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Black Garlic Extract as an Antiviral for Herpes Simplex Virus-2 in Lung Cells

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Abstract

Alliin, a sulfur compound, is known for providing garlic with its unique fragrance and taste, as well as for its antimicrobial properties. This is one of many antioxidant compounds found in garlic, along with polyphenols and flavonoids. When raw garlic is aged using high temperatures and high humidity levels, it develops a black color and a sweeter taste. Garlic in this form, referred to as black garlic, contains higher levels of antioxidants than fresh garlic. Antioxidants play a vital role in alleviating cellular stress during viral infections. Once a virus particle attaches and enters a host cell, a slew of mechanisms begin that create oxidative stress through the production of reactive oxidative species (ROS). A prolonged state of oxidative stress can result in cell death, DNA damage, and disease progression. In this study, black garlic extract (BGE) is evaluated for its ability to mitigate cytopathic effects and oxidative stress caused by Herpes Simplex Virus-2 (HSV-2) infections *in vitro*. Antiviral assays were performed to determine the percent of viral inhibition resulting from treatment with the BGE. ROS-Glo™ H₂O₂ assays were then completed to measure the post-infection ROS levels of BGE-treated virus and cells. The results thus far hint at promises of BGE providing viral inhibition along with decreased levels of oxidative stress. Future experiments will focus on refining the treatment and drug delivery method to maximize the effects that black garlic can have on viral infections.

MONTCLAIR STATE UNIVERSITY

Black Garlic Extract as an Antiviral for Herpes Simplex Virus-2 in Lung Cells

by

Jenna Rose Horowitz

A Master's Thesis Submitted to the Faculty of

Montclair State University

In Partial Fulfillment of the Requirements

For the Degree of

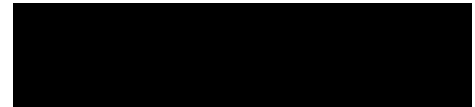
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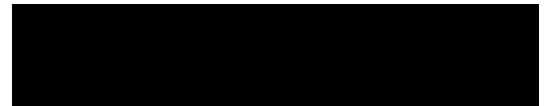
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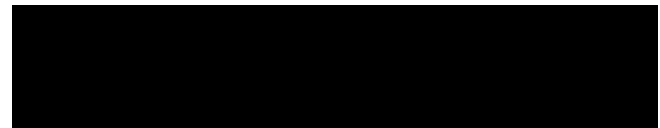
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BLACK GARLIC EXTRACT AS AN ANTIVIRAL FOR HERPES SIMPLEX VIRUS-2 IN
LUNG CELLS

A THESIS

Submitted in partial fulfillment of the requirements

For the degree of Master of Science

by

Jenna Rose Horowitz

Montclair State University

Montclair, NJ

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Introduction

Herpes simplex virus-2 (HSV-2) is an enveloped double-strand DNA virus responsible for lesions developing in the genital region, but they can also be found in the orolabial region (Grinde, 2013; Xu et al., 2019). This viral infection is contracted through contact with mucous membranes that are shedding the virus particles. Antivirals can be used to treat HSV-2 infections through episodic or suppressive regimens. Common medications prescribed are acyclovir and valacyclovir, but there are additional therapeutics available (Madkan et al., 2007). There are strains of HSV-2 that are resistant to specific antivirals, like acyclovir, due to genetic mutations (Hardy, 1992). Due to the rising rate of antiviral resistance, it is important to assess a variety of potential treatments. Compounds with high levels of antioxidants have been reported to successfully inhibit viral infections (Elias et al., 2021). Garlic, for example, has been used as an antimicrobial for many years due to the organosulfur compound, allicin (Mösbauer et al., 2021). Black garlic, a fermented form of garlic, contains an increased level of antioxidants, compared to raw garlic (Choi et al., 2014). Antioxidants can mitigate the oxidative stress caused by viral infections (Halliwell et al., 1995). While garlic has been studied previously for its antiviral properties, black garlic is new to the market and has not been assessed to the same extent. Due to its high levels of antioxidants, black garlic shows promise as an antiviral treatment. This study aims to analyze black garlic extract for its antiviral properties and its ability to inhibit HSV-2 from infecting A549 cells.

Review of Literature

Herpes Simplex Virus-2 (HSV-2) is a double-stranded DNA, enveloped virus a part of the *Herpesviridae* family (Xu et al., 2019). This family also includes Herpes Simplex Virus-1, Varicella-Zoster, Cytomegalovirus, Epstein-Barr Virus, and Kaposi's Sarcoma. Members of *Herpesviridae* can infect vertebrate animals including humans, birds, and reptiles. The alpha-herpesvirus, HSV-2, has its genetic material enclosed in an icosahedral capsid that is approximately 125 nm in diameter (Wang et al., 2018). Above the capsid is the tegument layer that contains 22 viral proteins while the viral envelope is composed of 16 membrane proteins. At the center of the virion sits the genome, which is 154kB long (Minaya et al., 2017). The genome codes for 74 genes.

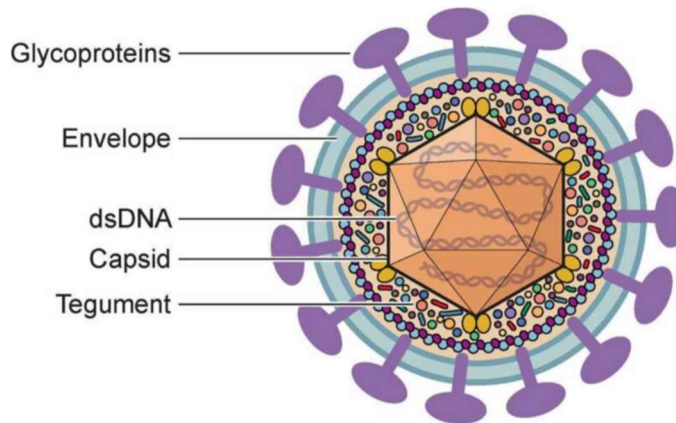


Figure 1: *Herpes simplex virus virion* (Zhu & Viejo-Borbolla, 2021)

To contract HSV-2, the host must be shedding the viral particles, oftentimes, through the lesions present. Through mucous membrane contact, another person may contract the virus. Members of the *Herpesviridae* family infect a specific type of tissue, dependent on the type of virus. Herpes simplex virus-2 targets epithelial and neuronal cells (Nicola et al., 2005). Glycoproteins B and C on the envelope of the virion particle bind to heparan sulfate or chondroitin sulfate proteoglycans on the host cell surface, in order to gain entry to the cell (O'Donnell & Shukla, 2008). The viral envelope then fuses with the cell membrane of the host

cell for the capsid to gain entry. Viral membrane glycoproteins gD, gB, gH, and gL are responsible for the fusion of viral and cellular membranes (Clarke, 2015). Once inside the cell, the viral nucleocapsid travels to the cell nucleus to deposit the viral genome through a nuclear pore (Newcomb et al., 2007). The HSV protein VP16 catalyzes the transcription of early genes by the cellular RNA polymerase II (Fan et al., 2020). The early proteins are responsible for DNA synthesis while the late proteins synthesized are structural proteins. The nucleocapsid is assembled inside the nucleus and travels to the Golgi apparatus and moves through the lumen to obtain an envelope (Wild et al., 2017). The virion is then encapsulated in a vesicle that will transport it to the plasma membrane and release it into the extracellular space (Roberts & Baines, 2010).

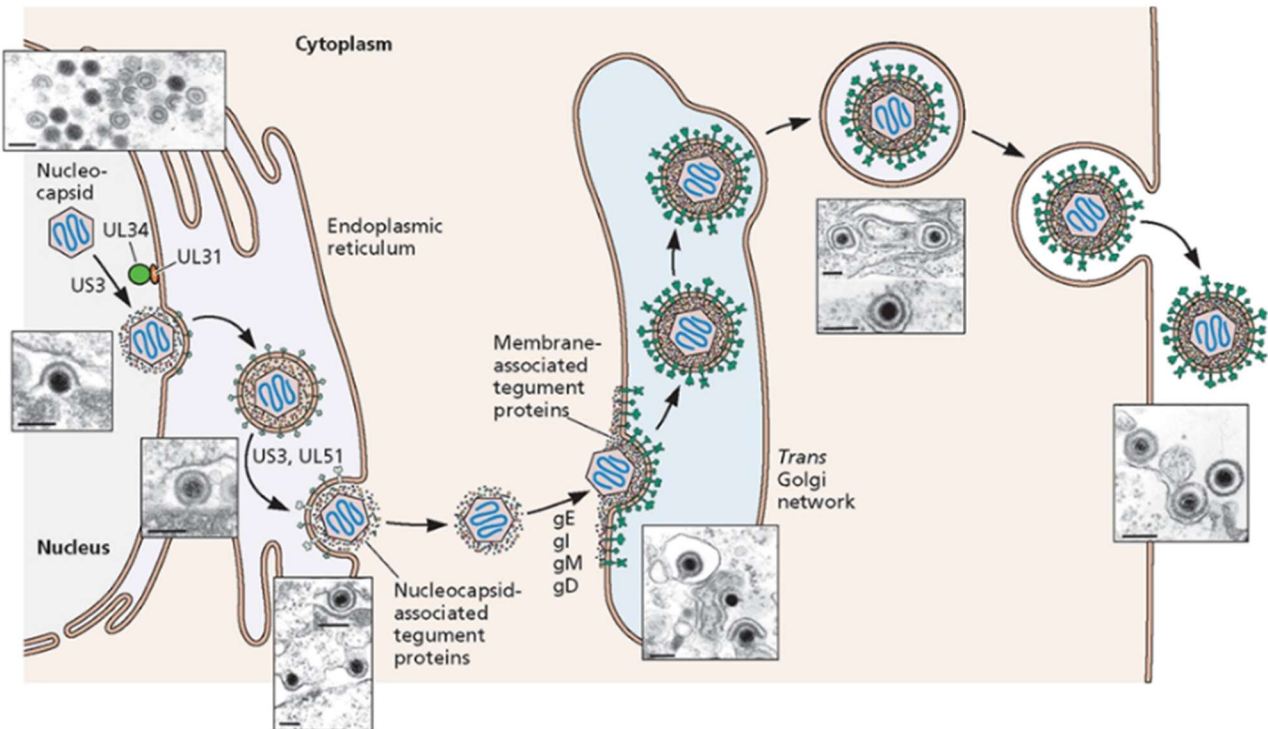


Figure 2: HSV virion assembly (Flint et al., 2020; Mettenleiter, 2002)

HSV-2 is most often known to produce lesions in the genital region, however, it is possible for it to be the cause of lesions located around the mouth as well (Grinde, 2013). In

addition to cold sores or genital herpes, an HSV-2 infection can result in meningitis or encephalitis as well (Bodilsen et al., 2022). The replication cycle of HSV-2 consists of two phases: lytic and latent. When lesions are not present, the virus is in the latent phase of its life cycle. During the lytic phase, the host is considered symptomatic and is shedding viral particles. The latent phase, however, is when the virus is not replicating. Even though it may not currently be shedding, disease progression can still take place. The virus regulates these two stages through lytic genes and latent genes. The early proteins, ICP0, ICP4, and ICP22, mentioned before are expressed during the lytic cycle (Pesola et al., 2005). During the latent cycle, the latency-associated transcripts (LATs) are the only genes transcribed and suppress the expression of the lytic genes. It is still questioned how the virus regulates between the lytic and latent cycle,

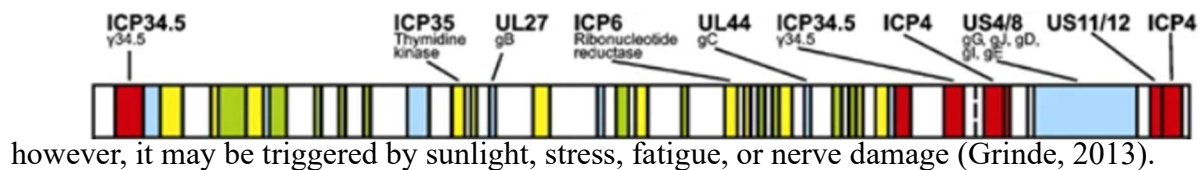


Figure 3: Genome of HSV (Argnani et al., 2005)

The disease caused by herpes simplex virus-2 is treatable with medications that are currently on the market. Acyclovir is one common treatment used to decrease the number and severity of outbreaks caused by HSV-2. This can include shortening the duration of episodes, the number of sores that form, as well as suppress transmission. Acyclovir is offered in a liquid, pill, or ointment and can inhibit viral replication by halting DNA synthesis (Gnann Jr. et al., 1983). The acyclovir molecule is converted intracellularly to acyclovir triphosphate, an analog for the nucleoside deoxyguanosine triphosphate. When DNA polymerase utilizes acyclovir triphosphate in DNA replication, it results in an absent 3' hydroxyl group causing additional nucleosides to no longer be attached. Acyclovir can be administered preventatively, through continuous

medication, or as needed. In a study of fifty-four patients, those who received acyclovir were found to experience less severe symptoms for a fewer number of days (Mertz et al., 1988). The virus was also shed for longer in the control group, up to 28 days, when compared to the acyclovir group of only six days (Mertz et al., 1988).

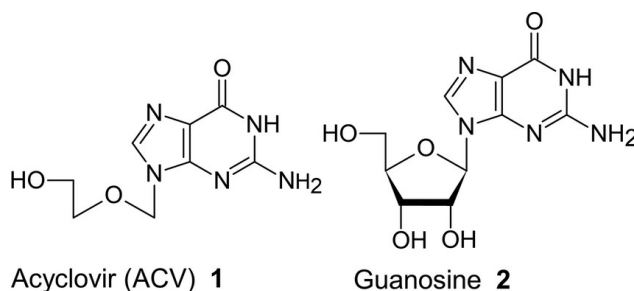


Figure 4: Chemical structure of acyclovir and guanosine (Ami & Ohru, 2021)

The effects of acyclovir are also dependent on the dosage and form of medication. While the Mertz et al. study (1988) found success with acyclovir capsules of 200 mg administered at five times per day over the course of 10 days, there are certain cases that support a different regimen. Acyclovir has also been provided in 400 mg capsules twice a day or even intravenously. The former used in recurrent episodes while the latter is reserved for more severe cases. Despite the dosage of acyclovir, the oral or intravenous treatments, are more effective than acyclovir administered topically. There is benefit in utilizing topical acyclovir at the first sight of lesions, however, it should be followed by an oral dosage as well.

Acyclovir is a widely received HSV-2 antiviral that is generally cost effective with low toxicity effects; however, it is not the only medication available. Valacyclovir, the L-valyl ester of acyclovir, is metabolized by the body and converted into acyclovir in the gastrointestinal tract (Madkan et al., 2007). The site of absorption results in a 54% bioavailability of the compound provided by valacyclovir, while acyclovir was found only to have a bioavailability of about 13-21% (Soul-Lawton et al., 1995). These results were determined when administering 350 mg of

intravenous acyclovir, the most effective method of medication, and 1,000 mg of oral valacyclovir. Despite the increased bioavailability of valacyclovir, there was little to no difference in the number of virus-shedding days in patients that were treated with high- or standard- doses of acyclovir and valacyclovir. However, the number of days of an episode of HSV-2 was found to be the same when comparing the standard-dose valacyclovir and high-dose acyclovir, suggesting a lower dose of valacyclovir is equivalent to a high dose of acyclovir. When comparing those who received a high-dose of valacyclovir to the low-dose group, the episode duration was shorter, the same was true for the number of HSV-2 shedding days between these groups (Johnston et al., 2012).

Penciclovir, an additional antiviral treatment for herpes simplex virus, is only available as a topical ointment. The standard dose for penciclovir is applying the 1% cream every two hours, during the day, for four days (Spruance et al., 1997). However, since it is a topical medication, it can only be applied to the orolabial region. Herpes simplex virus-1 is most commonly the cause of lesions on the orolabial region, but it is possible for HSV-2 to also be located there. HSV-1 and HSV-2 are able to be treated by similar types of antivirals (Spruance et al., 1997). Penciclovir functions in a similar manner as acyclovir. It is first phosphorylated by thymidine kinase to form penciclovir triphosphate. This nucleoside analog is then able to inhibit DNA polymerase from replicating by terminating both forward and reverse strands (Lin et al., 1998).

Since penciclovir is only available topically, its uses were minimal until the development of famciclovir. The antiviral famciclovir is a prodrug of penciclovir, therefore, it is metabolized into penciclovir intracellularly. Famciclovir is available in an oral form, allowing for an increased bioavailability of penciclovir in the body at 77% (Saltzman et al., 1994). Each of the previously mentioned antivirals have a similar percent of success in reducing the length and

severity of exposure for their recommended dosage. The most common side effects reported while taking these treatments have been headaches, nausea, and diarrhea, but otherwise, have been well-received (Saltzman et al., 1994).

In patients that have been treated with acyclovir intravenously, there have been cases of renal failure due to an accumulation of the antiviral molecule (Gunness et al., 2011). Acyclovir and the other mentioned nucleoside analogs are filtered from the bloodstream through the kidneys, therefore, patients with a history of renal dysfunction must be closely monitored while receiving acyclovir treatments. Those with normal functioning kidneys are not likely to experience irreversible kidney damage (Gunness et al., 2011). In addition to potential nephrotoxicity, there have been reports of neurotoxicity amongst patients with preexisting neurological complications (Marks et al., 2020). The symptoms of neurotoxicity are commonly present in patients that are also experiencing renal failure. High levels of an acyclovir metabolite, 9-carboxymethoxymethylguanine (CMMG), were found in patients experiencing neurological side effects (Helldén et al., 2003). These complications are likely due to the administration of a high dosage, suggesting that treatments must be tailored and adjusted as conditions develop in patients throughout the treatment course.

Acyclovir was the first medication available for the treatment of herpes simplex virus in 1982 (King, 1988). Due to the lengthy amount of time acyclovir has been available to those diagnosed with HSV, there has been a rising number of cases of acyclovir-resistant HSV. The resistance is due to a mutation in the viral genes encoding for thymidine kinase or DNA polymerase (Hardy, 1992). The mutations alter the viral enzymes necessary for acyclovir to function and cease viral replication, therefore causing the drug to be inactive. The strains of HSV-2 that are resistant to acyclovir will also be resistant to the additional nucleoside analogs

that have been mentioned as they have identical mechanisms of action, by requiring phosphorylation by thymidine kinase to become activated. Those with acyclovir-resistant HSV could be treated with foscarnet or cidofovir.

Foscarnet is not incorporated into the viral DNA strand and does not require intracellular activation like the previously mentioned antivirals. Instead, foscarnet is able to inhibit the viral DNA polymerase by binding to it noncompetitively and preventing further replication (Helgstrand et al., 1978). Foscarnet must be administered with care as it is likely to result in nephrotoxicity at high levels and in patients with pre-existing renal failure (Aweeka et al., 1999). Cidofovir, another alternative for acyclovir-resistant HSV, is a nucleotide analog of cytosine. Since cidofovir is already phosphorylated, it does not require viral enzymes to become activated. Once cidofovir is added to the viral DNA strand during replication, chain termination occurs (Xiong et al., 1996). Activation of cidofovir can occur in both HSV-2 infected and uninfected as it only requires cellular enzymes to be phosphorylated. Similar to foscarnet, the dose of cidofovir must be closely monitored to avoid nephrotoxicity (Lalezari et al., 1995). Foscarnet and cidofovir are only administered intravenously, making them less convenient than their capsule counterparts.

The presence of antiviral-resistant strains makes it necessary to continue developing treatments that are effective against HSV-2. In addition to the mentioned treatments, there have been studies analyzing various plant compounds for their antiviral properties. Epigallocatechin gallate stearate (EGCG) and the lipophilic modified EGCG-stearate, polyphenols derived from *Camellia sinensis* found in green tea, has demonstrated HSV-2 inhibition in Vero cells (Stamos et al., 2022). Cells infected with HSV-2 that had been treated with 75 μ M of EGCG-S did not display cytopathic effects compared to the untreated and infected Vero cells. EGCG and EGCG-

S both resulted in 99% inhibition when used as a viral treatment. In the same study, only 50 μM of EGCG and EGCG-S was required to see a reduction of viral penetration up to 57.3%.

Another form of EGCG, palmitoyl-EGCG, resulted in inhibition of HSV-1 at levels more effective than EGCG (de Oliveira et al., 2013). Most antivirals that can treat HSV-1 are capable of treating HSV-2 as well. Embelin, a benzoquinone derived from the plant *Embelia ribes*, has displayed antiviral effects against HSV-1 (Elias et al., 2021). When HSV-1 was treated with 54 μM of embelin, there was 100% of viral inhibition in Vero cells. This finding was supported through fluorescent microscopy images where cells infected with embelin-treated GFP-tagged HSV1-1 lacked the presence of virions, compared to the cells infected with untreated HSV-1. Binding and penetration assays were utilized to determine the mechanism of action of embelin. Concentrations as low as 20 μM of embelin were found to prevent the HSV-1 virions from attaching and infiltrating the host cell.

One common characteristic of EGCG and embelin is that they both display antioxidant properties (Caruso et al., 2018; Zhong et al., 2012). Antioxidants are compounds that can neutralize free radicals that could be caused by environmental factors like pollutants or pathogens (Halliwell et al., 1995). Free radicals are a form of reactive oxygen species (ROS) that include oxygen groups like superoxide and hydroxyl (Bayr, 2005). If ROS levels become too high in cells, oxidative stress can occur. Oxidative stress is the production of ROS at a faster rate than the body can neutralize them (Zhang et al., 2014). DNA damage, cellular apoptosis, and disease progression can occur if the body experiences extensive oxidative stress (Zhang et al., 2014).

Viral infections are one cause of oxidative stress due to excessive ROS levels. Reactive oxygen species are produced at a generally manageable level during mitochondrial oxidative

metabolism to produce ATP (van Hameren et al., 2019). When biological functions are no longer operating at a normal rate, ROS is produced more quickly. Viruses require the use of host machinery to replicate, creating an imbalance in the cell's metabolic status and encouraging the production of reactive oxygen species (Stehbens, 2004). HSV infections were found to cause a significant increase in ROS levels of kidney and dermal tissue, while also resulting in a decrease of nonenzymatic antioxidants (Sebastiano et al., 2016). Levels of the reactive oxygen species H₂O₂ were found to be significantly lower in Vero cells infected with embelin-treated HSV-1 (Elias et al., 2021). Additional research in the use of antioxidants as an effective antiviral demonstrates that a topical treatment, Ascoxal®, containing ascorbic-acid resulted in patients with shorter, and less, severe, non-genital herpes episodes (Hovi et al., 1995). Ascorbic-acid, or vitamin C, is an antioxidant that has displayed the ability to correct the imbalance of oxidative species induced by viral infections (Hoang et al., 2020). The evidence collected regarding antioxidants as potential antivirals has created an overwhelming argument for continuing research to locate alternatives for the classical medications provided for herpes simplex virus-2 infections.

Allium sativum, garlic, has been widely used as a medicinal treatment for thousands of years. Fresh, cooked, and extracted garlic have been used to alleviate ear infections, tuberculosis, and more in locations like Asia, Europe, and Ethiopia, respectively (Al Abbasi, 2008; Delaha & Garagusi, 1985). Allicin, the primary organosulfur compound in garlic, is responsible for the distinctive taste and smell that the herb is known for (Borlinghaus et al., 2014). In addition to people utilizing garlic as a treatment throughout history, research has provided evidence that allicin possess antibacterial properties through the inhibition of bacterial growth of *S. aureus* (Deresse, 2010). The antimicrobial effects of allicin do not stop at bacteria though and have been

reported with viruses as well. When Vero cells infected with SARS-CoV-2 were treated with 50 μM of allicin, the level of viral RNA present in the cells had decreased by up to 70% (Mösbauer et al., 2021). The success behind allicin's antimicrobial characteristics could be due to its antioxidant properties. Allicin was found to be capable of mitigating the byproducts of hydroxyl radicals, a type of ROS, by 32% in concentrations as low as 1.8 μg (Prasad et al., 1995). At 36 μg of allicin, the hydroxyl radical byproducts and decreased by 94%.

Rouf et al. propose three different potential mechanisms of action that garlic may have in inhibiting viral infection depending on the pathogen's classification. In positive-sense single-stranded RNA viruses, like HIV-1, the components of garlic have demonstrated the ability to inhibit the virus from entering the cell, block reverse-transcription, and prevent genome integration. For DNA viruses, like herpes virus, the garlic may block cell entry, prevent integration, and stop replication. Negative-sense single-stranded RNA are inhibited from entering the cell, uncoating the virion, producing positive-sense RNA, assembling the viral particle, as well as releasing the virus from the host cell (Rouf et al., 2020). Garlic's various modes of inhibition in different types of viral infections support the evidence for garlic as an antiviral. It is also vital to understand how components function while developing therapeutics for infections.

If the levels of antioxidants in garlic were increased, it can be hypothesized that the herb's ability to relieve oxidative stress caused by an excess of ROS would improve. There have been many studies on garlic for its health-improving properties, however, research on black garlic is still scarce. Black garlic is produced when fresh, raw garlic, is exposed to high temperatures and high humidity for an extended period of time (Choi et al., 2014). This causes the once white, sharp garlic cloves to turn black and have a sweeter taste as a result of the aging process altering the herb's chemical composition. The decomposition of the organosulfur

compound allicin creates the sweet taste that characterizes black garlic. Allicin can breakdown into various antioxidant compounds such as *S*-allylcysteine, tetrahydro- β -carboline, alkaloids, and flavonoids (Choi et al., 2014). Due to the increase in allicin byproducts found in black garlic, the levels of antioxidants increased from 13.91 mg/g to 58.33 mg/g, for polyphenols, and 3.22 mg/g to 15.37 mg/g, for flavonoids, by the 21st day in the aging process (Choi et al., 2014).



Figure 5: Black garlic fermentation process (Choi et al., 2014)

One way the antioxidant activity of black garlic has been assessed is through a radical scavenging activity assay. Black garlic was able to successfully scavenge the free radicals and undergo single electron transfer at a greater percentage than the raw garlic. Black garlic that had been aged for 21 days had 75.48% of free radical scavenging activity compared to the approximately 5% of the raw garlic's scavenging activity (Choi et al., 2014). Therefore, black garlic antioxidants operate at a higher efficiency than those found in the raw garlic. The black garlic in the previously mentioned study had been fermented at 70°C in 90% humidity, however, different conditions can result in varying levels of antioxidants. Over the aging period, the concentration of allicin decreased while the phenolic content continued to increase (Chang & Jang, 2021). On the 25th day, the amount of allicin in the black garlic had become negligible while the phenolic content peaked on day 35. Zhang et al. analyzed the effect that the temperature can have on the black garlic fermentation process. It was found that the higher temperature of 80°C or 90°C resulted in black garlic reaching a greater phenolic concentration more quickly than the low temperatures. However, when black garlic was fermented at lower

temperatures, such as 60°C, the phenolic content became equal to the garlic processed in 90°C temperatures by day 72 (Zhang et al., 2016). This evidence suggests that it would be beneficial to determine the most efficient conditions to produce black garlic with high antioxidant concentration without needing a long period of time.

With antiviral-resistance increasing, continued research into other sources of therapeutics becomes more necessary. Plant-derived antivirals have successfully inhibited viral infections through direct treatments or when combined with traditional medicines. When garlic extract was combined with vancomycin, to treat vancomycin-resistant *Enterococci*, it increased the susceptibility of the bacteria to the treatment (Jonkers et al., 1999). As there are multiple forms of garlic available, like oils, tablets, pills, nasal spray, or extract, the effective methods of treatments for certain infections should be further analyzed (Rouf et al., 2020). Garlic extract has displayed antiviral properties against herpes simplex virus-2, however, black garlic extract has not yet been tested explicitly (Weber et al., 1992). This study will assess black garlic extract for its ability to inhibit HSV-2 infection in A549 lung cells.

Materials and Methods

Cell Culture Maintenance

A549 lung epithelial cells (CCL-15) were used in this study because they are a continuous cell line and were able to be successfully infected by HSV-2 in previous experiments (Stamos et al., 2022). A549 lung cells were cultured in T25 flasks with F12-K media (American Type Culture Collection (ATCC) Manassas, VA). The media was complete with 10% Fetal Bovine Serum (FBS) and 1% Gentamicin. When cells reached confluency, the flask was washed with 1% Phosphate Buffer Solution (PBS) and subcultured using 0.25% Trypsin-EDTA. Seeded flasks were placed in a 37°C incubator at 5% CO₂.

Virus Propagation

Cells that reached 70 – 80% confluency were infected with Herpes Simplex Virus-2 VP26-GFP which expresses a fusion protein of VP26 and GFP (generously donated by Dr. Andrea Bertke, Virginia Tech University). The media was removed from the flask and 100µL of virus stock was added. The flask was incubated for one hour at 37°C with intermittent mixing. After the incubation period, the virus solution was removed and 5mL of fresh F12-K media was added. Once full cytopathic effects were observed, within 48 – 72 hours, the media was removed. The lysate was collected, centrifuged, and supernatant was stored at -80°C.

Black Garlic Extract Preparation

Solutions for treating the cells or virions were first prepared with 1 mL of F12-K media. The water-based and ethanol-based extracts each had a concentration of 3 parts black garlic to 1 part solvent, resulting in a 333mg/1mL solution. The necessary amount of extract for each treatment was calculated using a percent volume/volume solution. A vortex was used to ensure

even mixing throughout the solutions. The extracts used are a product of the HerbalTerra LLC company and were stored at 4°C. At the start of the project, lower percentages of extract solutions were tested (1 – 7%) but were no longer used when no signs of inhibition were exhibited. In later parts of the experiment, 9 – 17% solutions were ultimately used.

Cytotoxic Effects in Cells Treated with Black Garlic Extract

A six-well plate was seeded with A549 cells from a confluent T-25 flask. Once 70-80% confluency was reached, after approximately 24 – 48 hours, cells were treated with solutions containing 5 – 20% of BGE solutions. The plate was placed in an incubator at 37°C, 5% CO₂ for one hour. The solutions were then removed and 3mL of fresh F12-K media was added. After a 48-hour incubation period, the plate was observed for cytotoxicity.

MTS Cell Viability Assay

A 96-well plate was prepared as mentioned above. 100 µL of solutions at 5 – 20% BGE were added to five wells each. The plate was incubated for one hour at which time the solutions were removed and 100µL fresh F12-K media was added. After 48 hours, 20µL of MTS reagent (Promega Corp., Madison, WI) was added to each well for one hour of incubation at 37°C, 5% CO₂. A Tecan microplate reader was utilized to measure absorbance. According to the Promega user manual, the MTS reagent allows for quantification of viable cells as it contains tetrazolium salt. Through cellular respiration, the tetrazolium salt is reduced into formazan that can dissolve into the cell culture media. Formazan is a colored product whose absorbance can be measured at 490nm. The level of absorbance is proportional to the number of viable cells in the well.

MTS Antiviral Cell Viability Assay

Cell-Treated Assay

A 96-well plate was prepared and 5-20% black garlic extract solutions were made as previously stated. The media from the 70 – 80% confluent cells was aspirated and 100 μ L of the black garlic extract solutions were added into their respective wells. The plate was placed into a 37°C, 5% CO₂ incubator for 30 minutes. 100 μ L of HSV-2 virions was added to 1mL of F12-K media. This step was repeated ten times to prepare one solution for each column of cells. After the 30-minute incubation period, the BGE solutions were removed from the cells, and 100 μ L of the HSV-2 virion solution was put into each well. The plate was then incubated at 37°C and 5% CO₂ for one hour. After the incubation period, the solution was removed once again and 100 μ L of fresh F12-K media was added to each well. The plate was incubated for approximately 48 hours. Prior to incubating the plate for an additional hour, 20 μ L of MTS One Solution was added to cells. The plate was then put into the microplate reader and read for optical density at 490nm followed by calculation of percent of infectivity.

Virus-Treated Assay

BGE solutions were prepared at their respective volumes (5 – 20%) and 100 μ L of HSV-2 lysate was placed into each tube. The solutions were left at room temperature for 30 minutes. The media from the plate was aspirated and 100 μ L of the BGE treated HSV-2 solution was added to each well. The plate was allowed to incubate for 1 hour at 37°C and 5% CO₂. The solutions were removed, and fresh F12-K media was added. After a 48-hour incubation period, 20 μ L of MTS One Solution was added to each well and placed back in the incubator for an

additional 20 minutes. The Tecan microplate reader was used to measure the optical density of the plate at 490nm to calculate the percent of infectivity.

Cytopathic Effects in Cells with Black Garlic Extract-treated Virus

A six-well plate was prepared, along with BGE solutions, for cell-treated and virus-treated assays as mentioned above. Images of the cells were taken using an inverted microscope for qualitative analysis of cytopathic effects.

Antiviral ROS-Glo™ H₂O₂ Assay

Antiviral assays were performed as previously stated for both cell-treated and virus-treated procedures using the ethanol-based BGE in 9%, 13%, and 17% solutions. At the final step of the antiviral assay, 80µL of fresh F12-K media was added. 30 hours post-infection, 20µL of H₂O₂ substrate solution was added to each well. The H₂O₂ Substrate Solution was prepared for 50 wells by adding 12.5µL of H₂O₂ substrate to 1.0mL of H₂O₂ Substrate Dilution Buffer. The plate was then incubated for an additional six hours at 37°C and 5% CO₂.

At 36 hours post-infection, 100µL of ROS-Glo Detection Solution was added to cells in each well. The solution was prepared for 50 wells with 5mL of Reconstituted Luciferin Detection Reagent, 50µL of D-Cysteine, and 50µL of Signal Enhancer Solution. The plate was incubated at room temperature for 20 minutes. A microplate reader was then used to record the relative luminescence units (RLUs).

Fluorescent Microscopy

Coverslips were placed in dishes on six-well plates and seeded with A549 cells. BGE solutions at 9%, 13%, and 17% were used to perform cell-treated and virus-treated assays with

GFP-tagged HSV-2. The dishes were incubated at 37°C, 5% for 24 hours. After the incubation period, the media was removed and washed with 1X PBS. 300µL of 300nM DAPI was added to cells on each coverslip for 5 minutes at 37°C. The solution was then removed and a 1:1 methanol-acetone solution was added to fix the cells for 15 minutes at -20°C. The solution was aspirated, and the coverslips were removed. A 90% glycerol and 10% PBS solution was used as a mounting medium and the coverslip was fixed to the microscope slide with clear nail polish. DAPI and FITC images were taken with a microscope at 200x using epifluorescence.

Statistical Analysis

Percent proliferation, viability, and inhibition were calculated in Microsoft Excel using the following formulas.

$$\text{Percent proliferation and viability: } \% = \left(\frac{\text{Treatment-Blank}}{\text{Cells only-Blank}} \right) \times 100$$

$$\text{Percent inhibition: } \% = \left(1 - \frac{\text{Treatment-Cells only}}{\text{Cells only-Virus only}} \right) \times 100$$

The means and standard deviations were calculated using JMP software. One-way ANOVA with Dunnett's post-hoc test was utilized through JMP as well to determine statistical differences.

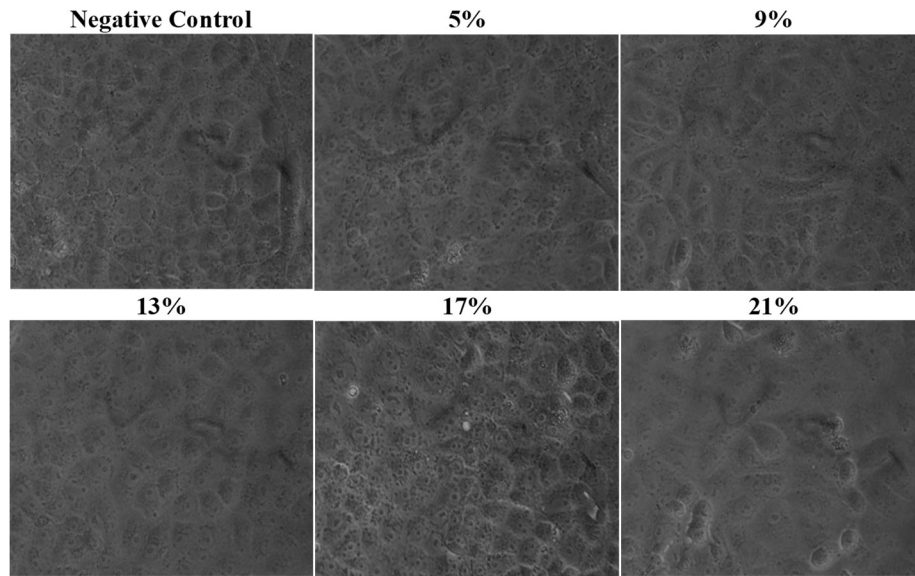
Figures were created using GraphPad Prism 9.

Results

A549 cells do not exhibit cytotoxic effects (CTE) when treated with less than 21% BGE solutions.

To determine if the water-based or ethanol-based BGE would negatively affect the proliferation of the A549 cells, the cells were first treated with a range of concentrations at 70-80% confluency for 48 hours and then observed for the presence of CTE. At 5% - 13% concentrations of the water-based BGE solutions, the cells' morphology did not appear to be affected by the treatment (Figure 6A). However, the 17% solution had cells present with rounder characteristics suggesting that they began to lift from the dish and lyse due to the high concentration of extract. There were signs of CTE for the 21% solution treatment group as there were fewer cells and an increased number of rounded ones. The ethanol-based BGE displayed similar results to the water-based one, where concentrations of 5% - 9% did not negatively affect the cell culture (Figure 6B). Cytotoxic effects were not observed until the 21% treatment as there were more rounded cells in the culture.

A.



B.

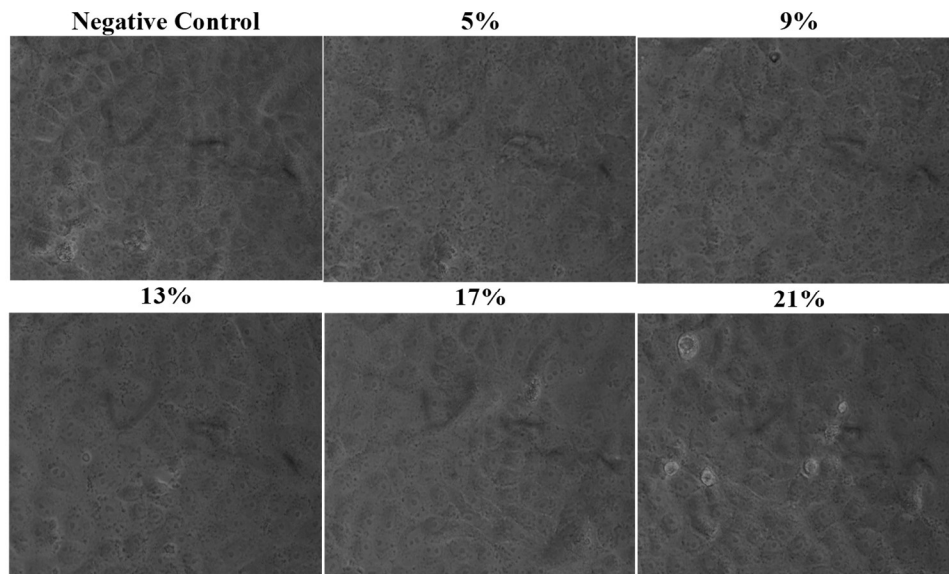
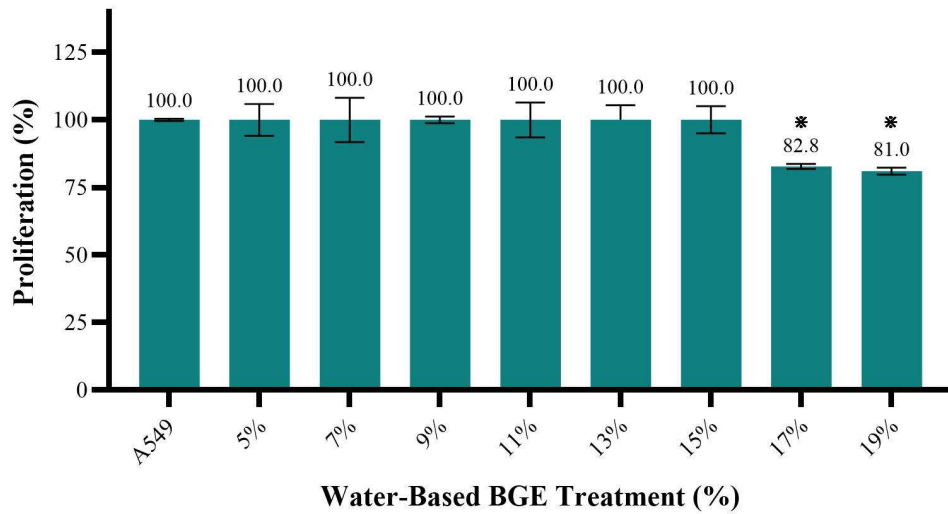


Figure 6: Cytotoxicity Assay. Images at 400x utilizing (A) water-based and (B) ethanol-based black garlic extract 48 hours post-treatment.

The qualitative results provided in Figure 6 were followed by an MTS assay to calculate the percent of proliferating cells present for each treatment. Cells that are no longer proliferating, and therefore not viable, will not be able to convert the tetrazolium salt in the MTS solution into formazan and produce a lower absorbance value during the assay. Cells treated with

concentrations of 5% - 15% water-BGE displayed 100% of cell proliferation (Figure 7A). At 17% of water-BGE, the percent proliferation decreased to 82.8%. The 19% concentration resulted in a percent proliferation of 81.0%. The percent proliferations of the 17% and 19% water-BGE concentrations were significantly different than the untreated control group. Those treated with 5% - 9% ethanol-BGE solutions resulted in 100% of cell proliferation. At 11% of ethanol-BGE, there is a slight decrease in proliferation from 100% to 98.3%. The proliferation continued to decrease to 83.8% at 13% of ethanol-BGE. There are significant differences in proliferation for the 17% and 19% ethanol-BGE, at 49.9% and 63.1%, respectively (Figure 7B). The results of the MTS completed with the ethanol-BGE support the cytotoxic levels found in Figure 6B, both demonstrating CTE beginning at concentrations of about 17%.

A.



B.

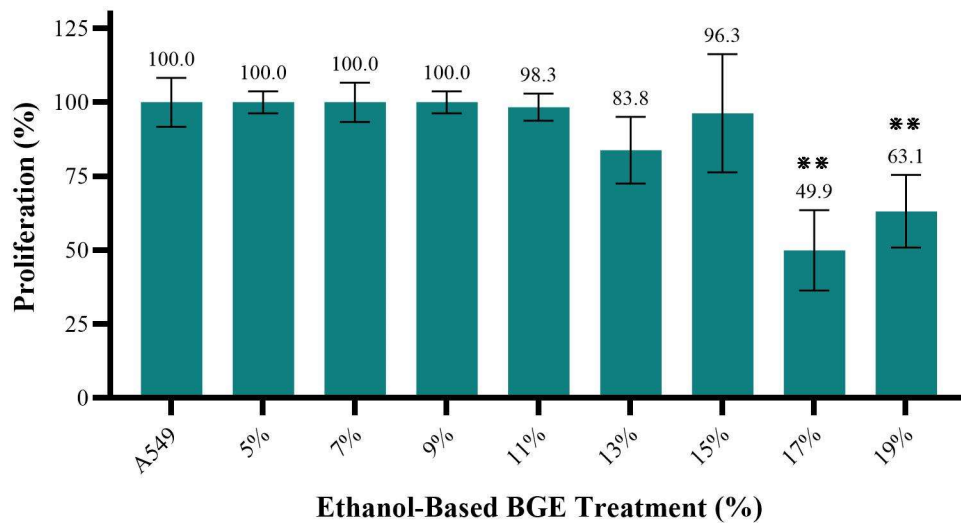


Figure 7: MTS Cytotoxicity Assay. (A) Water-BGE began to show cytotoxicity at 17%, with the lowest percent proliferation at 17%. (B) Ethanol-BGE began to show cytotoxicity at 13%, with the lowest percent proliferation at 17%. * $p < 0.05$, ** $p < 0.01$

Treatment with Ethanol-BGE decreased observable cytopathic effects (CPE) in HSV-2 infected A549 cells.

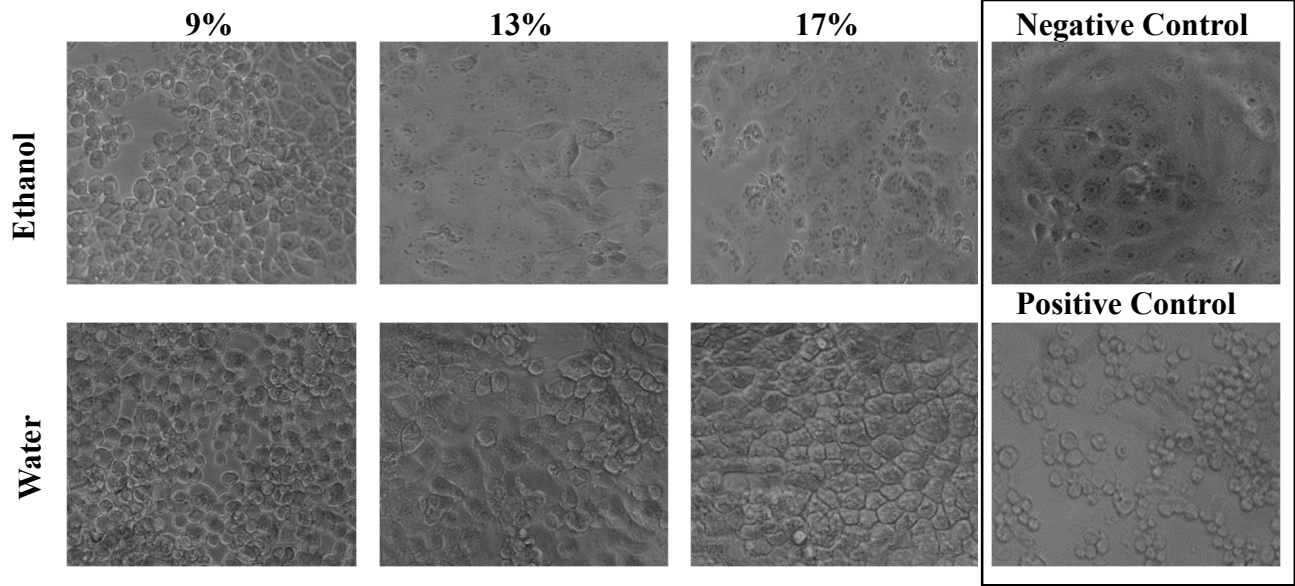
To determine the ideal method of treatment, two approaches were tested. The cell-treated assay involved exposing the cells to the BGE treatment prior to infection to determine if

treatment protected the cell from the virus. The virus-treated assay aims to observe the effect on the virus itself by applying the extract to the viral solution before infecting the cells. In addition to identifying the ideal mode of treatment, a wide range of BGE solutions, 1% - 17%, were used to narrow down the most effective dosage for viral inhibition.

When analyzing cell morphology, cells treated with ethanol-BGE displayed less rounding and lifting, when compared to those treated with water-BGE prior to HSV-2 exposure (Figure 8A). The cells treated with ethanol-BGE maintained qualities more closely resembling the cells found in the negative control group, suggesting that a lower number of cells were infected by the virus. The level of CPE observed in the cells treated with the ethanol-BGE decreased from the 9% solution to the 17% solution. However, the same effects were not observed for the cell-treated assay utilizing the water-BGE. As the treatment increased, from 9% to 17%, the CPE caused by HSV-2 were not alleviated. As presented in Figure 8A, the cells maintain a rounded conformation, more closely resembling the positive HSV-2 control group. The number of lifted cells appear to decrease as the treatment increases, but the typical cell morphology is still not present.

The virus-treated assay provided similar results for the ethanol-BGE. There were some healthy cells present in the 9% and 17% treatments, while the 13% solution contained the least number of lysed cells (Figure 8B). While lysed cells are not present in the 17% ethanol-BGE treatment, there are not as many cells, implying a lower number of surviving cells. The water-BGE virus-treatments resulted in similar cell morphology as the ethanol-BGE. The 13% solution contains some lysed cells, but a decreased number when compared to the 9% solution. The 17% water-BGE treatment contains an approximately equal number of lysed or damaged cells to healthy ones.

A.



B.

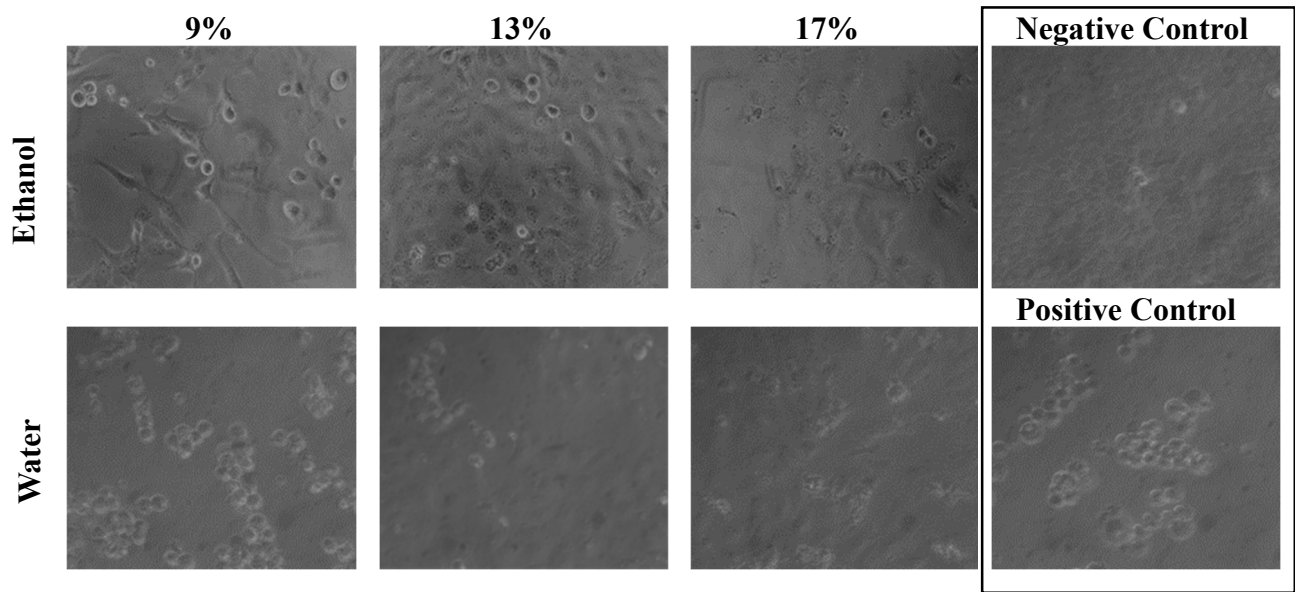


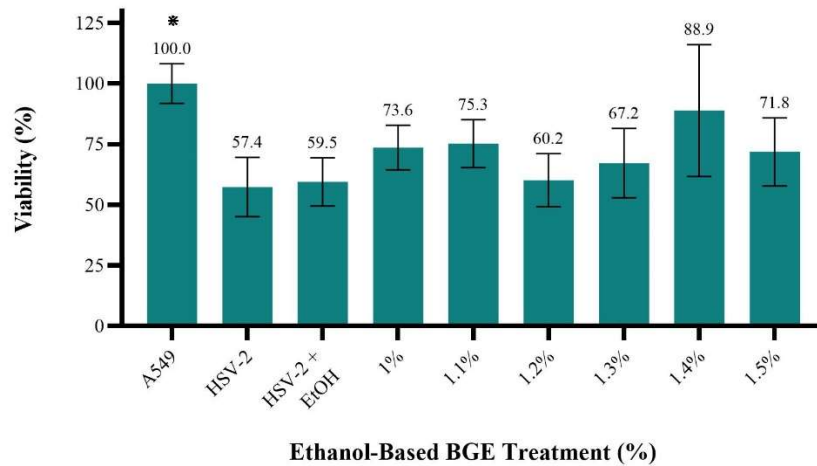
Figure 8: Cytopathic Assay. Images taken at 400x using ethanol-based or water-based black garlic extract in (A) Cell-Treated and (B) Virus-Treated assays.

Cells treated with an increased concentration of ethanol-BGE displayed a greater percentage of viable cells post-infection.

Due to the reliability and consistent results provided by the ethanol-BGE results, it was selected as the sole treatment for the remaining assays. Inverted microscopic images indicated more morphological changes to cells treated with water-BGE than ethanol-BGE (Figure 8). The cytotoxic results displayed in Figures 6 and 7 for treatments at 17% - 21% suggested that a lower percentage of the extract may be more effective at preventing viral infection. Utilizing MTS assays to perform cell- and virus-treated assays, the number of viable cells for each treatment was quantified. Ethanol-BGE solutions ranging from 1% - 1.5% were used to treat the A549 cells, which were then exposed to HSV-2. Although the percent of viable cells appear high, from 60.2% viability for the 1.2% treatment to 88.9% viability for the 1.4% treatment, there was no significant difference calculated between the treatment groups and the percent of viable cells for the HSV-2 only control group (Figure 9A).

While the percentage of viable cells in each treatment group appears to be relatively equal based on the results of Figure 9A, the percentage of inhibition suggests that the virus was prevented from infecting the cells. The absorbances from the MTS antiviral assay in Figure 9A were used to calculate the percent inhibition. The 1.4% treatment solution, that had the greatest percent of viability in Figure 9A, also has the highest percentage of inhibition at 73.9% (Figure 9B). The 1.2% solution treatment was calculated to have the lowest inhibition at 6.7%. The mock-treatment provided by the HSV-2 + ethanol treatment did not result in a difference in cell viability and had a low inhibition of 4.9%, indicating that the ethanol in the extract does not inhibit the virus from infecting the cell on its own.

A.



B.

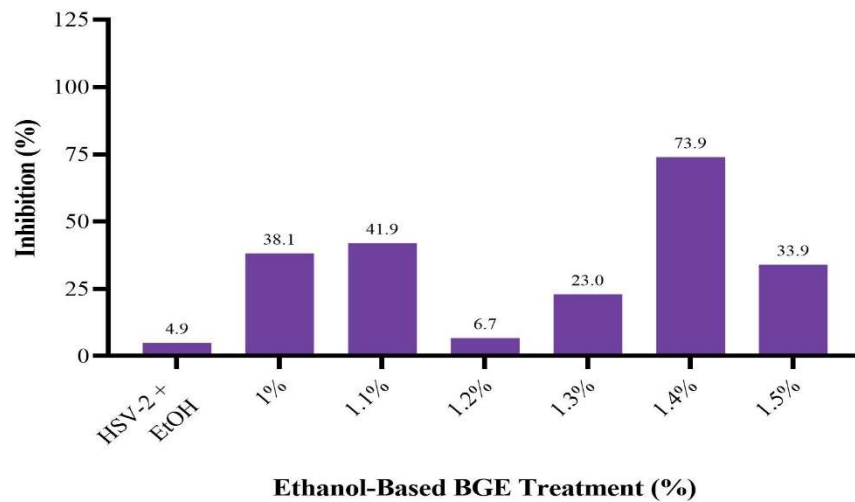


Figure 9: Cell-Treated MTS Antiviral Assays. (A) The percentage of viability of cells treated with low volumes of ethanol-based black garlic extract (1% - 1.5%) and (B) the percentage of inhibition for each treatment. * $p < 0.05$ when compared to the HSV-2 only control.

The treatment volumes that were administered in Figure 9 for the cell-treated assay, 1% - 1.5%, were then used in virus-treated assays. The percentage of viability for each treatment ranged from 20.6% - 24.9% and were approximately equal to the viability percentage of the HSV-2 control at 26.2% (Figure 10). Therefore, these concentrations of the ethanol-BGE treatment were not effective in preventing the virus from infecting the cells and increasing the

percent viability. The percent of inhibition was not calculated from this antiviral assay because the treatment values and the virus-control were too similar to indicate inhibition.

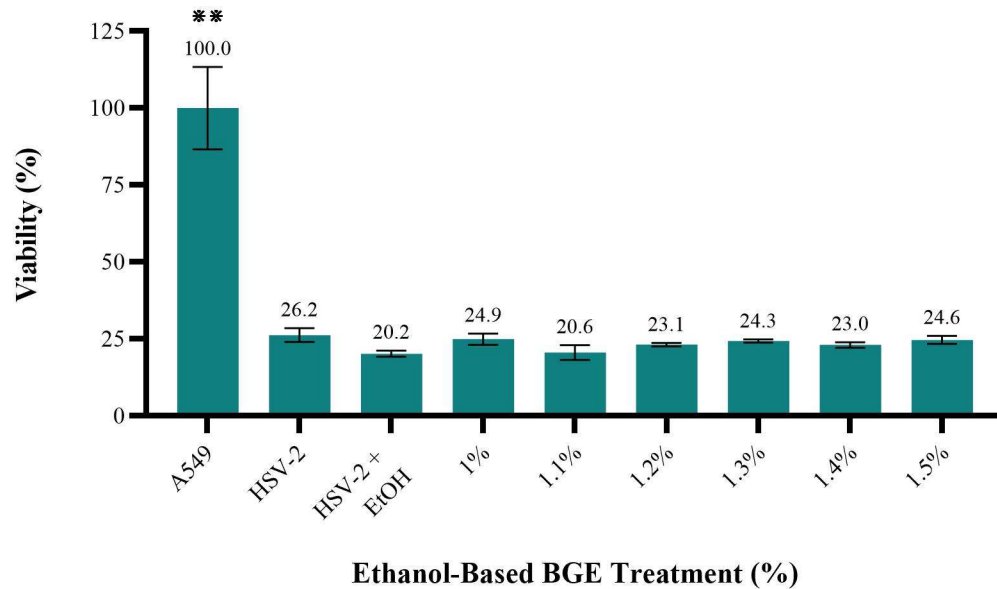


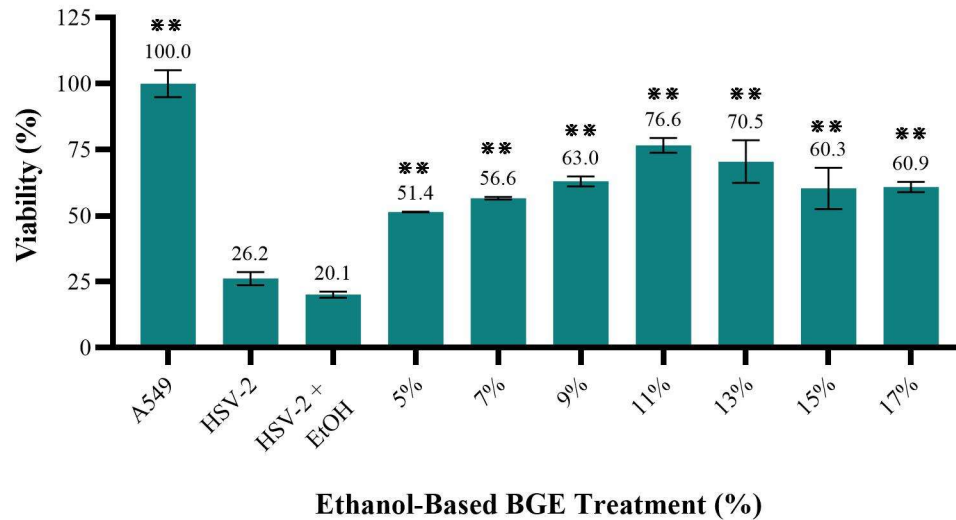
Figure 10: Virus-Treated MTS Antiviral Assay. The percent viability of cells infected with HSV-2 treated with low volumes (1% - 1.5%) of ethanol-based black garlic extract. ** $p < 0.01$ when compared to the HSV-2 only control.

The cell-treated antiviral assay displayed in Figure 9 did not present significant differences in the treatment groups for the percent viability, however, the percent of inhibition was more prevalent than compared to the virus-treated assay (Figure 10). The effectiveness of the cell-treated assays was further analyzed by increasing the concentration of the BGE to determine if that would result in an improved percent of viable cells. When comparing the percent viability of the treatment groups to the viral-control group, each treatment had a significant difference resulting in a p -value less than 0.01. The 11% treatment group resulted in the greatest percent viability at 76.6%, while the 5% treatment had the lowest percent of viable

cells at 51.4% (Figure 11A). It should be noted that as the concentration of the BGE increases, the viability percent decreases, while remaining greater than the virus-control.

The percent inhibition for the cell-treated assay at higher concentrations reflects the results in Figure 11A for the percent cell viability. The 11% treatment group was the most successful at inhibiting the virus at 68.3% inhibition while 34.1% was the lowest inhibition calculated belonging to the 5% treatment group (Figure 11B). These results suggest that the 11% solution of the ethanol-based BGE would be the most effective concentration to inhibit HSV-2 infection via the cell-treated method.

A.



B.

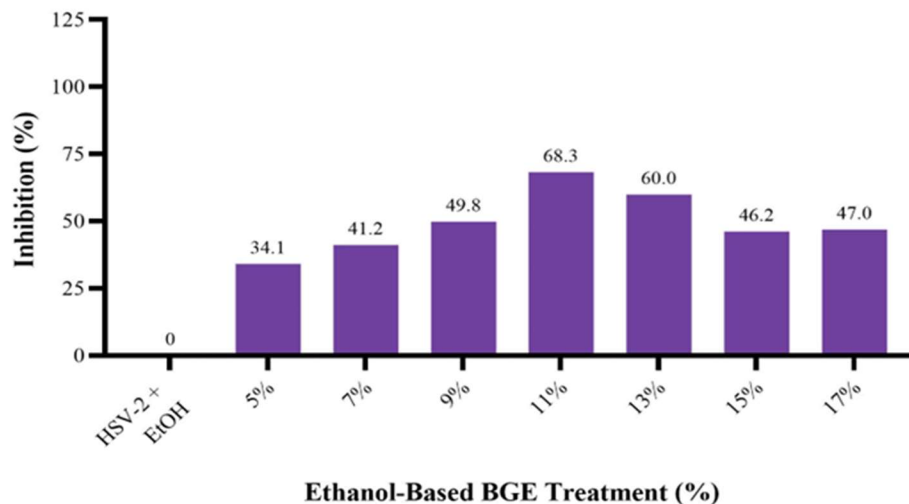


Figure 11: Cell-Treated MTS Antiviral Assay. (A) percent viability and (B) percent inhibition of cells treated with high volumes (5% - 17%) of ethanol-based black garlic extract. ** $p < 0.01$ when compared to the HSV-2 only control.

Cell morphology is affected by viral infection.

GFP-tagged HSV-2 allowed for the visualization of the number of infected cells for each treatment. The nuclei of the cells were counterstained with DAPI. The tegument protein of the HSV-2 virion contains the green-fluorescent protein, localizing the fluorescence to the cytoplasm of the infected cell. Cell-treated and virus-treated assays were completed using 13% and 17%

ethanol-BGE. The level of green fluorescence present correlates to the amount of virus in the cell. The 13% cell-treated group emitted the highest level of green fluorescence, relatively equal to the positive control group. The intensity of the fluorescence decreased with the 17% cell-treated group (Figure 12). The 13% and 17% virus-treated groups both had relatively low levels of fluorescence, similar to the negative control.

The background signal of green fluorescence found in the negative control image can be used to analyze the morphology of the cytoplasm of the infected cells. The cells of the negative control group generally maintain their rounded structure, while the cytoplasm of the infected cells appear to be elongated where there are greater levels of fluorescence, such as in the 13% cell-treated group. The DAPI counterstain also indicates potential nuclear damage to the infected cells. The 13% virus-treated group contains more cells with misshapen nuclei, when compared to the nuclei of the negative control group (Figure 12). The fluorescent images indicate that the virus-treated method was more successful at inhibiting HSV-2 from infecting the cells for both the 13% and 17% concentrations. However, the 17% concentration for the cell-treated method was able to reduce the level of fluorescence as well.

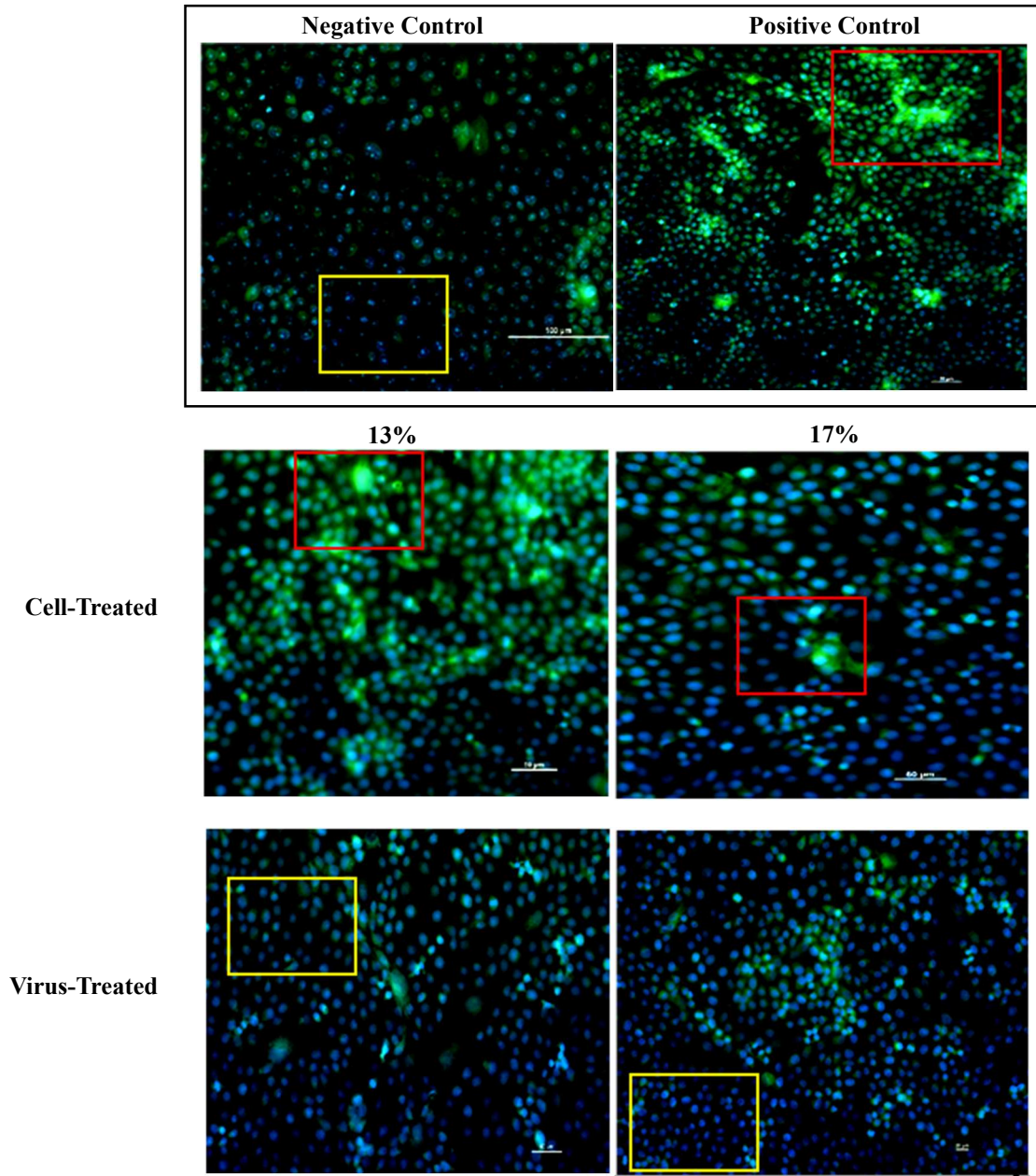


Figure 12: Fluorescent Microscopy. Images taken at 200x of DAPI-stained cells infected with HSV-2 tagged with GFP and ethanol-based black garlic extract. Red boxes indicate regions of high GFP, yellow boxes indicate regions of low GFP.

13% ethanol-BGE solutions alleviated oxidative stress in cell-treated and virus-treated groups.

The ROS-Glo H₂O₂TM Antiviral Assay measures the level of oxidative stress based on the release of hydrogen peroxide, a reactive oxygen species. Cells that are infected with HSV-2 undergo oxidative stress because viruses rely on the biosynthetic mechanisms of the host for viral replication. Concentrations of 9%, 13%, and 17% solutions of ethanol-BGE were utilized in cell-treated and virus-treated assays to determine if the antioxidants in black garlic can mitigate the oxidative stress caused by HSV-2 viral infection. The HSV-2 group had an average of 6916 RLU (Figure 13). While the 9% and 17% treatment groups did not indicate a significant difference when compared to the positive control group, the 13% treatment of cell and virus-treated groups did. The 13% cell-treated solution was found to have an average of 5480 RLU, while the virus-treated group had an average of 5546 RLU. The virus-treated ethanol group presented a significant difference with 5682 RLU as compared to the HSV-2 only control group. However, there was no significant difference between the cell-treated ethanol group and the HSV-2 only control group. Therefore, treatment with the 13% ethanol-BGE solutions was effective in mitigating the oxidative stress caused by HSV-2 via the cell-treated and virus-treated assays.

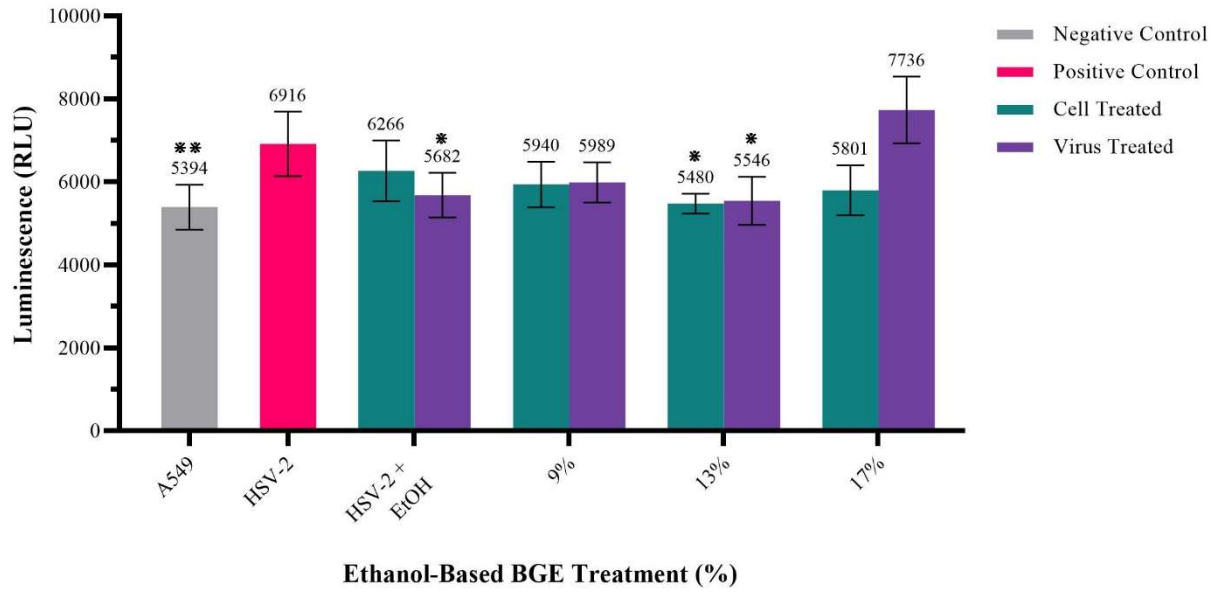


Figure 13: ROS-Glo H₂O₂[™] Antiviral Assay. Oxidative stress measured in RLU for cell and virus treated assays. * $p < 0.05$, ** $p < 0.01$ when compared to the HSV-2 only control.

Discussion

The World Health Organization estimated in 2016 that half a billion people around the globe have a Herpes Simplex Virus-2 infection, an incurable condition (James et al., 2020). While there are antivirals on the market to alleviate HSV-2 outbreaks, there are a growing number of drug-resistant strains due to genetic mutations (Hardy, 1992). Research is currently being conducted on a vaccine to prevent HSV-1 and HSV-2 infections, however, one has not yet been approved by the FDA (Awasthi & Friedman, 2022). This study aimed to analyze an alternative treatment for HSV-2 that would be able to inhibit viral infection and mitigate cellular damage.

Due to its antioxidant levels and the presence of the known antiviral component allicin, black garlic has the potential to be an effective treatment against viruses (Choi et al., 2014; Mösbauer et al., 2021). The cytotoxic assays performed demonstrated that concentrations of the extract at about 17% incited A549 cell damage (Figs. 6 & 7). It is vital that the substance used as a therapeutic is not harmful to the cells themselves. While studies have shown ethanol to have a cytotoxic effect at concentrations of 20%, the amount of black garlic extract used in this study for each treatment (Fig. 6-13) maintained low ethanol concentrations to prevent cell damage (Calderón-Montaña et al., 2018). The extract utilized contained 333mg of black garlic material per 1mL of extract. The solvent portion of the water-based solution is composed of 60% glycerin and 40% water, while the ethanol-based solution is made up of 48% alcohol, 47% water, and 5% glycerin (Herbal Terra LLC). Ethanol, along with methanol and dimethyl sulfoxide, is a commonly used treatment vehicle for *in vitro* studies (Nguyen et al., 2020).

Treatments for HSV-2 are either prescribed episodically or suppressively (Corey et al., 2007). Analyzing the black garlic extract for its most effective mode of application would

determine how to prescribe it as an antiviral. The antiviral tests performed assessed if the extract would protect the cell from infection during the cell-treated assays or alter the ability of the HSV-2 particles to infect the host cells in the virus-treated assays. Both low and high percentages of ethanol-BGE were more effective at increasing cell viability in the cell-treated antiviral assays as compared to the virus-treated assays (Figs. 9-11). There was no significant difference in the viability of the treated and untreated HSV-2 virions. The extract was ineffective at damaging the virus as seen by the percent cell viability between the untreated and treated HSV-2 infected A549 cells (Fig. 10). However, for the cell treated antiviral assays, there were significant differences between the 5% - 17% concentrations and the untreated HSV-2 infected A549 cells (Fig. 11A). The percent of inhibition in Figure 11B demonstrates that the ethanol-BGE treatment of cultured A549 cells inhibited HSV-2 infection.

Black garlic is well known for its sweet taste as well as its high antioxidant levels. The ROS-Glo Antiviral Assay was used to determine if the antioxidants present in the black garlic extract could reduce the level of oxidative stress. According to the results of the ROS-Glo assay, the RLU of the virus-treated ethanol group resulted in a significant difference when compared to the RLU of the HSV-2 only control (Fig. 13). However, the previous assays reported that the ethanol groups did not present a significant difference when compared to the results of the HSV-2 only control group (Figs. 9-11). The 13% concentration of ethanol-BGE treatment resulted in the lowest level of reactive oxygen species post-HSV-2 infection (Fig. 13). These results were true for both the cell-treated and virus-treated 13% ethanol-BGE assay, despite the previous reported failure to inhibit treated-virus particles from infecting the cells (Fig. 10). While the black garlic may not be potent enough to completely prevent infection, it could aid the cell by maintaining homeostatic conditions and reducing the oxidative stress caused by HSV-2. The

virus-treated methods, however, did indicate a reduction in GFP expression as seen in Figure 12. The 13% and 17% ethanol-BGE concentrations contained less GFP fluorescence, and therefore, decreased levels of HSV-2.

Due to the conflicting evidence provided by the cell-treated and virus-treated assays, it is difficult to determine the ideal dosage and method of application. While the results do hint at some potential viral inhibition, there is not enough data presented by this study to confirm that the ethanol or water-based black garlic extract is an adequate antiviral for herpes simplex virus-2. To increase the efficacy of the extract, it would be worthwhile to perform synergistic studies combining the BGE with existing antivirals. Since drug-resistance is on the rise for HSV-2, it is important to continuously develop novel treatments (Hardy, 1992). One study performed by Almehmady and Ali (2021) assessed a nano-emulsion composed of garlic oil and acyclovir, amongst other types of essential oils like tea tree, peppermint and thyme. The garlic oil was found to increase the bioavailability of the acyclovir nearly three times (Almehmady & Ali, 2021). A combination therapeutic, like the garlic oil-acyclovir emulsion, could result in a decreased dosage and delay antiviral resistance.

Antioxidants like phenolic acids eliminate reactive oxygen species through radical scavenging or by promoting the production of enzymatic antioxidants (Ahmad & Zeb, 2019). The high levels of antioxidants in the black garlic must be able to enter the cell to prevent oxidative stress from occurring. Current technology is improving the delivery of organic compounds into cells by using nano-based drug delivery systems (NDDSs) (Meléndez-Villanueva et al., 2019). Meléndez-Villanueva et al. (2019) synthesized gold nanoparticles that were reduced with garlic extract and performed antiviral assays for measles with the NDDSs. The results indicated a decrease in viral load in cells treated with the garlic extract-prepared

nanoparticles by 84% (Meléndez-Villanueva et al., 2019). Based on the TEM images of the treated measles virus showing the virion surrounded by NDDSs, the researchers hypothesized that the nanoparticle would bind to the receptors on the viral envelope and block the virus from contacting the cell receptors. While the measles virus is a RNA virus, *Paramyxoviridae* family, both measles and HSV-2 are enveloped viruses, and it would be worthwhile to explore this technology using black garlic extract and HSV-2.

The process that is used to create black garlic affects the chemical composition of it as well. Chang and Jang (2021) determined that the fermentation time and the temperature used affect the antioxidant levels of black garlic. Therefore, it is necessary to determine the ideal conditions to produce antioxidant-rich BG and ideally improve ROS-mitigation. Black garlic is commercially available in pill-form, powdered, bulbs, cloves, and pureed. It is possible a different type of black garlic could be more effective as an antiviral than the water and ethanol-based extracts that were tested in this study.

Conclusion

The current study determines that black garlic extract has potential antiviral properties. The data reported here suggest that treatments with 11% - 13% concentrations of the ethanol-based black garlic extract can inhibit viral infection and alleviate the subsequent oxidative stress. While black garlic is high in antioxidants, and the compound allicin has shown to be an effective antiviral, there were still some signs of cytopathic effects post-HSV-2 infection of treated-A549 cells. However, the percentage of inhibition found, the reduction in ROS levels, and the decrease in fluorescence indicating a lower presence of virions, suggests black garlic can inhibit HSV-2 infection. Future studies should analyze combination therapies, novel delivery systems, and other forms of black garlic to improve its efficacy as an antiviral.

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