since 0.5 mL of HSV-1 was used, the viral titer is calculated to be $8.2 \times 10^6$ PFU/mL.
Figure 8. Plaque assay for A549 cells treated with either (a) 10 mg/mL or (b) 1 mg/mL BTE prior to HSV-1 infection. No plaques resulted at any of the tested dilutions, $10^{-3}$ to $10^{-7}$, and thus, the viral titer is 0 PFU/mL.
Figure 9. Plaque assay for HSV-1 treated with either (a) 10 mg/mL or (b) 1 mg/mL BTE prior to inoculation of A549 cells. No plaques resulted at any of the tested dilutions, $10^{-3}$ to $10^{-7}$, and thus, the viral titer is 0 PFU/mL.
Fluorescent microscopy. To confirm the findings of the previous two experiments, fluorescent microscopy was employed to visually examine progeny virions in cells that were exposed to HSV-1 treated with 1 mg/mL BTE. Since the strain of HSV-1 being employed has GFP attached to the tegument protein, new virions that result from a successful infection will fluoresce. No viral fluorescence detected from either the control (cells treated with 10% FBS-media) (Figure 10) or cells inoculated with HSV-1 treated with BTE (Figure 11) at 12 hours post-infection. For cells infected with HSV-1, there was a significant amount of fluorescence (Figure 12) at this same time period. After another 12 hours (24 hours post-infection), there was still no viral fluorescence detected from the control (Figure 13), while there was a small amount of fluorescence from cells inoculated with HSV-1 treated with BTE (Figure 14) and still a significant amount of fluorescence from cells infected with HSV-1 (Figure 15).
Figure 10. Microscopy (400X magnification) of control A549 cells (treated with 10% FBS-media) taken 12 hours post-infection, depicting (a) fluorescence and (b) a merged image of fluorescent and phase contrast images.
Figure 11. Microscopy (400X magnification) of A549 cells inoculated with HSV-1, taken 12 hours post-infection, depicting (a) fluorescence and (b) a merged image of fluorescent and phase contrast images.
Figure 12. Microscopy (400X magnification) of A549 cells infected with HSV-1 treated with 1 mg/mL BTE, taken 12 hours post-infection, depicting (a) fluorescence and (b) a merged image of fluorescent and phase contrast images.
Figure 13. Microscopy (400X magnification) of control A549 cells (treated with 10% FBS-media) taken 24 hours post-infection, depicting (a) fluorescence and (b) a merged image of fluorescent and phase contrast images.
Figure 14. Microscopy (400X magnification) of A549 cells inoculated with HSV-1, taken 24 hours post-infection, depicting (a) fluorescence and (b) a merged image of fluorescent and phase contrast images.
Figure 15. Microscopy (400X magnification) of A549 cells infected with HSV-1 treated with 1 mg/mL BTE, taken 24 hours post-infection, depicting (a) fluorescence and (b) a merged image of fluorescent and phase contrast images.
DNA extraction and comparison. To compare the amount of DNA present in A549 infected cells exposed to either untreated or BTE treated HSV-1, A549 cells were either exposed to 10% FBS-media (mock-infected), untreated HSV-1 (HSV-1 mixed with 10% FBS-media) or HSV-1 treated with 1 mg/mL BTE. The DNA from these cell samples was extracted and compared using a spectrophotometer (Table 3). The cells infected with untreated HSV-1 were shown to have more DNA present (391.7 ng/μL) as compared to infected cells exposed to BTE treated HSV-1 (278.2 ng/μL). The mock-infected cells had the lowest amount of DNA (240.7 ng/μL) and served as a control.
Table 3

Comparison of DNA from Infected A549 Cells

<table>
<thead>
<tr>
<th>A549 Sample</th>
<th>Total DNA Present (ng/μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock-Infected</td>
<td>240.7 ± 10.8</td>
</tr>
<tr>
<td>Untreated HSV-1</td>
<td>391.7 ± 4.0</td>
</tr>
<tr>
<td>BTE Treated HSV-1</td>
<td>278.2 ± 10.2</td>
</tr>
</tbody>
</table>

*Note.* For each sample, the total DNA present represents a combination of cellular and any viral DNA, and is the average of three individual samples. Mock-infected A549 cells were not exposed to HSV-1, but to 10% FBS-media instead. Cells infected with HSV-1 were exposed to HSV-1 that was either untreated (mixed with 10% FBS-media) or treated with 1 mg/mL BTE. The spectrophotometer analyzed 2 μL of each sample to determine the amount of DNA present. As a blank, 2 μL of the elution buffer used to extract the DNA was utilized and registered as 0.0 ng/μL (data not shown). Values represent ± SD of the average of three samples.
**PCR and gel electrophoresis.** To confirm the previous findings, four sets of viral primers were used during PCR to amplify any viral DNA. Sequence-specific primers were used to amplify the DNA encoding the following regions: gB, gD, GFP and pUL46. Gel electrophoresis of the PCR products resulted in no visible bands for the mock-infected cells not exposed to HSV-1 (Figure 16), while bands on the gel corresponding to gD, GFP and pUL46 were apparent for untreated HSV-1 or HSV-1 treated with 1 mg/mL BTE (Figure 17); the former had a higher intensity than the latter.
Figure 16. Gel electrophoresis of PCR products extracted from A549 cells treated with 10% FBS-media (mock-infected) (columns 2 - 5). Column 1 contains the DNA ladder, with visible bands identified to the left in base pairs (bp). Columns 2, 3, 4 and 5 contain DNA amplified with primers for the HSV-1 gB, gD, GFP and pUL46 genes, respectively.
Figure 17. Gel electrophoresis of PCR products extracted from HSV-1 infected A549 cells either treated with 1 mg/mL BTE (columns 2 - 5) or untreated (columns 6 - 9). Column 1 contains the same DNA ladder seen in the previous gel (Figure 16); bands from the ladder corresponding to other bands on the gel are identified to the left in base pairs (bp). Columns 2 and 6, 3 and 7, 4 and 8, and 5 and 9 contain DNA amplified with primers for the HSV-1 gB, gD, GFP and pUL46 genes, respectively.
DNA comparison of PCR products. To confirm the results of the gel electrophoresis, each of the PCR products from the three samples was purified and then compared using a spectrophotometer (Table 4). The DNA concentration (in ng/μL) of the mock-infected PCR products (cells only) paralleled that of the A549 cells infected with HSV-1 treated with 1 mg/mL of BTE (treated HSV-1 infected cells), while that of the A549 cells infected with HSV-1 treated with 10% FBS-media (untreated HSV-1 infected cells) displayed a significantly higher amount as compared to the other two groups for three samples (Figure 18): gD, GFP and pUL46.
### Table 4

**Comparison of PCR Products**

<table>
<thead>
<tr>
<th>Sample</th>
<th>gB</th>
<th>gD</th>
<th>GFP</th>
<th>pUL46</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells Only</td>
<td>5.1 ± 1.1</td>
<td>5.3 ± 1.1</td>
<td>4.7 ± 1.0</td>
<td>6.2 ± 0.7</td>
</tr>
<tr>
<td>Treated HSV-1 Infected Cells</td>
<td>6.1 ± 0.2</td>
<td>6.4 ± 0.8</td>
<td>5.7 ± 0.8</td>
<td>11.2 ± 0.3</td>
</tr>
<tr>
<td>Untreated HSV-1 Infected Cells</td>
<td>7.0 ± 0.7</td>
<td>16.8 ± 2.5</td>
<td>25.0 ± 0.7</td>
<td>24.1 ± 1.4</td>
</tr>
</tbody>
</table>

*Note.* For each PCR product, the concentration represents the average of three individual samples. The spectrophotometer analyzed 2 μL of each sample to determine the amount of DNA present. As a blank, 2 μL of the elution buffer used to purify the DNA was utilized and registered as 0.0 ng/μL (data not shown). Values represent ± SD of the average of three separate samples.
Figure 18. Comparison of PCR products from three samples of infected A549 cells: mock-infected (cells only), cells infected with HSV-1 treated with 1 mg/mL of BTE (treated HSV-1 infected cells) and cells infected with HSV-1 treated with 10% FBS-media (untreated HSV-1 infected cells). PCR products were the same as those for the gel electrophoresis (Figures 16 and 17). As a control, 2 μL of the elution buffer used to extract the DNA was utilized and registered as 0.0 ng/μL (data not shown). For each PCR product, the concentration represents ± SD of the average of three individual samples.
Discussion

For many centuries, it has been believed that tea has therapeutic uses and benefits. In modern times, our advances in chemistry, biology and medicine have provided evidence on a molecular level for the beneficial properties of tea. Specifically, the polyphenolic compounds called flavanols, which can be found in all types of tea, have been shown to prevent certain illnesses and have high antioxidant characteristics (Khan & Mukhtar, 2007). While different types of teas have various types and amounts of flavanols, green tea is often purported to have the greatest benefits, thus leaving relatively few studies on black tea and its compounds to be conducted (Gupta et al., 2002).

One concern for human health is HSV-1. Infections caused by the herpesvirus are estimated to affect anywhere from 45% - 98% of the world population and up to 40% of these infected individuals are subject to recurrent outbreaks that most often result in lesions and ulcerations of the skin. While treatments, such as the drug acyclovir, are in use today, most rely on the presence of a viral protein, thymidine kinase, to inhibit viral replication; mutant viral strains lacking this enzyme are still infectious but do not respond to the available medications. In addition, current treatments can have side effects and often require frequent doses that can be expensive (Fatahzadeh & Schwartz, 2007; Thompson, 2006; Whitley & Roizman, 2001). Thus, alternative treatments to HSV-1 infections are necessary to alleviate the symptoms of infected individuals.

It was the aim of this study to determine if black tea extract enriched with theaflavins could inhibit HSV-1, in order to perhaps someday, after further study, be used as an alternative treatment for individuals afflicted with herpes infections. This hypothesis stems from the findings that black tea compounds have been shown to inhibit
bovine rotavirus and bovine coronavirus (Clark et al., 1998) and HIV-1 (Liu et al., 2005). In addition, a green tea catechin compound, EGCG, has already been shown to inhibit HSV-1; it is suggested that EGCG binds to glycoproteins on the envelope of the virus, thereby preventing viral entry into the host cell (Isaacs et al., 2008). Since black tea theaflavins are merely polymers of green tea catechins (Bonnely et al., 2003), it is possible that the former may also inhibit HSV-1. Yet, black tea theaflavins may inhibit HSV-1 through a different mechanism or at different concentrations or with fewer or less severe side effects as their green tea counterparts. The first step to resolving these treatment issues, and the purpose of this research, is to first determine if BTE containing theaflavins is capable of inhibiting HSV-1.

Viral inhibition depends on preventing some process that is required for the virus to make progeny, whether it is the first step of viral entry into the host cell or the last step of virion exit from the host cell. Regardless, the host cell must remain intact and alive, and thus, the first step to determine the inhibitory effect of BTE is to research its cytotoxic effect on the A549 cultured host cells. To this end, various concentrations of BTE were made and exposed to cells, which were then assayed using trypan blue to count the live and dead cells and WST-1 reagent to obtain a relative measurement of the amount of remaining living cells in the treated samples.

Overall, the concentrations of BTE tested, from 10 to $10^{-8}$ mg/mL, showed no cytotoxic effects on A549 cells, as the amount of living cells was comparable to the control group treated with 10% FBS-media (Figure 1). In fact, a study by Kundu et al. (2005) performed a similar experiment on human leukemia cells, showing that compared
to untreated cells, black tea extract at a concentration of 1 mg/mL resulted in only 16% reduction in cells, while lower concentrations of 100 µg/mL, 10 µg/mL and 1 µg/mL had an 8%, 0% and 0% reduction in cells, respectively. Similar results were found using pure theaflavins, as well as green tea extract (Kundu et al., 2005). In addition, a study by Babich et al. (2006) showed that increasing the amount of theaflavins decreased the cytotoxic effect on various cell types, indicating that green tea extract was more cytotoxic than black tea extract. Finally, the experimental results from this study indicating that 10 mg/mL BTE prevented cell death, as determined by trypan blue (Table 1; Figure 2) and WST-1 (Table 2; Figure 3) assays, can be explained by another study. It was shown that 5 mg/mL and 10 mg/mL of BTE included in the diet of fruit flies could extend their mean life span by approximately 10%, as compared to flies that did not ingest BTE (Peng, Chan, Li, Huang, & Chen, 2009). In short, BTE concentrations up to 10 mg/mL did not appear to be cytotoxic to A549 cells, and thus, the inhibitory effect of BTE on HSV-1 propagation was tested.

Inhibition was measured visually, through observations that utilized both phase contrast and fluorescent microscopy, as well as quantitatively, by determining viral titers with the plaque assay method and DNA concentration from infected cells with a spectrophotometer. Microscopic observations that utilized phase contrast were able to show the cytopathic effect that HSV-1 has on infected cells, which included rounding of the cells and detachment from the substrate; this effect was generally not seen in either the control or BTE treated groups (Figure 4). In addition to HSV-1 infected cells ballooning and degenerating, they can form multinucleated giant cells (Fatahzadeh &
Schwartz, 2007; Whitley & Roizman, 2001), although the latter was not observed.

Fluorescent microscopy of infected cells confirmed the presence of HSV-1, as the virus had GFP attached to its tegument (Willard, 2002). Viral fluorescence was mainly observed for cells inoculated with HSV-1 (Figures 11 and 14) and was absent from the control (Figures 10 and 13), treated only with 10% FBS-media; cells infected with HSV-1 treated with BTE had a minimal amount of fluorescence, indicating low infectivity (Figures 12 and 15). Confirmation of these results was supported by the data from the plaque assays, in which HSV-1 was shown to produce multiple plaques and have a viral titer of 8.0 x 10^6 PFU/mL (Figure 6), but when either the cells or the virus was treated with BTE, no plaques were visible and the viral titer dropped to 0 PFU/mL in the tested dilutions (Figures 8 and 9).

In addition, the amount of DNA in cells infected with BTE treated HSV-1 was significantly lower than that of cells infected with untreated HSV-1 (Table 3). While a large amount of the DNA present was cellular DNA from A549 cells, as indicated by the mock-infected control, the remainder of the DNA in HSV-1 infected cells was viral DNA produced through the replication of the HSV-1 genome. This was confirmed by gel electrophoresis of PCR products that included HSV-1 specific genes for gB, gD, GFP and pUL46. While mock-infected A549 cells displayed no bands (Figure 16), untreated HSV-1 infected cells displayed clear bands for gD, GFP and pUL46, as did cells infected with BTE treated HSV-1, though the bands were less intense (Figure 17). To compare the amounts of PCR products from the gels, purified samples were analyzed with a spectrophotometer. As with the gel, all three concentrations for the gB product were
similarly and relatively low (Table 4), possibly explaining why it was not observed for the positive control in the results for electrophoresis. The results of the gel also parallel those of the spectrophotometer, as the mock-infected A549 cells had the lowest concentrations for gD, GFP and pUL46, while untreated HSV-1 infected cells displayed the highest and cells infected with BTE treated HSV-1 had a level intermediary to the two, though more closely resembled those of the former than the latter (Figure 18). The combined results of the DNA concentrations and gels indicate that there was a higher concentration of viral DNA present in cells infected with untreated HSV-1 as compared to cells infected with BTE treated HSV-1, indicating that either more virions entered or more viral replication occurred in the former group as compared to the latter, once again supporting the hypothesis that BTE is capable of inhibiting HSV-1. Thus, BTE at the tested concentration of 1 mg/mL is sufficient to inhibit HSV-1, while not being cytotoxic to the host A549 cells. Now that the inhibitory effect of BTE has established, the mechanism by which BTE hinders the propagation of HSV-1 can begin to be studied.

While the results of this study clearly indicate, through various experiments, the inhibitory capacity of BTE on HSV-1, several important questions remain to be answered and further studies are required in order to resolve these issues. While a concentration of 10^0 mg/mL was shown to be effective at preventing HSV-1 propagation, lower concentrations may also be sufficient to accomplish the same task or there may be a dose-dependent inhibitory effect. Determining the effectiveness of a range of lower concentrations of BTE on inhibiting HSV-1 could be accomplished by merely repeating the same set of methods utilized in this study with the different BTE concentrations.
While an applicable treatment may be far in the future, by finding the lowest concentration of BTE necessary to effectively inhibit HSV-1, side effects for a potential treatment for humans can be minimized.

It also remains unclear as to which compound or compounds in BTE are responsible for inhibiting HSV-1. While the BTE used in this study was enriched with theaflavins, that alone is not sufficient to attribute the effects of BTE solely to theaflavins. Some compounds have been found to have an enhanced inhibitory effect when paired with other compounds, such that the effect of all the compounds together is far greater than the sum of the individual effects of the same compounds (Amoros, Simoes, & Girre, 1992; Gescher, Hensel, Hafezi, Derksen, & Kühn, 2011a). Thus, individual theaflavins should be tested to determine their effectiveness in inhibiting HSV-1; if the inhibitory effect of a single theaflavin is less than that of BTE, it is possible that two or more of the theaflavins or theaflavins with other compounds have a synergistic effect in inhibiting HSV-1. Again, the methods employed in this study can be replicated to determine the effectiveness of each compound or combination of compounds at inhibiting HSV-1. By determining exactly which compound or compounds are required to inhibit HSV-1, not only will the mechanism of inhibition be less elusive, but when developing a treatment, ineffectual and thus unnecessary compounds, which may only serve to add to undesired side effects, can be omitted.

Finally, the mechanism by which BTE inhibits HSV-1 has not been identified. There are many steps in the viral replication cycle that BTE can interrupt, but three main processes are viral entry into the host cell, viral replication and synthesis of viral proteins.
While the results of this study indicated that a lower amount of viral DNA and fewer virions were produced when HSV-1 was treated with BTE, this could be accomplished by merely stopping virus particles from entering the cells, thereby leaving fewer cells to produce viral DNA and progeny or it could be that viral replication is somehow slowed or prevented, possibly through the inhibition of proteins or their production. In addition, viral egress could be inhibited, also explaining the results of fewer infected cells and therefore, less viral DNA present. The theaflavin compounds found in BTE have a structure that consists of a multiple hydroxylation of complex aromatic rings, which is thought to be a key characteristic of inhibition for these compounds; similarly structured compounds, such as proanthocyanidins, have been shown to affect different aspects, and in some instances, multiple aspects of viral propagation (Gescher et al., 2011a; Gescher, Kühn, Hafezi, Louis, Derksen, Deters, Lorentzen, & Hensel, 2011b), which may allude to a mechanism of inhibition for theaflavins. The first step in determining how BTE affects HSV-1 propagation would be to conduct binding assays to determine if BTE allows the virus to attach to and/or fuse with the cell. If binding assays indicate that viral propagation is inhibited by BTE through a reduction in visible plaques, then BTE may affect the binding of one of the viral glycoproteins necessary to attach to and/or fuse with the cell; different binding assays can be employed to determine if any or both of these two processes are affected. If binding assays do not entirely or only partially explain the ability of BTE to inhibit HSV-1, then BTE may be inhibiting another process of the viral lytic cycle, such as viral replication, protein synthesis or egress. Clearly, further study is required to elucidate the mechanism of BTE inhibition of HSV-1.
In conclusion, BTE enriched with theaflavins was sufficient to inhibit HSV-1 in A549 cells at the tested concentrations/dilutions. While the minimal concentration, necessary compound(s) and mechanism of BTE required to inhibit HSV-1 still remain to be determined through further studies, the prospect of BTE and its compounds to eventually treat a number of individuals around the world affected with HSV-1 remains promising.
References


Yang, Z., Tu, Y., Xia, H., Jie, G., Chen, X., & He, P. (2007). Suppression of free-radicals and protection against H$_2$O$_2$-induced oxidative damage in HPF-1 cell by oxidized phenolic compounds present in black tea. *Food Chemistry*, 105, 1349-1356.