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

Jenna R. Horowitz, Lee H. Lee, Sandra D. Adams*

Department of Biology, Montclair State University, Montclair, NJ, USA
Email: *adamssa@montclair.edu

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Abstract

Allicin, an antioxidant, is known for providing garlic with its unique fragrance and taste, as well as for its antimicrobial properties. Black garlic, a fermented form of garlic, contains higher levels of antioxidants than fresh garlic. Antioxidants play a vital role in alleviating cellular stress during viral infections. Viral infections result in 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™ H_2O_2 assays were then completed to measure the post-infection ROS levels of BGE-treated virus and cells. The results thus far suggest that BGE may inhibit viral infection and decrease levels of oxidative stress.

Keywords

Black Garlic, Herpes Simplex Virus-2, Antioxidant, Antiviral, Allicin

1. 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 [1] [2]. This family also includes Herpes simplex virus-1, Varicella-zoster virus/Human herpes virus (HHV)-3, Cytomegalovirus/HHV-5, Epstein-Barr virus/HHV-4, and Kaposi's sarcoma herpesvirus/HHV-8. 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 [3]. 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 [4]. Genetic mutations resulted in strains of HSV-2 that are resistant to specific antivirals, like acyclovir [5]. It is important to assess a variety of potential treatments because of the rising rate of antiviral resistance. Compounds with high levels of antioxidants have been reported to successfully inhibit viral infections [6]. In addition to acyclovir, there have been studies analyzing various plant compounds for their antiviral properties. Epigallocatechin gallate (EGCG) and the lipophilic modified EGCG-stearate (EGCG-S), polyphenols derived from *Camellia sinensis* found in green tea, demonstrated HSV-2 inhibition in Vero cells [7]. Cells infected with HSV-2 treated with 75 μM 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 reduced viral penetration up to 57.3%. Most antivirals that can treat HSV-1 are capable of treating HSV-2 as well. Embelin, a benzoquinone derived from the plant *Embelia ribes*, displayed antiviral effects against HSV-1 [6]. When HSV-1 was treated with 54 μM of embelin, there was 100% viral inhibition in Vero cells. One common characteristic of EGCG and embelin is that they both display antioxidant properties [8] [9].

Garlic, for example, has been used as an antimicrobial for many years due to the organosulfur compound, allicin [10]. Black garlic, a fermented form of garlic, contains an increased level of antioxidants, compared to raw garlic [11]. Antioxidants can mitigate the oxidative stress caused by viral infections [12]. 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.

Antioxidants are compounds that can neutralize free radicals that could be caused by environmental factors like pollutants or pathogens [12]. Free radicals are a form of reactive oxygen species (ROS) that include oxygen groups like superoxide and hydroxyl [13]. 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 [14]. DNA damage, cellular apoptosis, and disease progression can occur if the body experiences extensive oxidative stress [14].

Viral infections are one cause of oxidative stress due to excessive ROS levels. 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 [15]. 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 [16]. Levels of the reactive oxygen species H_2O_2 were found to be significantly lower in Vero cells infected with embelin-treated HSV-1 [6].

Allium sativum, garlic, has been widely used as a medicinal treatment for thousands of years. Allicin, the primary organosulfur compound in garlic, is responsible for the distinctive taste and smell that the herb is known for [17]. Research has provided evidence that allicin possesses antibacterial properties

through the inhibition of bacterial growth of *S. aureus* [18]. The antimicrobial effects of allicin are not limited to 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 decreased by up to 70% [10]. For DNA viruses, like herpes virus, the garlic may block cell entry, prevent integration, and stop replication [19]. 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 [20]. At 36 μg of allicin, the hydroxyl radical byproducts and decreased by 94%.

Black garlic shows promise as an antiviral treatment due to its high levels of antioxidants. Black garlic is produced when fresh, raw garlic, is exposed to high temperatures and high humidity for an extended period [11]. This causes the once white, sharp garlic cloves to turn black and have a sweeter taste because 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 break down into various antioxidant compounds such as *S*-allylcysteine, tetrahydro-*β*-carbolines, alkaloids, and flavonoids [11]. 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 (**Figure 1**) [11].

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 [21]. 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 [19]. Garlic extract has displayed antiviral properties against herpes simplex virus-2, however, black garlic extract has not yet been tested explicitly [22]. This study will assess black garlic extract for its ability to inhibit HSV-2 infection in A549 lung cells.

Figure 1. Black garlic fermentation process [11].

2. Materials and Methods

2.1. Cell Culture Maintenance

A549 lung fibroblast cells were cultured in T25 flasks with F12-K media (Amer-

ican 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. Cells were subcultured every 7 - 10 days. Seeded flasks were placed in a 37˚C incubator at 5% CO_2.

2.2. 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 100 µL 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 5 mL of fresh F12-K media was added. Flasks were observed using an inverted microscope for evidence of cytopathic effects (CPE). CPE was indicated by rounding and lifting of cells from the monolayer. 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.

2.3. Black Garlic Extract Preparation

Solutions for treating the cells or virions were first prepared with 1 mL of F12-K media. This approach followed the method described in previous studies to dilute the extract to non-cytotoxic concentrations [6] [7]. The water-based extract and ethanol-based extracts (HerbalTerra LLC) had a concentration of 3 parts black garlic to 1 part solvent, resulting in a 333 mg/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 solute. The extracts were stored at 4˚C.

2.4. Alterations of Cell Morphology

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% CO_2 for one hour. The solutions were then removed and 3 mL of fresh F12-K media was added. After a 48-hour incubation period, cells were observed for morphological changes. Morphological changes would include rounding, lifting, and changes in membrane permeability as evidenced by swelling or shrinking of cells. Cells were examined, using an inverted microscope, at 400× magnification to observe cytopathic effect in the cells.

2.5. Cell Proliferation

The CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS) kit (Cat#G3580, Promega Corp., Madison, WI) was used to quantify cell viability. A

96-well plate was prepared from a confluent T-25 flask. 100 µL of solutions at 5% - 20% BGE were added to five replicates for each treatment concentration. Five replicates of A549 cells were treated with 100 µL of F12-K media only as a control. The plate was incubated for one hour at which time the solutions were removed and 100 µL fresh F12-K media was added. After 48 hours, 20 µL of MTS reagent was added to each well for one hour of incubation at 37˚C, 5% CO_2. The MTS reagent allows for quantification of viable cells as it contains tetrazolium salt. Through cellular respiration, the tetrazolium salt in the MTS reagent is reduced to formazan, a colored product whose absorbance can be measured at 490 nm in a Tecan microplate reader. The level of absorbance is proportional to the number of viable cells in the well. The absorbance levels were normalized (min/max values) to the A549 control. The average absorbance for each treatment was used to calculate the percent proliferation.

$$\text{Percent proliferation}:\% = \frac{\text{Treatment} - \text{Blank}}{\text{Cells only} - \text{Blank}} \times 100$$

2.6. MTS Cell Viability

Cell-Treated Assay

The CellTiter 96 Aqueous One Solution Cell Proliferation Assay was used to quantify cell viability. A 96-well plate was prepared, media from the 70% - 80% confluent cells was aspirated and 100 µL of the BGE (5% - 20%) were added into their respective wells. Concentrations of 5% - 20% BGE were selected based on results of previous assays. Concentrations lower than 5% were not successful at inhibiting viral infection (reported in **Figure 5**), while concentrations higher than 20% were potentially cytotoxic (reported in **Figure 2**). Five replicates of cells were treated with only fresh F12-K media. An additional five replicates were treated with an ethanol solution prepared by adding 22.5 µL of ethanol to 1 mL of F12-K media. The plate was incubated at 37˚C, 5% CO_2 for 30 minutes. To prepare one solution for each column of cells (set of replicates), 100 µL of HSV-2 virions was added to 1 mL of F12-K media. This step was repeated ten times. After the 30-minute incubation period, the BGE solutions were removed from the cells, and 100 µL of the HSV-2 virion solution was put into each well. Five replicates received the untreated HSV-2 solution. The plate was then incubated at 37˚C and 5% CO_2 for one hour. After the incubation period, the solution was removed once again and 100 µL 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 (as per manufacturer's instructions), 20 µL of MTS One Solution was added to cells. The plate was then put into the microplate reader and read for optical density at 490 nm followed by calculation of percent of viability.

$$\text{Percent viability}:\% = \frac{\text{Treatment} - \text{Blank}}{\text{Cells only} - \text{Blank}} \times 100$$

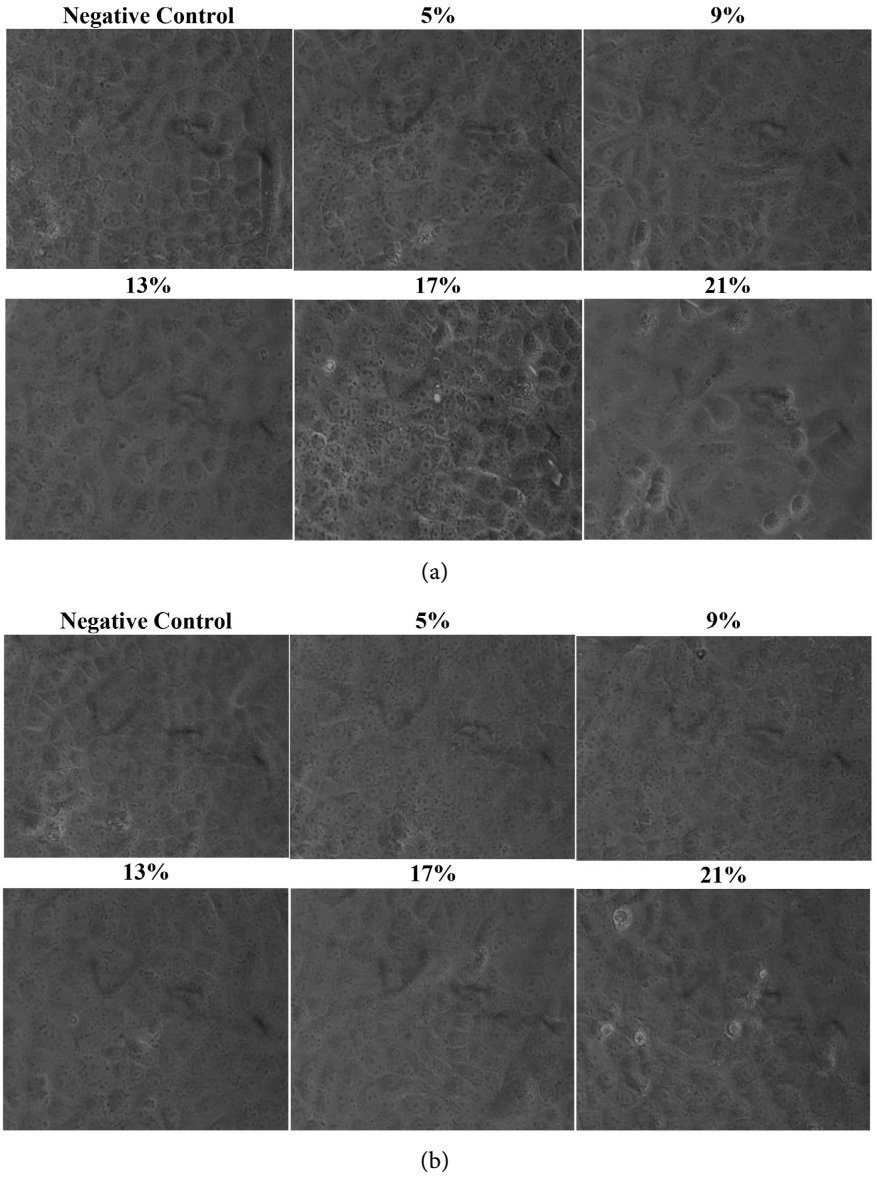


Figure 2. Cytotoxicity Assay. Images at 400× utilizing (a) water-based and (b) ethanol-based black garlic extract 48 hours post-treatment.

Virus-Treated Assay

BGE solutions were prepared at their respective volumes (5% - 20%) and 100 µL of HSV-2 lysate was placed into each tube. Three additional tubes for controls were prepared containing F12-K media only (negative control), HSV-2 in F12-K media (positive control), and HSV-2 in the ethanol-media solution (vehicle), respectively. The solutions were left at room temperature for 30 minutes. The media from the plate was aspirated and 100 µL 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% CO_2. The solutions were removed, and fresh F12-K media was added. After a 48-hour incubation period, 20 µL 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 490 nm to calculate the percent of viability.

$$\text{Percent viability}:\% = \frac{\text{Treatment} - \text{Blank}}{\text{Cells only} - \text{Blank}} \times 100$$

2.7. Inverted Microscopic Observation

A six-well plate was prepared, along with BGE solutions, for cell-treated and virus-treated cell viability assays. Cells were examined, using an inverted microscope at 400× magnification for qualitative analysis of cytopathic effects.

2.8. Antiviral Ros-Glo™ H_2O_2 Assay

Antiviral assays were performed 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, 80 µL of fresh F12-K media was added. 30 hours post-infection, 20 µL of H_2O_2 substrate solution was added to each well. The H_2O_2 Substrate Solution was prepared for 50 wells by adding 12.5 µL of H_2O_2 substrate to 1.0 mL of H_2O_2 Substrate Dilution Buffer. The plate was then incubated for an additional six hours at 37˚C and 5% CO_2. At 36 hours post-infection, 100 µL of ROS-Glo Detection Solution was added to cells in each well. The solution was prepared for 50 wells with 5 mL of Reconstituted Luciferin Detection Reagent, 50 µL of D-Cysteine, and 50 µL 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).

2.9. 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 1× PBS. 300 µL of 300 nM 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 200× using epifluorescence.

2.10. Statistical Analysis

Percent proliferation, viability (reported in 2.6), and inhibition were calculated in Microsoft Excel.

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

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.

3. Results

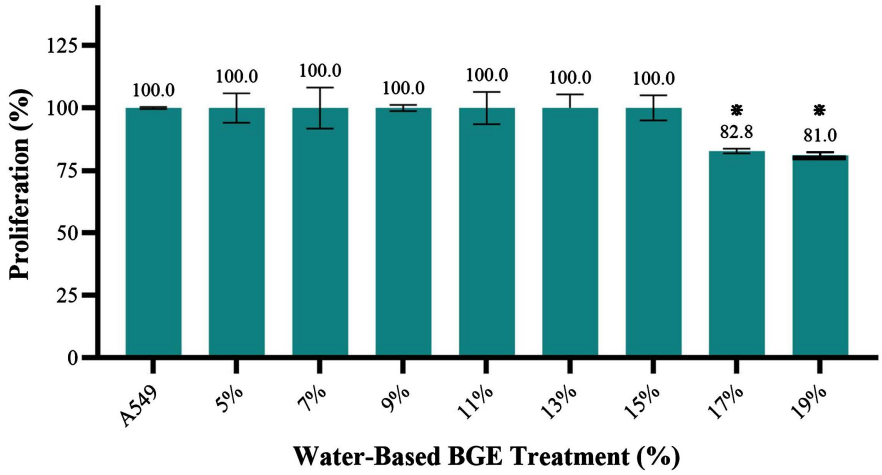
3.1. A549 Cells Do Not Exhibit Cytotoxic Effects (CTE) When Treated BGE Solutions Less Than 21%

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 2(a)**). 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 2(b)**). Cytotoxic effects were not observed until the 21% treatment as there were more rounded cells in the culture.

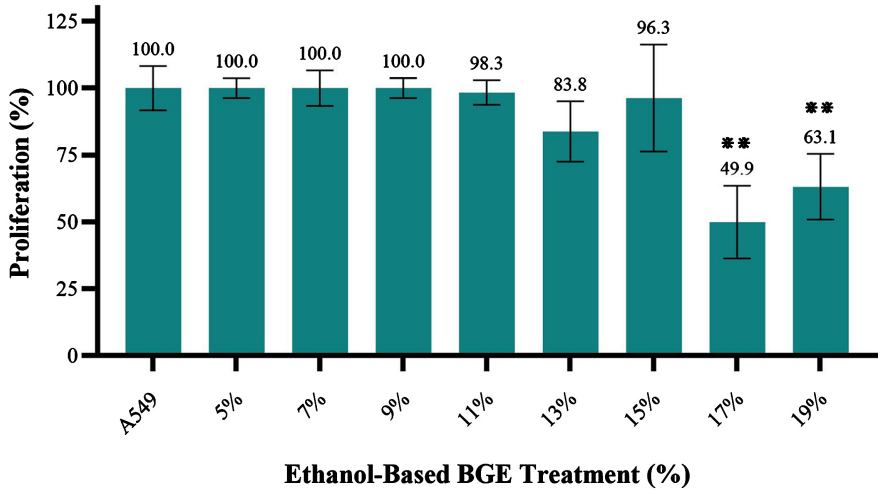
The qualitative results provided in **Figure 2** were followed by an MTS assay to calculate the percent of proliferating cells present for each treatment. Cells that are no longer proliferating, and therefore not viable, will not be able to convert the tetrazolium salt in the MTS solution into formazan and produce a lower absorbance value during the assay. Cells treated with concentrations of 5% - 15% water-BGE displayed 100% of cell proliferation (**Figure 3(a)**). 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% 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 3(b)**). The results of the MTS completed with the ethanol-BGE support the cytotoxic levels found in **Figure 2(b)**, both demonstrating CTE beginning at concentrations of about 17%.

3.2. 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



(a)



(b)

Figure 3. 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%. * $p < 0.05$, ** $p < 0.01$.

infection to determine if treatment protected the cell from the virus. The virus-treated assay determined 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 4(a)**). 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 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 4(a), the cells maintain a rounded conformation, more closely resembling the positive HSV-2 control group. The number of lifted cells appears 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 4(b)). 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.

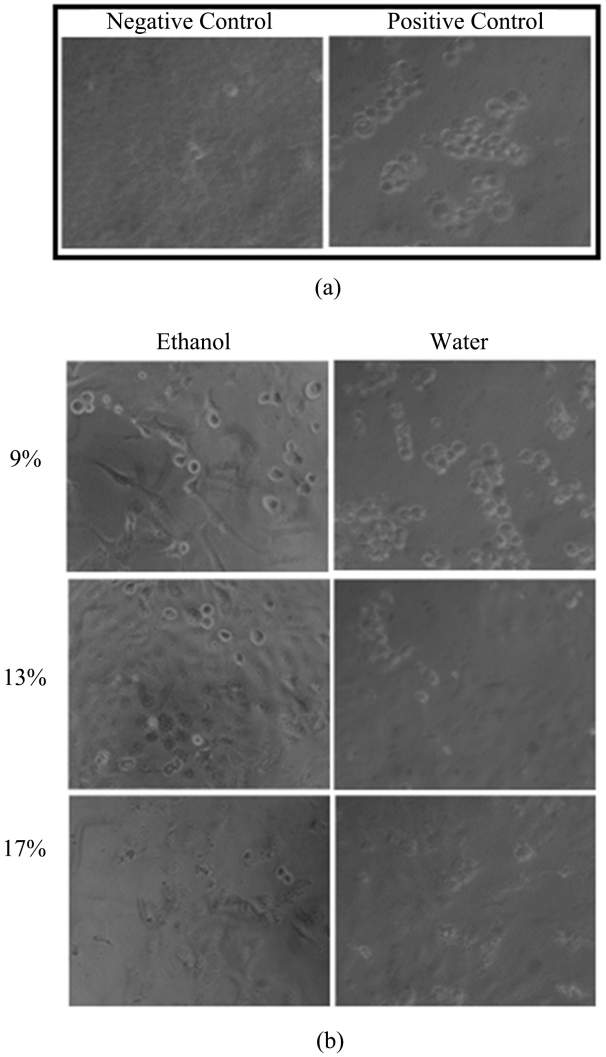
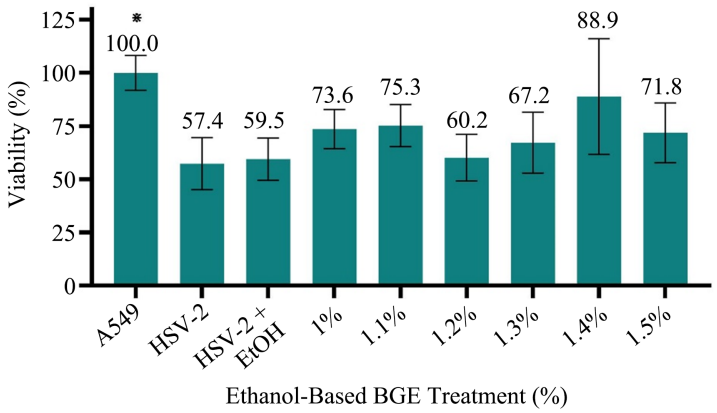


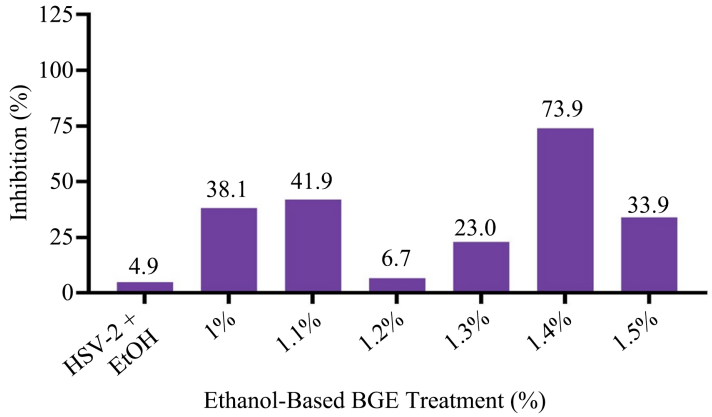
Figure 4. Cytopathic Assay. Images taken at 400× using ethanol-based or water-based black garlic extract in (a) Cell-Treated and (b) Virus-Treated assays.

3.3. 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 4). The cytotoxic results displayed in Figure 2 and Figure 3 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 appears 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 5(a)).



(a)



(b)

Figure 5. 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.

While the percentage of viable cells in each treatment group appears to be relatively equal based on the results of Figure 5(a), the percentage of inhibition suggests that the virus was prevented from infecting the cells. The absorbances from the MTS antiviral assay in Figure 5(a) were used to calculate the percent inhibition. The 1.4% treatment solution, that had the greatest percent of viability in Figure 5(a), also has the highest percentage of inhibition at 73.9% (Figure 5(b)). 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.

The treatment volumes that were administered in Figure 5 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 was approximately equal to the viability percentage of the HSV-2 control at 26.2% (Figure 6). 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 percentage of inhibition was not calculated from this antiviral assay because the treatment values and the virus-control were too similar to indicate inhibition.

The cell-treated antiviral assay displayed in Figure 5 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 6). 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 percentage 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 7(a)). It should be noted that as

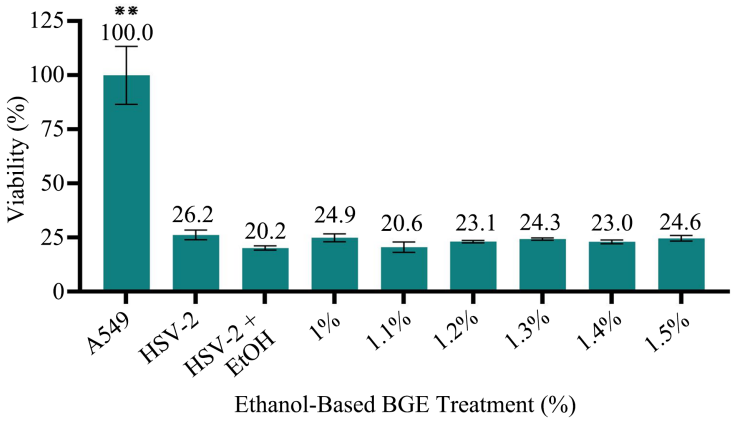


Figure 6. Virus-Treated MTS Antiviral Assay. The percent viability of cells infected with HSV-2 treated with low volumes (1% - 1.5%) of ethanol-based black garlic extract. ** $p < 0.01$ when compared to the HSV-2 only control.

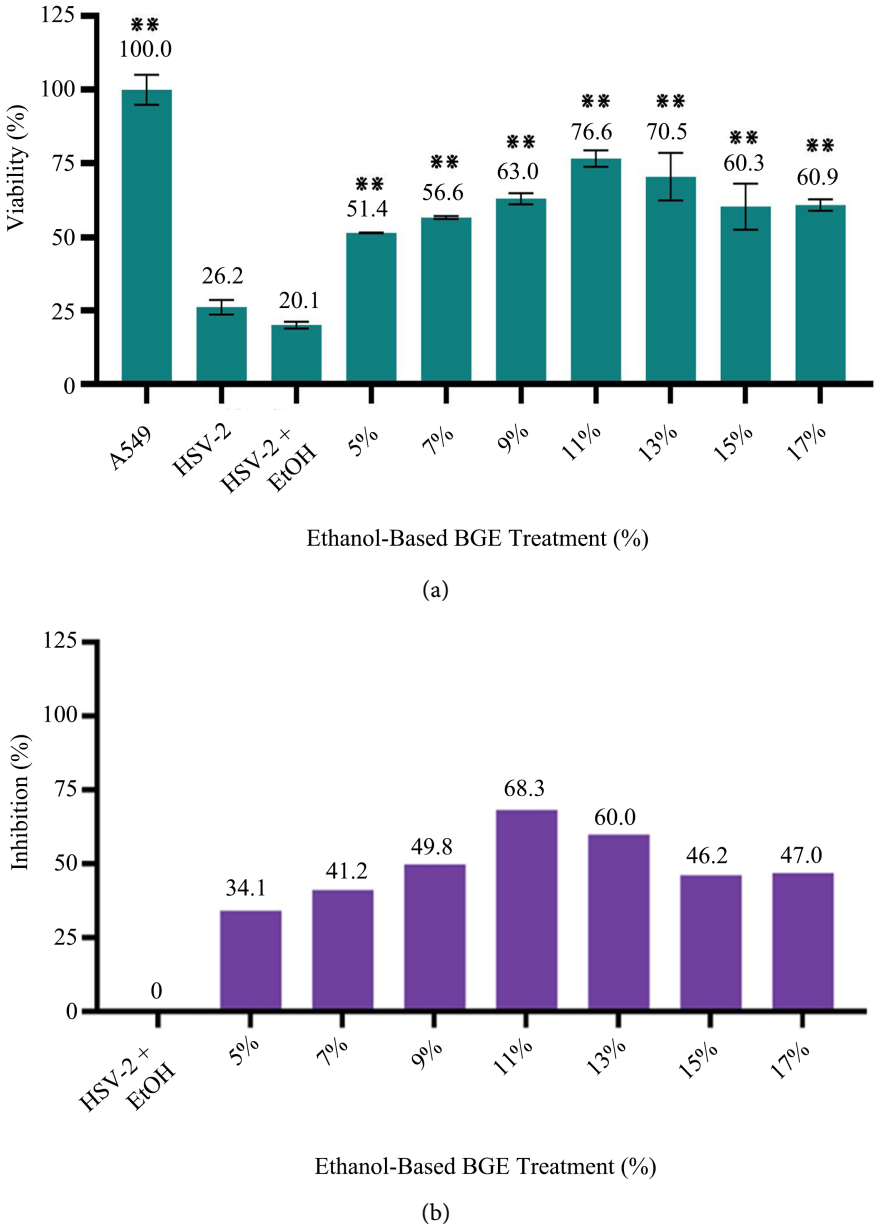


Figure 7. Cell-Treated MTS Antiviral Assay. (a) percent viability and (b) percent inhibition of cells treated with high volumes (5% - 17%) of ethanol-based black garlic extract. ** $p < 0.01$ when compared to the HSV-2 only control.

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 7(a)** 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 7(b)**). 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.

3.4. Cell Morphology Is Affected by HSV-2 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 8**). The 13% and 17% virus-treated groups both had relatively low levels of fluorescence, like 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 appears 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 8**). 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.

3.5. 13% Ethanol-BGE Solutions Alleviated Oxidative Stress in Cell-Treated and Virus-Treated Cells

The ROS-Glo $H_2O_2^{TM}$ 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 9**). 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.

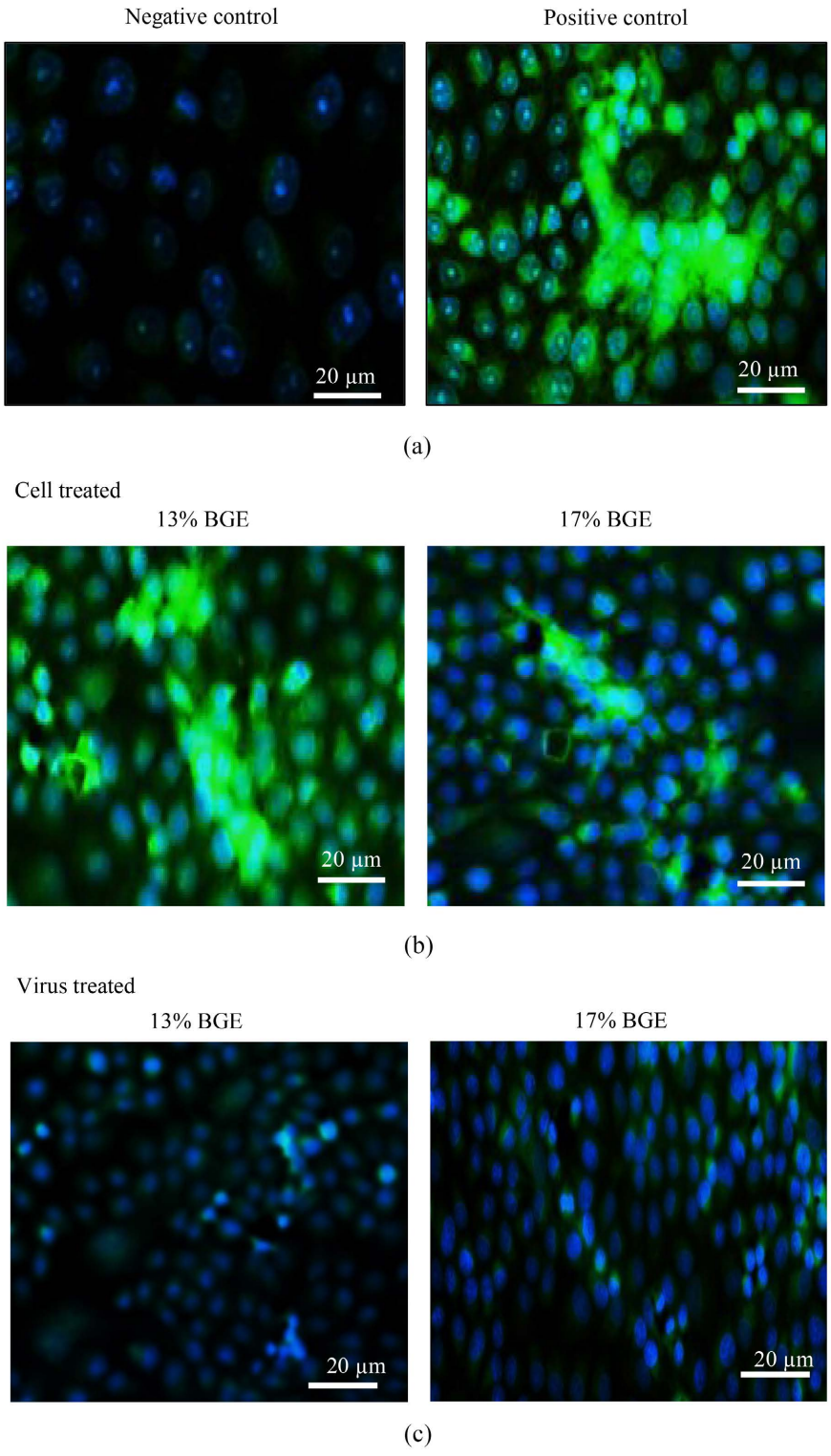


Figure 8. Fluorescent Microscopy. Images taken at 200× of DAPI-stained cells infected with HSV-2 tagged with GFP and ethanol-based black garlic extract. Red boxes indicate regions of high GFP, yellow boxes indicate regions of low GFP. (a) Fluorescent images of uninfected and HSV-2 infected A549 cells; (b) Images of A549 cells treated with 13 and 17% BGE, respectively, before infection with HSV-2; (c) Images of A549 cells infected with 13% and 17% BGE infected virions, respectively.

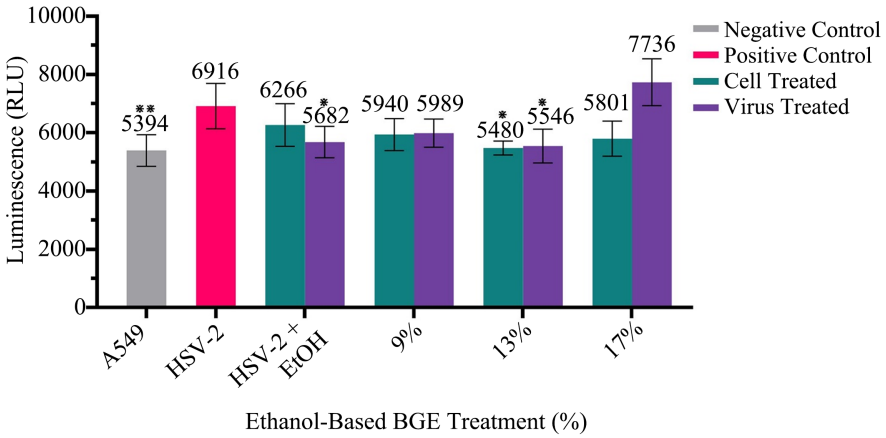


Figure 9. ROS-Glo H_2O_2™ Antiviral Assay. Oxidative stress measured in RLU for cell and virus treated assays. * $p < 0.05$, ** $p < 0.01$ when compared to the HSV-2 only control.

4. 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 [23]. 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 [5]. 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 [24]. 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 [10] [11]. The cytotoxic assays performed demonstrated that concentrations of the extract at about 17% incited A549 cell damage (**Figure 2** & **Figure 3**). 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 (**Figures 2-9**) maintained low ethanol concentrations to prevent cell damage [25]. The extract utilized contained 333 mg of black garlic material per 1 mL 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 [26].

Treatments for HSV-2 are either prescribed episodically or suppressively [27]. 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 (**Figures 5-9**). There was no significant difference in the viability of the treated and untreated HSV-2 virions (**Figure 6**). However, for the cell treated antiviral assays, there were significant differences between the 5% - 17% concentrations and the untreated HSV-2 infected A549 cells (**Figure 7(a)**). The percent of inhibition in **Figure 7(b)** 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 (**Figure 9**). 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 (**Figures 5-7(b)**). The 13% concentration of ethanol-BGE treatment resulted in the lowest level of reactive oxygen species post-HSV-2 infection (**Figure 9**). These results were true for both the cell-treated and virus-treated 13% ethanol-BGE assay, despite the previously reported failure to inhibit treated-virus particles from infecting the cells (**Figure 6**). 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 results of this study suggest potential viral inhibition. To increase the efficacy of the extract, it would be worthwhile to perform synergistic studies combining the BGE with existing antivirals. Due to drug-resistance for HSV-2, it is important to continuously develop novel treatments [5]. 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 [28]. 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 [29]. 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) [30]. 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% [30]. 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 re-

ceptors. While the measles virus is an 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, powdered, bulbs, cloves, and pureed forms. 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.

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Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

JRH, LHL, and SDA designed the study. SDA supervised JRH in the laboratory. JRH conducted the experiments and performed all statistical analyses. JRH, LHL, and SDA drafted the manuscript. All authors read and approved the final manuscript.

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