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Effects of Myrrh on HSV-1 Using Plaque Assay

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EFFECTS OF MYRRH ON HSV-1 USING PLAQUE ASSAY

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science.

By

BADRIEAH ALAMRI

B.S., University of Tabuk, 2009

2017

Wright State University

WRIGHT STATE UNIVERSITY
GRADUATE SCHOOL

Dec 05, 2016

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ABSTRACT

Alamri, Badrieah. M.S. Microbiology and Immunology Graduate Program, Wright State University, 2017. Effects of Myrrh on HSV Type 1 Using Plaque Assay.

Herpes simplex virus type 1 (HSV-1) is a highly infective human pathogen which infects a wide range of population in North America and worldwide. HSV-1 infection has two phases, lytic and latent. Recurrence of HSV-1 is a major challenge to clinicians to control the infection especially in immune depleted individuals. Acyclovir (ACV) is an antiviral drug used to treat HSV-1 infection. Low solubility of ACV in water, mutation of viral thymidine kinase, and mutation of viral DNA polymerase are major problems that cause usage limitations of ACV. Myrrh has been used as an analgesic and anti-inflammatory natural product in middle eastern countries for centuries. Recently Myrrh has shown a promising therapeutic action against fungal and parasitic infections. In the current study, low concentrations of Myrrh treatment increased cell survival of HSV-1 infected Vero cells. Prolonged exposure to Myrrh was found to be toxic to viable cells. Myrrh treatment of HSV-1 infected Vero cells was compared with ACV treatment of HSV-1 infected Vero cell. Myrrh treatment increased cell survival of infected Vero cells, similar to the effect of ACV treatment. These results provide evidence that Myrrh exerts antiviral effects against herpes virus infection.

Hypothesis

Myrrh (Extract) will protect Vero cells from the lytic effect of HSV-1 as determined by increased cell survival of Myrrh-treated HSV-1 infected Vero cells compared to untreated Vero infected cells.

Specific Aims:

In the present study,

- The effect of Myrrh extract on cell survival of Herpes Simplex Virus Type-1 infected Vero cell was evaluated and compared with cell survival with Acyclovir treatment.
- The most effective concentration for Myrrh extract as inhibitor of HSV-1 replication was determined using plaque assay.

Table of Contents

Introduction.....	1
Literature Review	4
Herpes Simplex Virus-1	4
Life Cycle of the Virus	6
Entry of the Host Cell	6
Replication of HSV-1	7
Latency Phase	9
ACV	11
ACV Limitations and Resistance	12
Myrrh	14
Materials and Methods.....	17
Plaque Assay	17
Vero Cells	17
Herpes Simplex Virus-1.....	18
Acyclovir.....	18
ACV Treatment before Infection.....	18
ACV Treatment after Infection.....	18
Myrrh Extract.....	19
Myrrh Treatment before Infection	19
Myrrh Treatment after Infection	19
Cell Viability.....	19
Statistical Significance.....	20

Results	21
References	38

List of Figures

Figure 1: Structure of Herpes Simplex Virus.	4
Figure 2: Entry to the host cell.....	6
Figure 3: Resemblance between Acyclovir and Guanosine chemical structure.	13
Figure 4: Mechanism of ACV.....	13
Figure 5: Pictures for Myrrh Tree from South Region of Saudi Arabia.....	14
Figure 6: Toxicity of Myrrh on Vero cells.....	24
Figure 7: Effect of different concentrations of Myrrh treatment 2 hours before HSV-1 infection.....	25
Figure 8: The number of plaques formation after two-hours of Myrrh treatment prior to HSV-1 infection.....	26
Figure 9: Comparison of Myrrh added 2 hours after HSV-1 infection	27
Figure 10: Comparison of HSV-1 cytopathic effects of Vero cells treated with Myrrh 4 hours before infection.....	28
Figure 11: Comparison of HSV-1 cytopathic effects of Vero cells treated with Myrrh 4 hours following infection	29
Figure 12: Comparison of percent cell viability between 2 and 4 hours of Myrrh pretreatment.....	30
Figure 13: Comparison of cell survival between treatment with Myrrh or ACV of HSV-1 infected cells (2 Hours of treatment before infection).....	31
Figure 14: Comparison of cell survival between Myrrh or ACV treatments of HSV-1 infected	

cells (2 Hours of treatment after infection)32

Figure 15: Comparison of cell survival between Myrrh or ACV treatments of HSV-1 infected

cells (4 Hour treatment) 33

List of Abbreviations

HSV-1	Herpes Simplex Virus
ACV	Acyclovir
TK	Thymidine Kinase
gB	glycoprotein B
gC	glycoprotein C
gD	glycoprotein D
gH	glycoprotein H
gL	glycoprotein L
HS	Heparan Sulphate
HVEM	Herpes Virus Entry Mediator
VHS	Virion Host Shut Off
VP16	Virion Protein 16
EI	Early Immediate Genes
LAT	Late Gene
ER	Endoplasmic Reticulum
CNS	Central Nervous System
UV	Ultraviolet Light
MC	Methyl Cellulose
dH2O	Distilled Water
DMEM	Dulbecco's Modified Eagle's Medium
FBS	Fetal Bovine Serum
PBS	Phosphate buffered saline

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DEDICATION

This thesis is dedicated to my father, who encouraged and supported my education since I was a child. It is also dedicated to my mother, who believes in me more than I believe in myself. I would not stand here if it were not for their support. Their encouragements, love, and prayers every single day and night made it possible for me to keep up my dedication to this project. I would also like to extend my deepest gratitude to the University of Tabuk for their contributions in aiding me to complete my educational experience.

Introduction

Ninety percent of the human population has been infected by different types of herpes viruses (Kukhanova et al., 2014). HSV-1 is the most common type of herpes virus and mainly affects the epidermal cells and epithelia of the oral mucosa (Fatahzadeh and Schwartz, 2007). HSV-1 causes lesions and cold sores on the lips, skin, mouth cavity, and eyes (Whitley and Roizman, 2001; Grünewald et al., 2003). About 300,000 HSV ocular infections are recorded in the USA every year (Whitley and Roizman, 2001). HSV-1 is a large, double-stranded DNA (Manservigi et al., 2010). The HSV-1 virion's structure includes a capsid surrounding the viral DNA core and an envelope with abundant glycoprotein spikes which are critical for the attachment of the virus to the host cell. (Whitley and Roizman, 2001). Tegument proteins occupy the space between the capsid and the envelope (Fatahzadeh and Schwartz, 2007). Tegument proteins play essential roles in the virus replication cycle (Kim et al., 2012; Thompson et al., 2009).

In humans, HSV-1 has two distinct phases (Akhtar and Shukla, 2009). The first phase of infection is the lytic phase and second is the latent phase (Kim et al., 2012; Akhtar and Shukla, 2009). The virus replicates during the lytic phase, when the symptoms of infection appear (Gilden et al., 2009; Thompson et al., 2009). The latent phase of the virus follows the lytic phase, during which the virus stays inactive inside the host cell (Feldman et al., 2002). During the latent phase, the virus infiltrates the sensory neurons and stays as a dormant virus in the sensory ganglia (Maillet et al., 2006; Yao, 2014; Kukhanova et al., 2014). Reactivation of the infection can occur later in life, causing damage and inflammation to the host (Feldman et al., 2002).

Acyclovir (ACV) is an anti-viral drug which is prescribed as a treatment for HSV-1 infection (Tyring et al., 2002). ACV has a suppressive effect on HSV-1 due to its ability to inhibit virus replication (Viggosson, 2011). ACV has two mechanisms of action. ACV is activated by thymidine kinase (TK) of the virus, inhibits the DE novo synthesis of viral DNA, and terminates the growing viral DNA chain (Viggosson, 2011; Bacon et al., 2003). Failure to produce viral TK or any mutation in DNA polymerase lessens the activity of ACV (Bacon et al., 2003). Poor solubility, low oral bioavailability, and lack of absorption limit ACV usage (De Clercq and Field, 2006; Al-Subaie et al., 2015). Natural medicines have garnered increased attention recently (Shuaib et al., 2013). The low cost and higher safety of usage of natural medicines makes them a viable choice to replace many synthetic medicines that have the same effects (Shalaby and Hammoud, 2014; Shuaib et al., 2013). Myrrh is a natural product that consists of water-soluble gum, alcohol soluble resin, and about 3–8% essential oil (Ashry et al., 2010; Abdul-Ghani et al., 2009; Boffa et al., 2015; Su et al., 2011). Myrrh is one of the earliest medicines in recorded history. It is an essential element used in perfume and incense in addition to its uses for medicinal purposes (Shen et al., 2012; Guyatt, 2002). Due to its anti-inflammatory and cytotoxic effects, it has become a powerful treatment for various types of infections (Tipton et al., 2003; Shuaib et al., 2013; Langhorst, et al., 2013). Mixing Myrrh with toothpaste and mouthwash is a useful inhibitor for inflammation of oral fissures (Boffa et al., 2015; Guyatt, 2002; Tipton et al., 2003). Different species of myrrh have anti-tumor and anti-proliferative effects that have been observed by scientists investigating myrrh's effects on breast and colon cancers (Boffa et al., 2015; Abdul-Ghani et al., 2009; Shen et al., 2012; Tipton et al., 2003). Myrrh oil (MO) has been applied to cure paw swelling in mice (Shalaby and Hammoud, 2014). Reduction of skin infection, obesity, wounds, and pain are among the most promising

consequences of myrrh product application (Shuaib et al., 2013; Shalaby and Hammoud, 2014; El-Sherbiny and El Sherbiny, 2011; Nomicos, 2007).

Literature Review

Herpes Simplex Virus -1 (HSV-1)

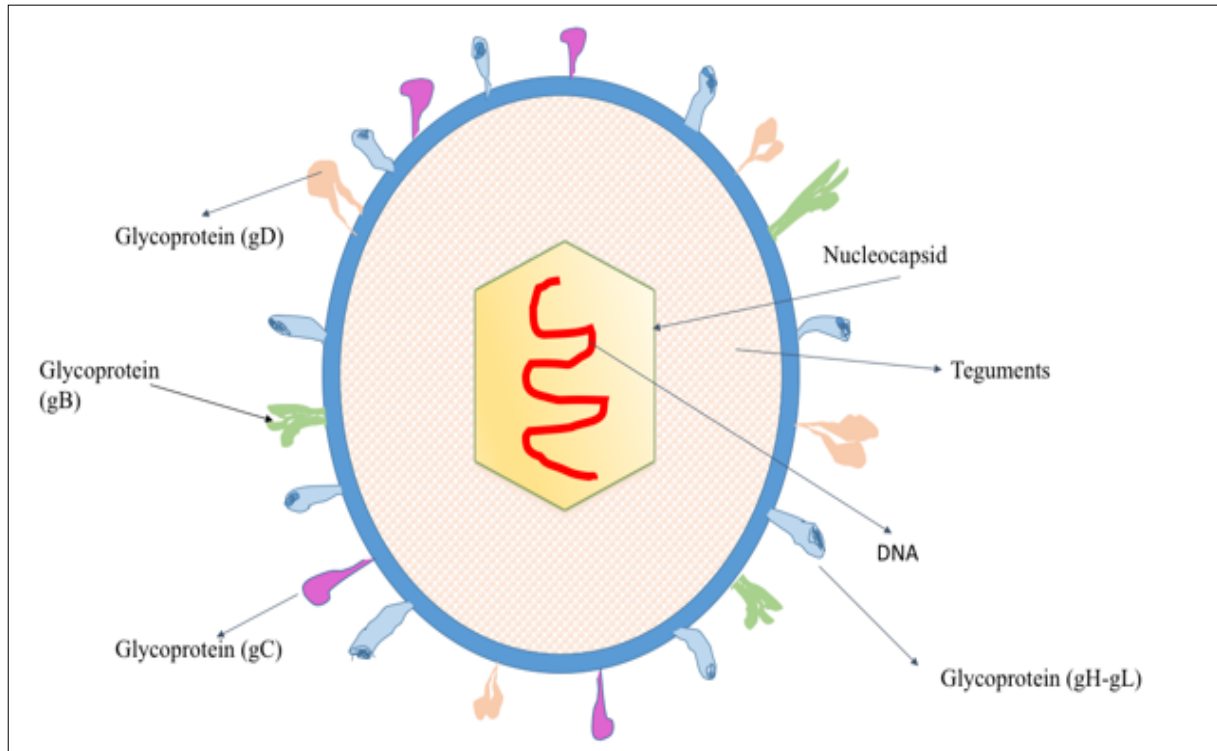


Figure 1: Structure of Herpes Simplex Virus. Double stranded DNA surrounded by nucleocapsid. Teguments fill the space between the capsid and the envelope Adapted from Manservigi et al., 2010.

Herpes simplex virus-1 (HSV-1) is an alpha herpes virus (Woo and Challacombe, 2007). It contains a large double-stranded DNA core. The virion consists of a DNA core, a capsid, and an envelope covered by spiky glycoprotein (Manservigi et al., 2010). The glycoproteins play an essential role for attachment between the virus and host cell receptor (Whitley and Roizman, 2001). Teguments fill the space between the capsid and the envelope of the virus (Fatahzadeh

and Schwartz, 2007). The virion has a unique structure that protects its genetic material from the extracellular environment. This structure allows for easier cell penetration so the genome can be efficiently released into the host cell nucleus (Figure 1) (Spear, 2004).

HSV-1 infections appear on the lips, skin, mouth, eye, and mucosal membranes (Whitley and Roizman, 2001). There are a number of different ways the virus can be distributed to an unaffected person. Direct exposures to an open sore, respiratory droplet, or body fluids are all associated with viral transmission (Fatahzadeh and Schwartz, 2007).

HSV-1 manifests itself on the gums, cheeks, or tongue, causing oral lesions and cold sores (Woo and Challacombe, 2007; Grünewald et al., 2003). The lesions are characterized by burning, itching, and painful sensations (Whitley and Roizman, 2001). Mucosal discharge is another way to disseminate repetitive HSV-1 infection (Woo and Challacombe, 2007). An HSV-1 infected mother can transmit the virus to her infant during birth, causing neonatal herpes. The virus can also be transmitted to the fetus through cross contamination (Fatahzadeh and Schwartz, 2007).

Herpetic whitlow is another type of HSV-1 lesion that occurs on the fingers or nails of medicinal specialists who handle medical equipment while caring for infected patients (Whitley and Roizman, 2001). Three hundred thousand HSV ocular infections are discovered annually in the USA (Whitley and Roizman, 2001). Scratching an open sore and then touching the eyes can disseminate the herpes to the eyes and, over time, can cause a lesion over the cornea (Woo and Challacombe, 2007).

HSV-1 can also target the trigeminal nerve. The virus migrates along the nerve to the brain and causes encephalitis. If left untreated, encephalitis causes death in more than 70% of

cases, while only 2.5% of survivors regain normal neurological activity (Whitley and Roizman, 2001)

Life Cycle of the Virus:

Entry to the host cell:

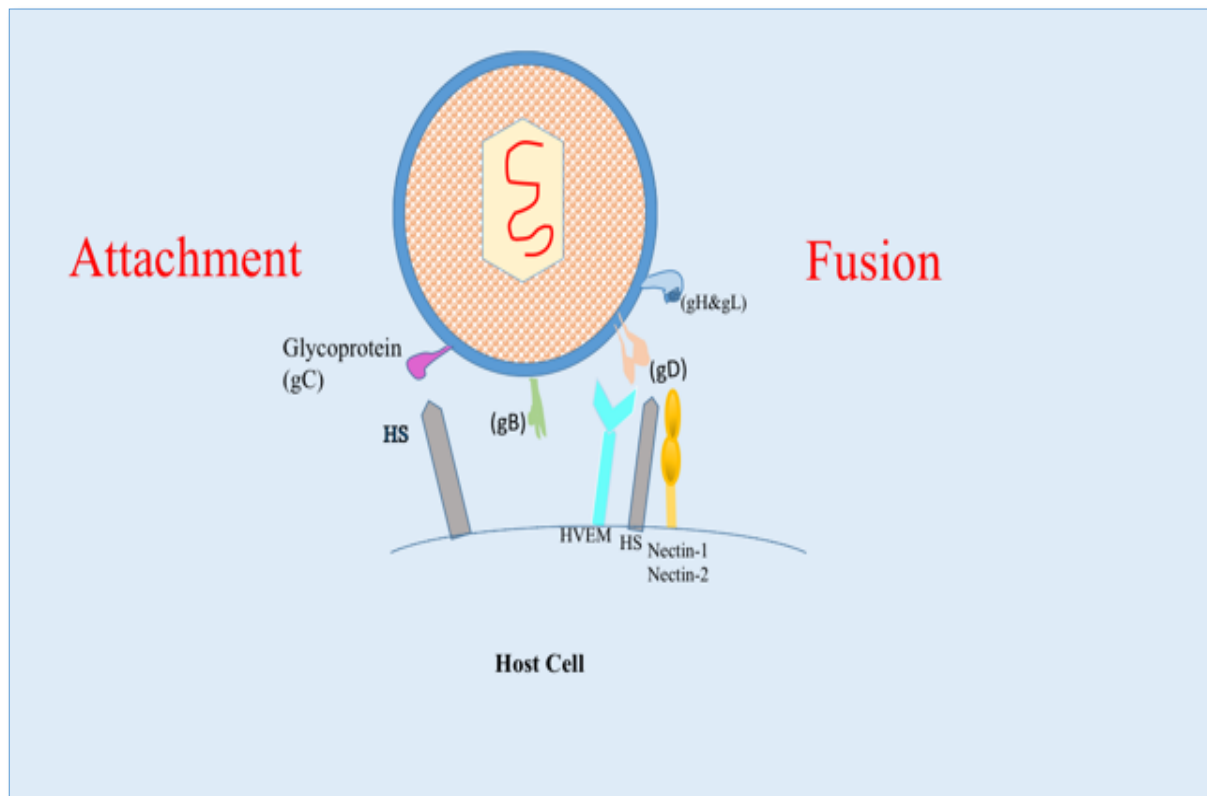


Figure 2: Entry to the host cell. Essential host cell receptors and viral glycoproteins that are required for HSV entry. The viral envelope has been embedded with several types of glycoproteins (gB, gC, gD, gH and gL) essential for the penetration. Binding of gB or gC to heparan sulphate (HS) facilitates the attachment of HSV-1 to cells, allowing for gD to interact with its receptors: HVEM, nectin-1 and nectin-2, and 3-O-sulfotransferases. Binding of gD to these receptors promotes fusion of the virus with a cell membrane. gB and gH-gL heterodimers along with gD and a gD receptor are mandatory for the fusion process. Adapted from Spear, 2004.

As previously mentioned, HSV-1 primarily enters external surface of skin or mucosa via glycoproteins on the virion's envelope interacting with the receptors found on the cell surface (Akhtar and Shukla, 2009). Glycoproteins gB, gC, gD, gH, and gL are required for HSV1 entry (Shelly et al., 2012; Akhtar and Shukla, 2009). Nectin-1 and -2, herpes virus entry mediator (HVEM), and 3-O heparan sulfate are also important receptors involved with viral entry (Akhtar and Shukla, 2009; Krummenacher et al., 2002). Heparan sulfate (HS) chains have a significant role in facilitating HSV attachment. Binding of the virus occurs when HS interacts with gC and gB (Bender et al., 2005). Nectin-1 and -2 and HVEM use gD to mediate entry of the virus (Krummenacher et al., 2003). T lymphocytes and dendritic cells tend to use HVEM receptors, while nectin-1 is used by neuronal and epithelial cells (Spear, 2004; Campadelli-Fiume et al., 2002). Interaction between gD and its receptor is a mandatory step for the fusion of the envelope with the cell membrane (Akhtar and Shukla, 2009). Furthermore, multi-glycoprotein combinations of gB, gH and gL heterodimers can carry out the fusion process (Akhtar and Shukla, 2009; Shelly et al., 2012; Spear, 2004; Bender et al., 2005).

Replication of HSV-1:

Replication of herpes simplex virus has multiple phases (Akhtar and Shukla, 2009). The first phase is called the lytic phase, while the second phase is called the latent phase. One prominent aspect of HSV1 is the ability to switch between production, replication, and latency (Kim et al., 2012). Following entry into the host cell, the virus uncoats its nucleocapsid and teguments are released into the host cell's cytoplasm (Xing et al., 2013; Akhtar and Shukla, 2009). The herpes virus capsid migrates towards the nucleus with the aid of microtubules to

deliver the viral genome to the nucleus of the host cell (Akhtar and Shukla, 2009; Padeloup et al., 2009; Nicoll et al., 2012). Within the host cell cytoplasm, a variety of tegument proteins are released (Nicoll et al., 2012; Kim et al., 2012; Thompson et al., 2009). Two of these proteins play roles in the replication cycle of a virus. One is virion host shut off (VHS) and the other is VP16 (virion protein 16) (Nicoll et al., 2012; Mossman et al., 2000). Release of these tegument proteins into the cytoplasm is a very important step in the viral replication cycle (Kim et al., 2012; Thompson et al., 2009). VHS shuts off the transcription machinery of the host cell and also blocks the translation of the host cell messages by degrading the host mRNA in the cytoplasm (Mossman et al., 2000; Weir, 2001; Dauber et al., 2014; Matis and Kudelova, 2001). Mutations in viral VHS decrease their ability to terminate host cell protein synthesis (Weir, 2001). On the other hand, VP16 passes through nuclear pores to the nucleus and helps in viral transcription by activating transcription of some early immediate genes (EI) that are required for the replication of the viral genome (Ghosh et al., 1996; Xing et al., 2013). When VP16 promotes transcription of EI genes that are used for replication, the mRNA of early genes travels out to the cytoplasm and is translated into early proteins (Xing et al., 2013; Mossman et al., 2000; Padeloup et al., 2009). The early proteins that are produced return to the nucleus to replicate viral DNA (Kim et al., 2012; Thompson et al., 2009; Mossman et al., 2000). After replication is complete, there are several varieties of transcriptions as early genes start to produce early and late genes (Mossman et al., 2000). This is the period in which lytic infection or lysogenic infection occurs (Nicoll et al., 2012). In this period, late genes start to express mRNA which is translated to generate the late structural protein. Once the structural protein and DNA (genetic material) are produced, they will form a virion (Turner and Jenkins, 1997). HSV1 makes its capsid inside the host cell's nucleus; after the capsid is complete, it bulges out from the nucleus

with the nuclear membrane surrounding it (primary envelopment) (Johnson and Baines, 2011; Mettenleiter et al., 2006). It then moves into the Endoplasm Reticulum, where viral proteins are produced (Leuzinger et al., 2005). The capsid leaves with some of these protein components which are involved in the formation of spikes (Leuzinger et al., 2005). The virion is transported to the Golgi apparatus. Inside the Golgi apparatus, the protein undergoes secondary envelopment and the glycoproteins of the virus are modified (Granzow et al., 2001; Mettenleiter, 2004). After modification, the virus is ready to leave the cell taking a part of the cell membrane as the virus is budding out (Owen et al., 2015). Once the HSV1 has its virion DNA, capsid and spiked envelope, the virus is fully mature (Johnson and Baines, 2011; Zeev-Ben-Mordehai, 2014).

Latency Phase:

The absence of viral replication and failure of lytic gene expression lead to a potential virus phase where the host cells harbor lifelong infection (Feldman et al., 2002; Ma et al., 2014; Bertke et al., 2011; Thompson et al., 2009). One of the most fundamental features of HSV-1 infection is that it can reactivate causing recurrent infections (Yao, 2014; Kukhanova et al., 2014).

After maturation, the virion progressively spreads to the neighboring cells (Nicoll et al., 2012). The virus travels through the sensory nerve until it reaches the trigeminal ganglion of the central nervous system (CNS) (Webre et al., 2012; Nicoll et al., 2012; Lee et al., 2015). The virus establishes a dormant infection in the root dorsal ganglion where the genome persists in the cell's nucleus (Maillet et al., 2006; Yao, 2014; Kukhanova et al., 2014). During the latency phase, there is no active virus generation and symptoms do not appear in the infected patient (Feldman et al., 2002; Lee et al., 2015; Al-Dujaili et al., 2011). HSV-1 can reactivate spontaneously or due to stress, UV light, or immunosuppression (Lee et al., 2015; Feldman et al.,

2002; Webre et al., 2012). As explained before, VP16 has an important role in IE genes during the lytic phase (Xing et al., 2013; Mossman et al., 2000; Padeloup et al., 2009). Therefore, VP16 is important in order to reactivate HSV-1 (Thompson et al., 2009). Thompson et al., 2009 found that VP16 is required to regulate the termination of the latent cycle, re-entering the lytic cycle. HSV-1 migrates through the neural process back to the epithelial cell where the virus starts its productive cycle again (Thompson et al., 2009; Nicoll et al., 2012). The start of the viral replication cycle in epithelial cells causes lesions in the mouth, skin, and nasal canal which illustrates the reason behind patients recurrently getting cold sores in the same location (Lee et al., 2015; Ma et al., 2014).

Acyclovir:

Acyclovir (ACV) is an anti-viral drug used to treat infection with herpes simplex virus (HSV1), varicella zoster (chickenpox), and herpes zoster (shingles) (Gopal et al., 2013; Al-Subaie et al., 2015; Viggosson, 2011). ACV has been used as a potent and reliable suppressor of herpes simplex virus (HSV1) for more than 25 years (Piret and Boivin, 2011; Tyring et al., 2002; Viggosson, 2011). ZoviraxTM is its commercial name (Viggosson, 2011). ACV should be used five times a day in order to be effective (Tyring et al., 2002). The most beneficial characteristic of ACV is that it is a safe treatment for HSV1 (De Clercq and Field, 2006; Al-Subaie et al., 2015; Tyring et al., 2002). ACV has the ability to attack virally infected cells while leaving the uninfected cells untouched (Krishnan et al., 2010; De Clercq and Field, 2006; Viggosson, 2011; Tyring et al., 2002). Thymidine kinase (TK) is a specific enzyme that exists only in the infected cell, which makes it possible to activate ACV and make it functional (Bacon et al., 2003; Viggosson, 2011; Tyring et al., 2002). Due to the similarity of the chemical structure of Acyclovir and the nucleoside guanosine (Viggosson, 2011), ACV competes with the guanosine nucleosides to inhibit viral replication (De Clercq and Field, 2006). In HSV-1 infected cells, ACV is converted to acyclovir monophosphate by viral thymidine kinase (TK) (Piret and Boivin, 2011; Viggosson, 2011; Tyring et al., 2002; De Clercq and Field, 2006). The second phosphorylation, which is mediated by guanosine monophosphate kinase, adds two more phosphates to the last acyclovir monophosphate to form acyclovir triphosphate (the active form) (Piret and Boivin, 2011; Tyring et al., 2002; Viggosson, 2011; De Clercq and Field, 2006). The active form of the drug works as an inhibitor of DNA polymerase (Piret and Boivin, 2011; Viggosson, 2011; De Clercq and Field, 2006; Krishnan et al., 2010; Bacon et al., 2003). Triphosphate ACV suppressed DNA polymerase about ten to thirty fold (Viggosson, 2011).

Furthermore, ACV is considered an obligate DNA terminator when it is incorporated into the herpes virus DNA chain (De Clercq and Field, 2006; Viggosson, 2011). ACV's active form does not have a hydroxyl group, which is normally located in position 3, thus blocking chain elongation (Piret and Boivin, 2011).

ACV limitation and resistance:

There are two common reasons for ACV resistance by HSV1. Mutation of the viral TK causes insufficiency of the ACV phosphorylation process, which prevents its therapeutic value (Tyring et al., 2002; Bacon et al., 2003). Mutation of the DNA polymerase of the virus can be another reason for a lack of ACV activity (Bacon et al., 2003). This leads to the inability to integrate acyclovir triphosphate in the DNA molecules (Tyring et al., 2002). Moreover, ACV has limitations due to its low solubility in water so it can not be used as a muscular injection or in eye drops. Lack of absorption by transdermal application is another factor restricting ACV usage (De Clercq and Field, 2006; Al-Subaie et al., 2015).

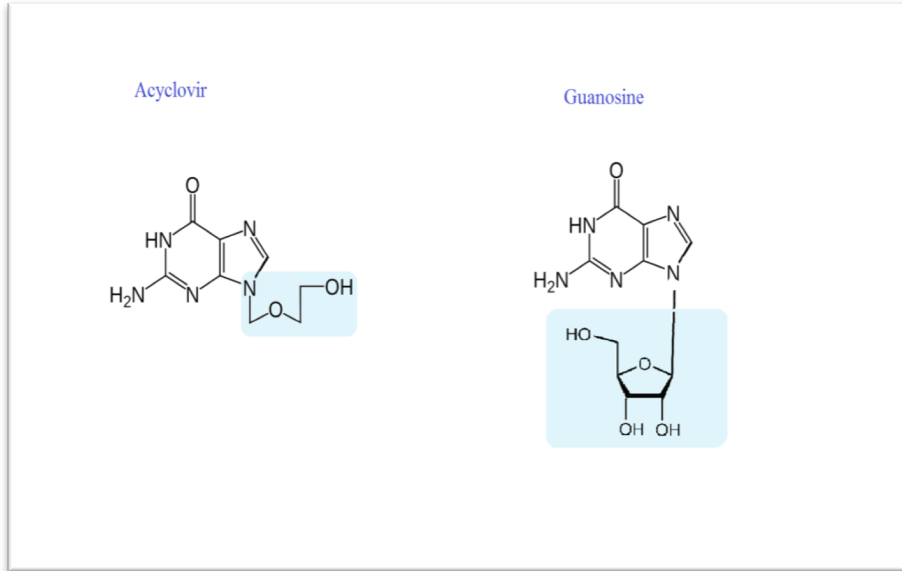


Figure 3: Resemblance between Acyclovir and Guanosine chemical structure. Adapted from Viggosson, 2011.

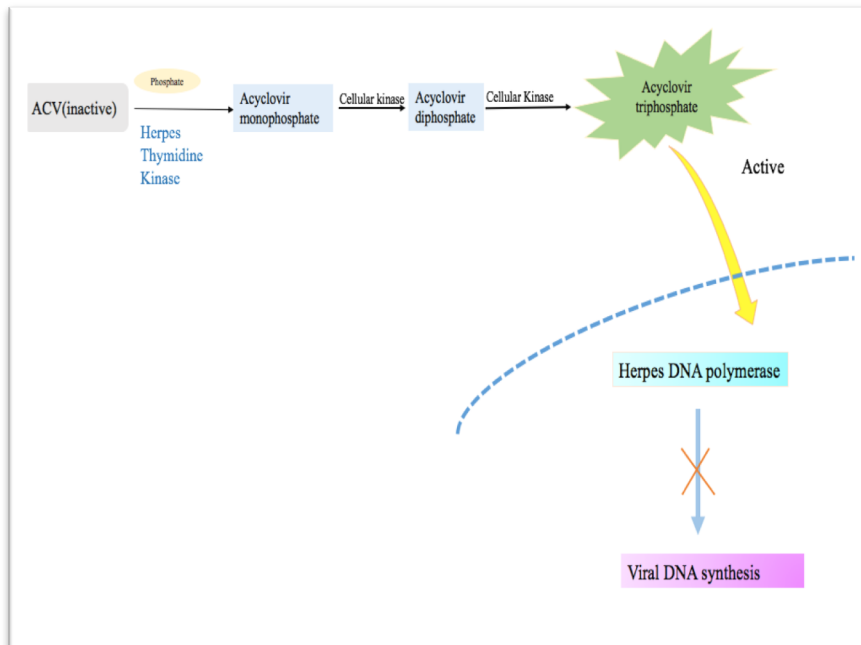


Figure 4: Mechanism of ACV. Inactive ACV is phosphorylated by HSV-1 Thymidine Kinase. Two more phosphorylations activate ACV. Acyclovir triphosphate inhibits HSV-1 replication. Adapted from Piret and Boivin, 2011.

Myrrh



Figure 5: Pictures for Myrrh Tree from South Region of Saudi Arabia.

The repetitive use of antibiotics to treat bacterial disease can cause antibiotic resistance. This has led to a search for medical herbs as an alternative medicine to avoid many different side effects. Medicinal herbs have been used around the world since ancient times (Shuaib et al., 2013). Natural medical extracts can be more viable, cheaper, and safer compared to synthetic drugs. The World Health Organization reports that 90% of the world's population relies on traditional medicine (Shalaby and Hammoud, 2014; Shuaib et al., 2013).

Myrrh has been confirmed in the USA by the Food and Drug Administration as a safe substance for use in foods and drinks and as a fragrance in beauty agents (Shalaby and Hammoud, 2014; Abdul-Ghani et al., 2009). Myrrh is a yellow resinous exudate which is harvested from the trunk of the small Myrrh tree (Su et al., 2011; Shalaby and Hammoud, 2014;

Guyatt, 2002). Many previous studies identify Myrrh as one of the oldest herbs utilized in perfumes and medicines (Abdul-Ghani et al., 2009; Boffa et al., 2015). Water-soluble gum (30–60%), alcohol soluble resin (20–40%), and essential oils (3–8%) are the main substances that come from Myrrh (Ashry et al., 2010; Abdul-Ghani et al., 2009; Boffa et al., 2015; Su et al., 2011). Myrrh has been used in perfume and incense commercially as well as medically (Shen et al., 2012; Guyatt, 2002). Due to its anti-inflammatory, cytotoxic, and bacterial activities, myrrh has been used to cure many infections (Tipton et al., 2003; Shuaib et al., 2013; Langhorst, et al., 2013). Myrrh reduces inflammation of the oral cavity (Tipton et al., 2003) and has been added to toothpaste and mouthwash for this purpose (Boffa et al., 2015; Guyatt, 2002). For more than 50 years, mixing myrrh with chamomile flowers and coffee charcoal has been a useful treatment for diarrhea (Langhorst, et al., 2013; Nomicos, 2007). According to Shalaby and Hammoud, (2014) Myrrh extract has analgesic and anti-inflammatory activity, controls weight gains and adjusts blood lipid levels in rats. In additional experiments, myrrh oil inhibited paw swelling in rats with a 100 mg/kg dose after 4 hours (Su et al., 2011). Different species of myrrh have anti-tumor and anti-cell proliferation effects. Myrrh has been used to treat breast and colon cancer (Boffa et al., 2015; Abdul-Ghani et al., 2009; Shen et al., 2012; Tipton et al., 2003). The average number of intestinal schistosomiasis eggs decreased from 181 eggs per gram to only 4.0 eggs per gram in patients treated with Myrrh (Tipton et al., 2003; Abdul-Ghani et al., 2009). Myrrh has also been used to treat trauma, skin infections, and fevers (Shuaib et al., 2013; Shalaby and Hammoud, 2014; El-Sherbiny and El Sherbiny, 2011; Nomicos, 2007). Both gram-negative and gram-positive bacterial strains are inhibited by Myrrh (Boffa et al., 2015; Abosalif et al., 2015; Shuaib et al., 2013). Recently, scientists examined the bioactivity of Myrrh on fresh salad as a natural preservative. Myrrh extended the shelf life of the product by lowering bacteria growth (Boffa et

al., 2015). A commercial drug called Mirazid containing 300 mg of purified myrrh is confirmed as an effective drug against many parasites (Shalaby and Hammoud, 2014; Boffa et al., 2015; Shen et al., 2012). Mirazid is an anti-parasitic which kills all worms of *S.mansoni* in four weeks and eliminate most of the symptoms associated with the parasite within one week (Abdul-Ghani et al., 2009; Yakoot, 2010; Shen et al., 2012). Yakoot (2010) reported a cure rate of more than 90% for most experiments using Mirazid.

Materials and Methods

Herpes simplex virus-1

Herpes simplex virus-1 was initially obtained from Dr. Nancy Sawtell, Children's Hospital Medical Center, Cincinnati, OH. Vero cells were infected at 90-100% confluency with 1 ml HSV-1 at 0.1 MOI and once the cells were rounded, the supernatant was harvested, aliquoted and stored at -80°C. Virus was tittered before being used for further experimentation.

Plaque Assay

The plaque method has been used to quantify infectious virus through counting of plaques (zones of killed cells) on virus-infected Vero cells. Vero cells were grown in 12 well plates until they reached 90-100% confluence. HSV-1 was added and the plate was incubated for two hours to ensure that the Vero host cell absorbed the virus. Following virus infection, 1 ml of methyl cellulose (MC) was added to each well. MC is used to prevent the diffusion and spread of virus. Following the overlaying of MC, plates were incubated for 3-5 days. Monolayers were fixed using 4% formaldehyde and kept at room temperature overnight. The fixative was then removed and the monolayers were stained using 5% Crystal Violet for about 10- 15 minutes. All wells were gently washed with distilled water (dH₂O) and the plate was allowed to dry at room temperature and plaques counted. The following equation was used to calculate the viral titer.

$$\frac{\text{Average \# Plaques}}{D \times V} = \text{PFU/ml}$$

$$D \times V$$

D=dilution V=Volume of diluted virus added to the plate

Vero cell

The Vero cell line is used as a host cell for the virus growth. The cell line was derived from the kidney of an African green monkey (CCL-81, ATCC). The culture medium was prepared using 90% of Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin. The cell growth was monitored every day and cells were split every 2-3 days. DMEM, FBS and antibiotics were purchased from Fisher Scientific.

Acyclovir (ACV) treatment before infection

Vero cells were cultured using 12 well tissue culture plates (Fisher Scientific) for 24 hours. The cells were treated with ACV using different doses (2, 1, and, 0.05 µg /ml of medium) and incubated for 2 and 4 hours. Then cells were infected with HSV-1 at 0.1 MOI and incubated for 2 hours at 37°C in 5% CO₂. Following virus infection, 1 ml of methyl cellulose (MC) were added. Monolayers were fixed with 4% formaldehyde overnight. The fixative was removed and monolayers were stained using 5% Crystal Violet for about 10-15 minutes. Plates were washed with distilled water (dH₂O) and the plaques were counted.

ACV treatment after infection

Vero cells were grown in 12 well plates with 10% DMEM overnight. The next day, cells were infected with HSV-1 at 0.1 MOI for 2 hours. After the virus in medium was aspirated, plates were incubated in culture medium containing ACV for 2 and 4 hours. The effect of virus was examined using plaque assay.

Myrrh treatment before infection

Vero cells were cultured in 12 well tissue culture plates. After 24 hours, Myrrh extracts were added at different concentrations (1%, 2%, 0.5%) for 2 and 4 hours. After that, cells were infected with HSV-1 at 0.1 MOI and incubated for 2 hours at 37°C. One ml Methylcellulose was added to each well and the dishes were incubated for 3-5 days. Monolayers were fixed with 4% formaldehyde overnight then stained with 5% Crystal Violent for 10-15 min. Plates were washed with distilled water (dH2O) and the plaques were counted.

Myrrh treatment after infection

Vero cells were plated in 12 well tissue culture plates with 10% DMEM and incubated overnight. Then, cells were infected with HSV-1 at 0.1 MOI for 2 hours. After the virus was aspirated, plates were incubated in culture medium containing different doses of myrrh extract for 2 and 4 hours. The effect on virus infectivity was examined using plaque assay.

Cell viability

Vero cells were grown until 90- 100% confluence. They were treated with Myrrh for 2 or 4 hours either before or after virus infection. Infected, untreated cells were used as a control. Vero cells were washed using Phosphate-buffered saline (PBS). Trypsin was added to the cells. After cells were detached, 10% DMEM was added. Detached cells were centrifuged at 1500 rpm (4°C) for 5 minutes. One ml of fresh medium was added to the cell suspension. 20 µl of the suspended cells were mixed with 20 µl Trypan blue dye (Fisher Sciences) to analyze cell viability. Aemocytometer was used to count viable cells. The following equation was used to count cell viability:

Cell Viability (%) = total viable cells (unstained) ÷ total cells (stained and unstained) × 100

Statistical Significance:

All experiments were repeated three times to confirm the results. Data were analyzed by one-way ANOVA (Sigma Plot 12.0, YSTAT). Data are represented as mean ± SEM.

RESULTS

Toxicity of Myrrh on Vero cell

Inhibition of the viral cytopathic effect of Myrrh on the Vero cell by Myrrh was examined using different concentrations of Myrrh. Infected Vero cells were treated with 20% or 10% concentrations of Myrrh. High concentrations of Myrrh were toxic as shown in Figure 6A. However, infected Vero cells treated with lower concentrations of Myrrh (2%, 1%, and 0.5%) survived as shown in Figure 6B. Toxicity was determined visually by cell density of the infected treated cells compared with the controls infected, untreated Vero cells.

Effect of Myrrh extract on HSV-1 infection (2 hours before infection):

Cultured Vero cells at 90% confluence were treated with 2%, 1%, and 0.5% concentrations of Myrrh two hours before HSV-1 infection. Cell density reached highest value at 2% concentration ($P \text{ value} \leq 0.001$) while 0.5% concentration of Myrrh was the lowest ($P \text{ value} \leq 0.01$), compared with the untreated, virus-infected cells (see figure 7).

Effect of Myrrh extract on HSV-1 infection (2 hours after infection)

Cultured Vero cells (at 90- 100% confluence) were treated with different concentrations of Myrrh (2%, 1%, and 0.5%) at 2 hours post HSV-1 infection. At two hours, there was a significant reduction ($P \text{ value} \leq 0.001$) in the number of plaques formed at all Myrrh concentrations compared to the control (virus infected cells). Myrrh was most effective at 2% concentration on virus infected cells compared to other concentrations (Figure 8). Analyzing cell density using Image J gave the same results as shown in Figure 9. Densities of the treated cells decreased with the reduction of the concentrations of Myrrh compared with the control (virus

infected cell).

Effect of Myrrh extract on HSV-1 infection (4 hours before infection)

Plaque assays were used to detect the concentration of Myrrh that most effectively inhibited virus replication. Before HSV-1 infection, Myrrh treatment was added for 4 hours using different concentrations (2%, 1%, and 0.5%). A 4 hour treatment with 0.5% concentration of Myrrh before virus infection displayed a significant result (p value .031) compared to virus infected cell (control), while (1% and 2%) concentrations of Myrrh showed a slight reduction compared with the control Figure 10.

Effect of Myrrh extract on HSV-1 infection (4 hours after infection)

Vero cell were grown to approximately 90-100% confluence. They were treated with Myrrh 4 hours after virus infection and the cells were stained with crystal violet to determine the cells that survived the infection. Concentrations of Myrrh 2% and 1% led to insignificant increase in cell survival density compared to the control (infected Vero cells). As shown in Figures 11 there was a significant difference (p value .008) between the 0.5% concentration and the control (infected untreated cells).

Cell Viability comparison between 2 and 4 hours treatment of Myrrh

There was notable decrease in the cell viability percentage at 2% and 0.5% concentrations between 2 hrs and 4 hrs of Myrrh treatment. Cell viability percentage was significantly increased higher at 2 hrs treatment compared to 4 hrs treatment (P value ≤ 0.001) as shown in Figures 12 A and C. However, 1% concentration of Myrrh treatment led to a less significant difference then 2% and 0.5% (P value 0.048) Figure 12 B.

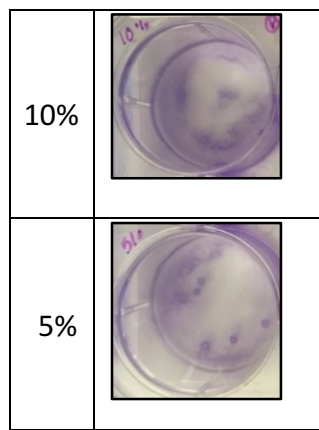
Comparison of ACV or Myrrh treatment at 2 hours before and after infection

The scanned pictures were analyzed using Image J. Pixel densities for each of the three random wells were analyzed using Sigma Plot statistic. No significant differences in cell survival were found using a one-way analysis of variance between the ACV and Myrrh treatments at 2%, 1% and 0.5% concentrations at 2 hours before infection (Figure 13).

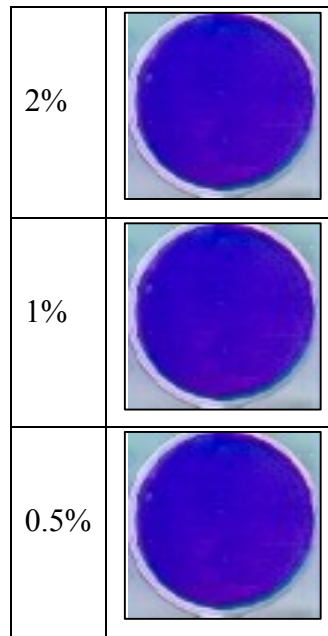
After 2 hours of the ACV or Myrrh treatments followed by HSV-1 infection, infected Vero cells treated with ACV showed greater survival rates (P value ≤ 0.001) relative to infected Vero cells treated with Myrrh in all concentrations (Figure 14).

Comparison of ACV or Myrrh treatment at 4 hours before and after infection:

At 4-hours the Myrrh treatment displayed significant results only for the 0.5% concentration. As shown in Figure 15, cell survival with ACV at a 0.5% treatment significantly increased survival compared to the Myrrh treatment before infection (P value ≤ 0.001) and after infection (P 0.01).



(A)



(B)

Figure 6. Toxicity of Myrrh on Vero cells after infection with 0.1 MOI of virus. **(A)** 10% and 5% concentrations of myrrh caused cell death while lower concentrations of 2%, 1% and 0.5% protect against cytopathic effect **(B)**.

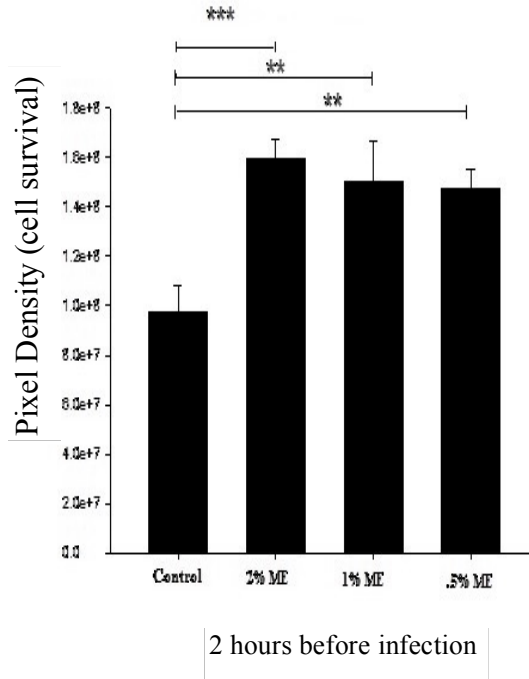


Figure 7. Effects of different concentrations of Myrrh treatment 2 hours before HSV-1 infection. Pixel Density of cell survival of infected treated cells using different concentrations (2%, 1%, and 0.5%) significantly increases compared with control untreated infected cells. Vero cells infected with HSV-1 that were treated with 2% Myrrh concentration 2 hours before infection, have a greater survival rate compared cells treated with other Myrrh concentrations. (ME: Myrrh Extract; *** =P value ≤ 0.001 ; **=P value ≤ 0.01)

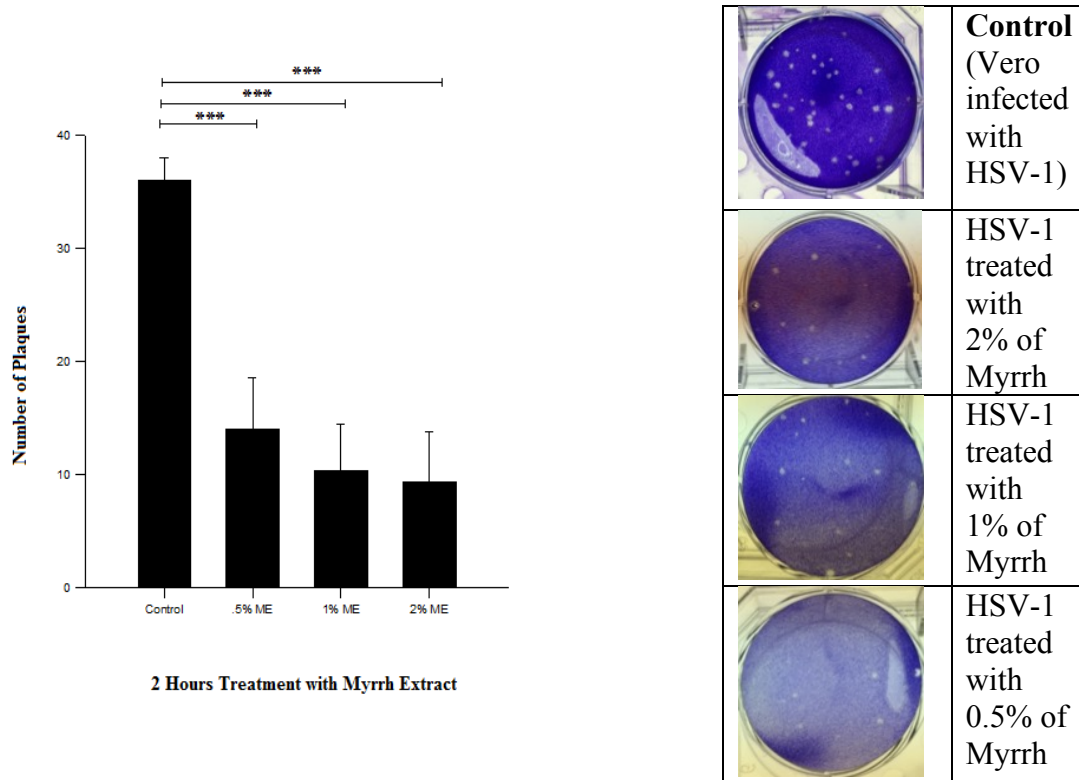


Figure 8. (A) The number of plaques formatted after two-hours Myrrh treatment prior to HSV-1 infection. The number of plaques for the treated cells decreased significantly at all concentrations compared with the control (untreated HSV-1 infected Vero cells). (ME: Myrrh Extract; *** =P value \leq 0.001). **(B)** Plaque Assay plates at two-hours treatment with different concentrations of Myrrh following infection. There was a substantial reduction on the formation of plaques at all Myrrh treatment concentrations compared with the control (Vero infected cells).

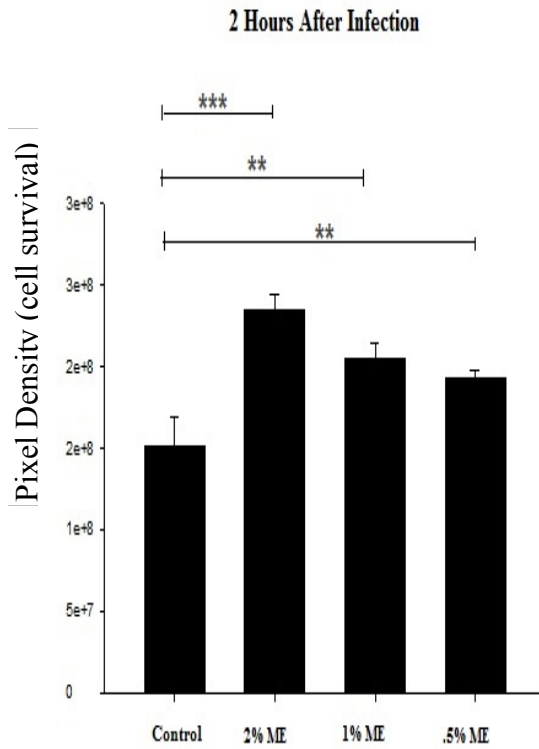


Figure 9. Comparison of Myrrh added 2 hours after HSV-1 infection 2 hours to infected untreated cells (control). Myrrh treatment for 2 hours after virus infection, resulted in a significant increase in cell survival at all concentrations compared with the control untreated infected cells. (ME: Myrrh Extract; *** =P value \leq 0.001; **=P value \leq 0.01)

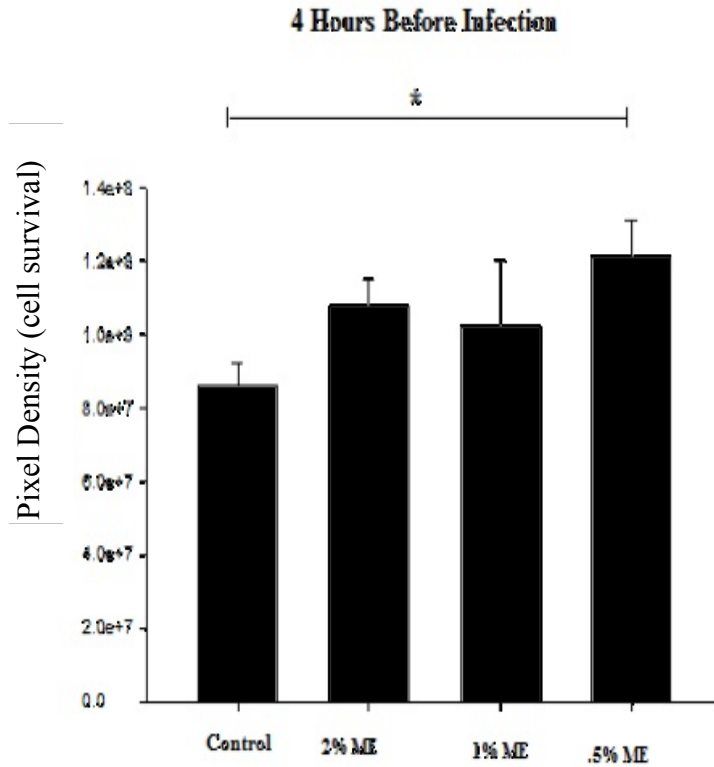


Figure 10. Comparison of HSV-1 cytopathic effects of Vero cells treated with Myrrh 4 hours before infection. Pixel Density (cell survival) of scanned images of cells treated with different concentrations of Myrrh. There was a significant increase in cell survival at 0.5% of Myrrh compared to the control (infected, untreated cells) (* P=value .031; ME: Myrrh Extract).

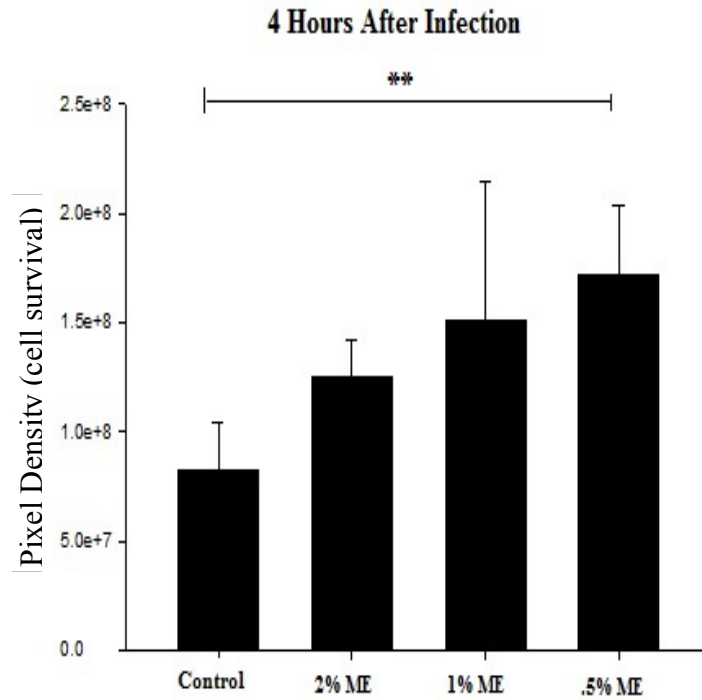


Figure 11. Myrrh effect on infected Vero cells added 4 hours following infection. Cell survival significantly increased only at 0.5% concentration (** P value 0.008) compared to the control (infected, untreated cells) (ME: Myrrh Extract).

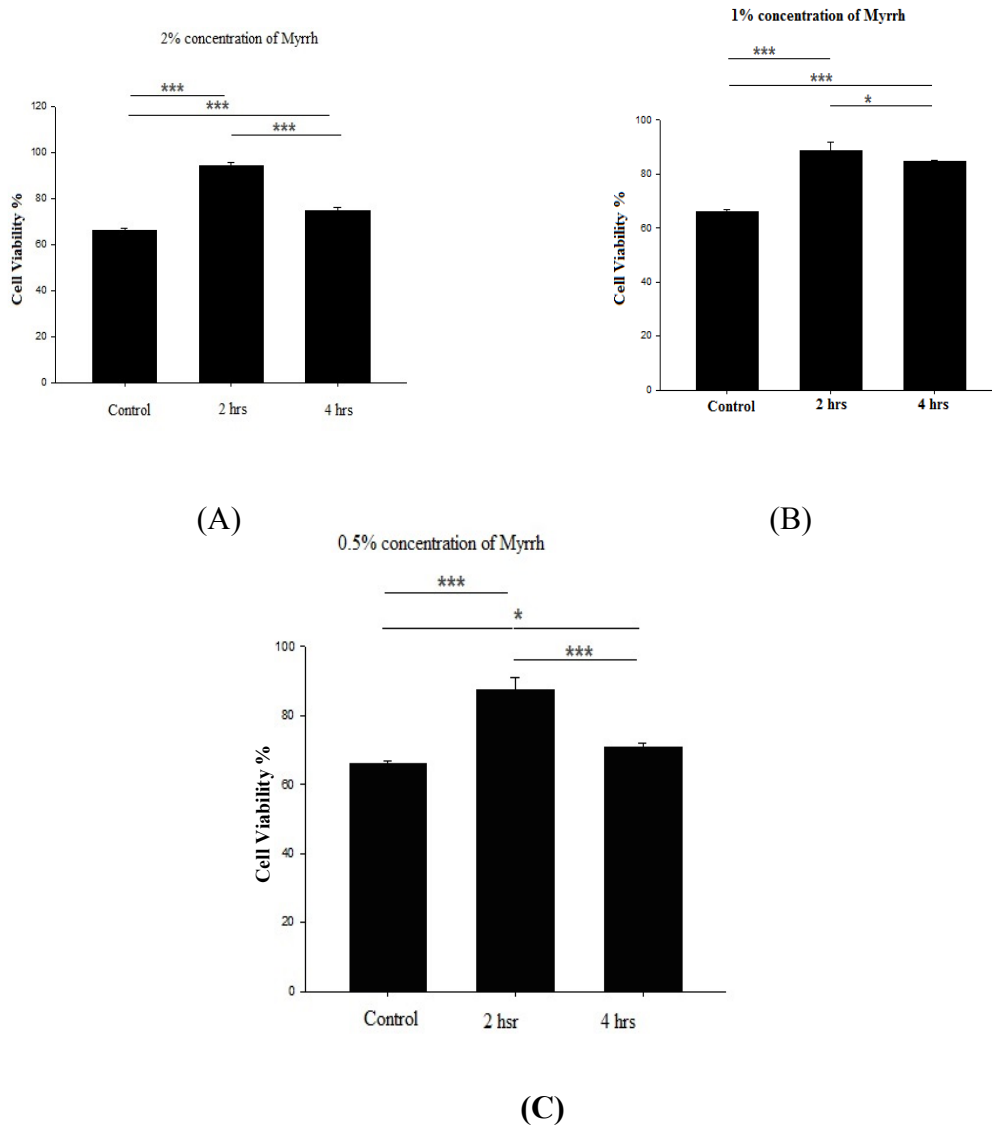


Figure 12: Comparison of present cell viability between 2 and 4 hours of Myrrh pretreatment. (A) Cell viability percentage increased significantly at 2hrs (P value < 0.001) and at 4 hrs (P value < 0.001) of 2% myrrh treatments compared with the control (infected, untreated cells). Pretreatment with 2 hours led to a significantly higher survival than the 4 hours treatment (P value < 0.001). Figure (B) Cell viability percentage increased significantly at 2hrs (P value < 0.001) and 4 hrs (P value < 0.001) compared with the control. However, the differences between 2 hrs and 4 hrs of 1% of Myrrh were nearly insignificant (P value = 0.048). (C) At 0.5% concentration of Myrrh cell survival was greater at 2hrs compared to 4 hrs of treatment (P value < 0.001) (ME: Myrrh Extract).

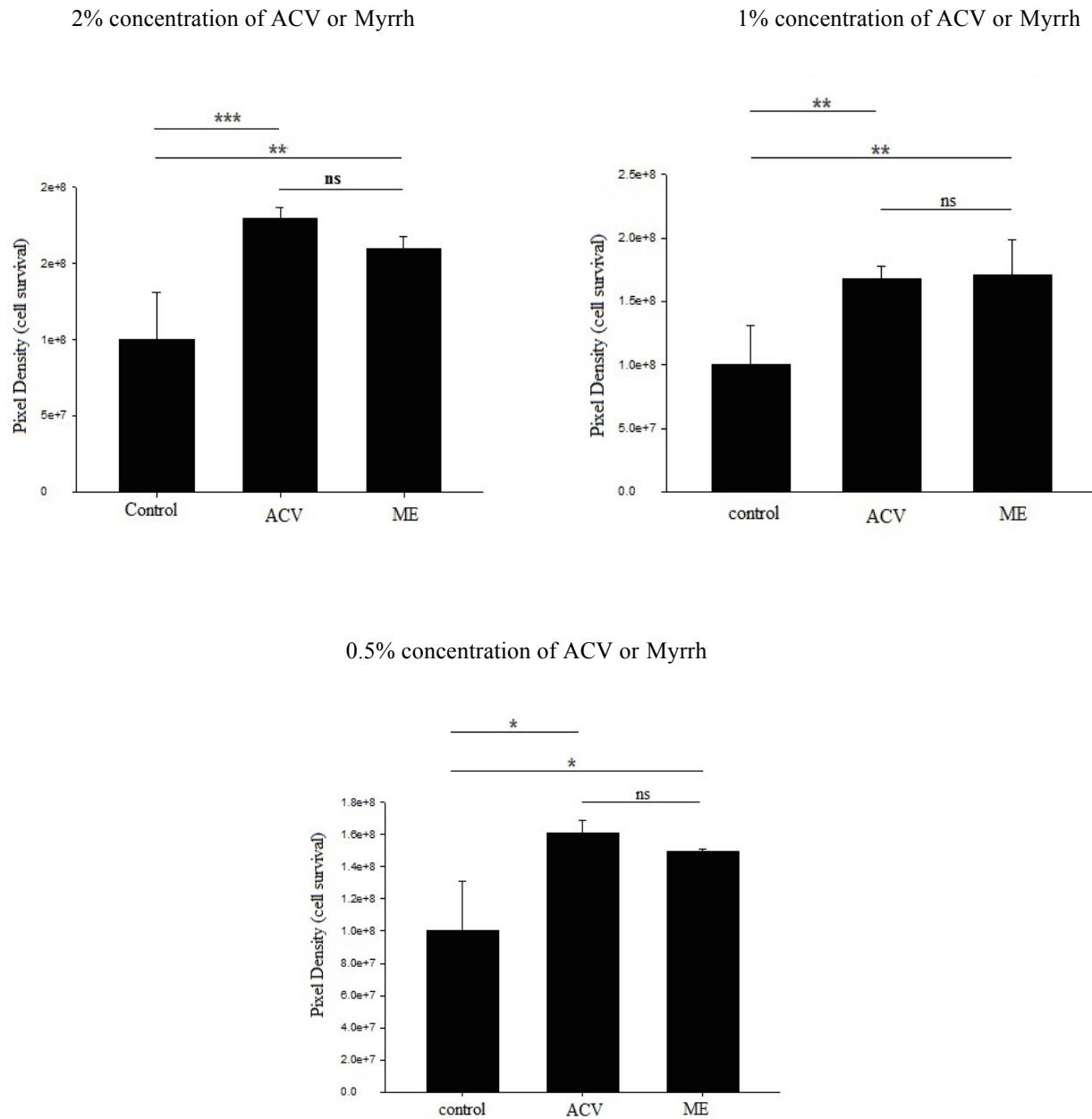


Figure 13. Comparison of cell survival between Myrrh or ACV treatments 2 hours before infection with HSV-1. There was a significant difference in cell survival in ACV and Myrrh compared to the control (infected untreated cell) (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$). There was no significant difference between ACV and Myrrh at any concentration (ns= not significant) (ME: Myrrh Extract).

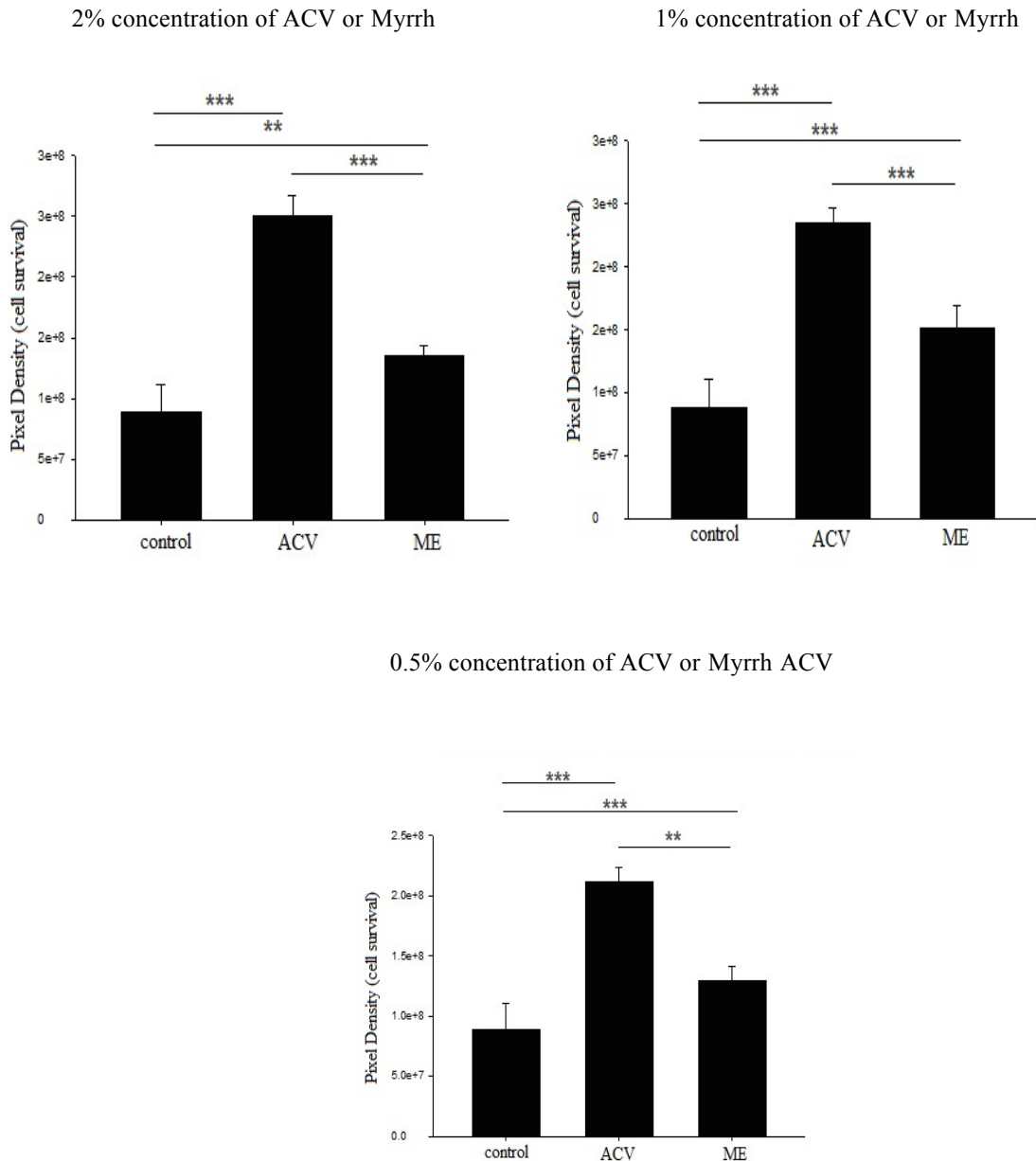
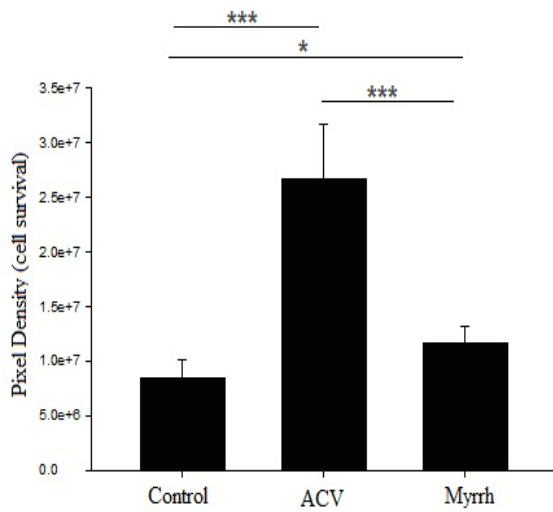


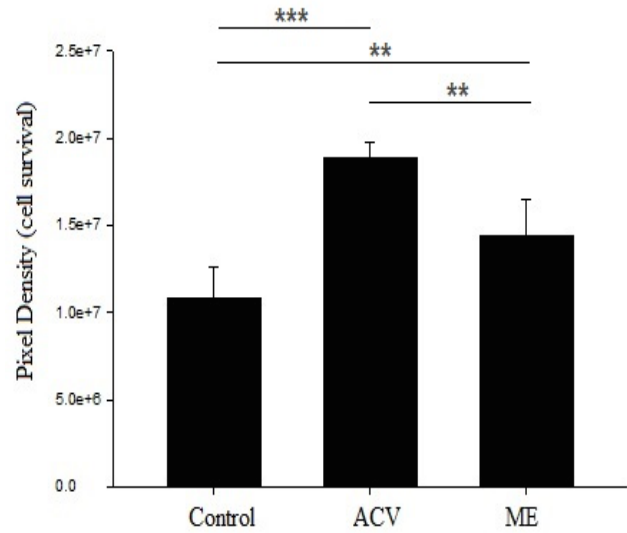
Figure 14. Comparison of cell survival between treatment with Myrrh or ACV of HSV-1 infected cells (2 Hours treatment after infection). There was a notable increase in cell survival between the ACV and Myrrh treatment compared to the control (infected untreated cell). Also infected Vero cells treated with ACV showed greater survival rate relative to infected Vero cells treated with Myrrh in all concentrations. (** $P \leq 0.01$; *** $P \leq 0.001$) (ME: Myrrh Extract).

0.5% concentration of ACV or Myrrh



(A)

0.5% concentration of ACV or Myrrh



(B)

Figure 15. Comparison of cell survival between Myrrh or ACV treatments of HSV-1 infected cells (4 Hours treatment). (A) At 4 hours treatment with ACV before infection led to a significant increase in cell survival compared to Myrrh (P value < 0.001). (B) Treatment with ACV at 4 hours after infection significantly increased cell survival compared to Myrrh ($P \leq 0.01$) (ME: Myrrh Extract).

Discussion

Myrrh has been used in Middle Eastern countries for centuries, not only as a foaming material but also as a medication. Recently, researchers have demonstrated that myrrh has antibacterial effects against gram positive bacteria such as *Bacillus subtilis* and *Staphylococcus aureus*. Myrrh application has shown promising action against pathogenic Gram- negative bacteria such as *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Pseudomonas aeruginosa*. Myrrh also effectively inhibits fungal pathogenic growth of *Candida albicans* and *Apergillus niger* (Abosalif et al., 2015). Myrrh is used as an anti-parasitic agent to treat human trematode infections. Researchers have examined the anti-parasitic effect of myrrh with exploring its mode of action, safety, and efficacy on parasite infections (Abdul-Ghani et al., 2009).

In the current study, Myrrh extract's effect was examined on Vero cells infected with Herpes Virus Type-1. High concentration (10% and 5%) of Myrrh extracts were toxic to the Vero cells, while lower concentrations (2%, 1%, and 0.5%) were protective for Vero cells against the cytopathic effect of HSV-1.

Application of Myrrh Extract for 2 hours at lower concentrations of 2%, 1% and 0.5% showed significant reduction in the HSV-1 viral replication. The results were compared with infected untreated cells using plaque assay and confirmed by testing cell viability.

NF-kB is activated in HSV-1 infected Vero cells leading to increase secretion of pro-inflammatory cytokines secretion including IL6, IL8, TNF- α , and interferons (Li et al., 2006). Epithelial cells' ability to act as accessory immune cells is a possible explanation for the

significant reduction in the virus' cytopathogenic effect on Myrrh treated Vero cells (Tipton et al., 2003). The treated Vero cells may lead to a decrease in the production of IL-1B, which in turn can reduce the production of cytokines caused by HSV-1, such as IL6 and IL8 (Tipton et al., 2003). This hypothesis is worthy of further investigation in future studies.

At 4 hours of Myrrh treatment, the 0.5% is the only concentration that showed significant reduction in cytopathogenicity before and after virus infection. 2% and 1% concentrations of Myrrh resulted in insignificant reductions. This phenomenon could be due to prolonged exposure to high toxic concentrations of Myrrh Extract that led to normal cell death. This explains why in middle eastern countries, Myrrh extract is applied to the area surrounding the wound and prohibited over the lesion.

One possible mechanism of Myrrh treatment on HSV-1 infected cells could be that myrrh blocked the virus entry to the Vero cell and this could explain the effect that myrrh treatment before infection shows a significant increase in cell survival in all tested concentrations. This effect could inhibit new progeny of virus to enter new Vero cells when myrrh treatment is used after infection. The other possibility could be Myrrh treatment has effects on metabolic activity, cell membrane integrity, and IL-1B stimulated production of IL-6 and IL-8 by that lead to cytotoxic effects on infected Vero cells.

Future Study

Myrrh application is a very promising natural medicine that has been applied in ancient countries as an anti-inflammatory treatment for many infection diseases. Myrrh products have been used as mouthwashes and for wound cleaning in many countries. It will be beneficial to

investigate myrrh's therapeutic effects on many other pathogenic micro-organisms such as Lactobacillus microbiota (a causative agent of bacterial vaginosis), candida (the causative agent of candidiasis), chlamydia trachomatis (the causative agent of epididymal infertility and eye infection) among others.

Peppermint Oil is an approved inhibitor of HSV-1 (Schuhmacher et al., 2003).

Examination of Myrrh Oil and comparison of its effects with those of the Myrrh Extract used in our study will broaden the spectrum of applications of Myrrh on many pathogenic organisms.

Myrrh treatment inhibits the secretion of pro-inflammatory cytokines of microphages (Kim et al., 2012). It would be beneficial to examine Myrrh effects on HSV-1 infected microphages M1 and M2 by studying the differences in cytokine and interferon levels before and after Myrrh treatment. Myrrh treatment elevated leukocyte levels even in the absence of injury; this suggest that Myrrh acts as a foreign body and initiates an immune response (Haffor et al., 2010). Future studies can investigate Myrrh effects on both T and B lymphocytes in the HSV-1 infected model animal.

Application of Myrrh with ACV treatment in many patients may lead to a decrease in the therapeutic dose and side effects associated with ACV such as nausea, diarrhea, dizziness or headache. It could also minimize the unusual bruising or bleeding that occurs in some individuals. Myrrh also could be beneficial in patients who develop a resistance to ACV or those with decreased absorption or abnormal TK activity.

Application of Myrrh extract to HSV-1 infected Vero cells showed promising results in our experiment. Future studies should investigate the possibility of similar effects on human epithelial cell lines because of the close similarities between both tissues.

The affect of Myrrh on viruses using different entries mechanisms should be examined. Labeling some virus components such as glycoproteins (gB, gC, gD,gH, or gL) may help clarify the effect of Myrrh treatment in inhibiting the virus invasion and or transit within infected host cells. HSV-1 uses F-actin to enter the host cell and microtubule to travel through the neuron; it would be interesting to label the microtubule associated proteins such as dynein and kinesin with a florescent dye and further examine Myrrh effects on blocking the movement of a virus.

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