

AMERICAN UNIVERSITY OF BEIRUT

ASSESSMENT AND CHARACTERIZATION OF AN ANTI-  
EBV COMPOUND EXTRACTED FROM SOIL-DWELLING  
BACTERIA

by  
MARWAN MOUNIF SAADEH

A thesis  
submitted in partial fulfillment of the requirements  
for the degree of Master of Science  
to the Department of Experimental Pathology, Immunology, and Microbiology  
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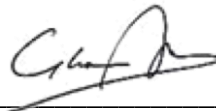


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# ABSTRACT OF THE THESIS OF

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for

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Title: Assessment and Characterization of an Anti-EBV Compound Extracted from Soil-Dwelling Bacteria

**Introduction:** The Epstein Barr Virus (EBV) is thought to infect >90% of the population world-wide; the virus then establishes latency in resting memory B-cells. The virus is associated with infectious mononucleosis (IM), malignancies, autoimmune disorders and organ transplant rejection. Currently, there are no available drugs/vaccines to treat or prevent EBV efficiently. The overall objective of the study at hand is to successfully identify, purify, and isolate an active compound with limited cytotoxicity that can inhibit EBV replication. Given the fact that natural products are a good and novel source of antimicrobials, our group, in a previous study identified that secondary metabolites produced by the QSB-12 strain of the *Pseudomonas* genus grown in the Vegetative (Veg) medium have a potent and minimally cytotoxic anti-EBV effect *in vitro*. The main objective of this study was to further purify the QSB-12 Veg fractions and to isolate and characterize an effective anti-EBV compound with minimal cytotoxicity in these fractions.

**Methods:** High-performance liquid chromatography (HPLC) was performed on the combined QSB-12-acquired ethyl acetate and chloroform subfractions that were most effective and minimally cytotoxic in our previous study. To assess for the antiviral effects of the post-HPLC fractions,  $0.1 \times 10^6$  P3HR-1, EBV producer cells were incubated per well in a 96-well culture plate with 65 ng/mL Phorbol 12-myristate 13-acetate (PMA) and a concentration of 0.08 mg/mL of the fractions for 5 days, at 37°C in a humidified incubator. The extent of viral replication (EBV load) was assessed by quantitative real-time PCR. Additionally, the percentage of cell viability after incubating with the fractions was assessed using the 0.4% trypan blue exclusion assay. Total live and dead cells were determined in triplicates. Cytotoxicity was assessed after an incubation with the fractions under the same conditions mentioned above but excluding PMA, the inducer of EBV production. After determining the most effective and least cytotoxic fraction, liquid chromatography-mass spectrometry (LC-MS) was performed to characterize the EBV-inhibitory compound and determine its molecular formula. Half maximal inhibitory concentration (IC<sub>50</sub>) was performed by serially diluting the fraction of interest and incubation with the EBV-producer cells followed by viral load quantification by real-time PCR.

**Results:** Six post-HPLC fractions were assessed for their potential anti-EBV effect. Five fractions were able to significantly decrease the EBV viral load in culture supernatants. However, fraction QSB-C showed the most potent inhibition with minimal cytotoxicity. All fractions were further characterized using LC-MS. The latter identified compounds of similar mass and formula across all fractions. A compound of interest was identified with a specific molecular mass of 245.1271 for an [M+H] ion and a formula of C<sub>12</sub>H<sub>15</sub>N<sub>5</sub>O with 98% purity in the QSB-C fraction. Therefore, the IC<sub>50</sub> of the QSB-C fraction was assessed and a value of 7.854 ± 1 µg/mL was determined to decrease the EBV viral load in culture supernatants by 50%.

**Conclusion:** Our findings have determined that a compound of interest produced by the QSB-12 bacterial isolate of the *Pseudomonas* genus has potent anti-EBV activity; this compound seems to have minimal cytotoxicity when assessed *in vitro* in the P3HR-1 cell line. Its 2D-structure and particular mechanism of action is yet to be fully determined; however, based on its molecular formula, and since it was assessed in EBV-positive P3HR-1 cells, the compound is likely inhibiting the stage of DNA synthesis and replication in the viral life cycle.

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# CHAPTER 1

## INTRODUCTION

Epstein Barr Virus (EBV), also referred to as *Human Herpes virus 4* (HHV-4), is a member of the herpes viruses. EBV is widely distributed around the world; the virus is thought to infect around 90% of the global population at least once throughout their lifetimes. It is the most common causative agent of infectious mononucleosis (IM). It is associated with malignancies such as Burkitt's lymphoma (BL), nasopharyngeal carcinoma (NPC), Hodgkin's and Non-Hodgkin's lymphoma, and post-transplant lymphoproliferative disease (PTLD). EBV establishes latency in memory B cells, and is associated with autoimmune and chronic inflammatory disorders such as rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE). EBV is mainly transmitted via saliva/fluid exchange; it spreads to a much lesser extent through blood transfusions, organ transplants, and via semen during sexual intercourse. Following exposure, EBV primarily infects epithelial cells followed by B-lymphocytes. The virus can establish latency in memory B-lymphocytes, which facilitates its persistence in infected individuals. Afterwards, the virus may reactivate, actively replicate, and is shed in the saliva where it can potentially be transmitted to another host. Various therapeutic options that may work against EBV were explored yet none have been able to completely clear an infection nor ameliorate EBV-related symptoms. Currently, there are no available drugs/vaccines to treat EBV efficiently. Therefore, it is essential to study and test novel anti-EBV drugs.

Natural products are old and vital sources of original drugs against emerging microbial, public health threats. Antivirals from natural sources that have been explored

so far are extracted from medicinal plants, fungi, algae, and bacteria (Sagaya Jansi et al., 2021). Secondary metabolites and bioactive molecules of these terrestrial and marine organisms have been successfully studied and used against infectious and non-infectious entities. Our group has previously acquired biologically active extracts from pure, soil-dwelling bacterial isolates. Some of these extracts and their fractions were effective in their anti-EBV activity and were promising due to their low levels of cytotoxicity. The aim of the study at hand was to proceed with purifying and identifying the anti-EBV fractions post high-performance liquid chromatography (HPLC) and to assess the active compound that is effective and least cytotoxic. Furthermore, we aimed at elucidating the half maximal inhibitory concentration (IC<sub>50</sub>) of the fraction of interest as well as its atomic mass and molecular formula. The latter would offer hints about the compound responsible for the anti-EBV activity and its biochemical properties.

## CHAPTER 2

### LITERATURE REVIEW

#### **A. Epstein Barr Virus**

##### ***1. Identification***

While studying tumor cells from Burkitt's lymphoma (BL), EBV was first discovered by Anthony Epstein and Yvonne Barr in 1964 and was named accordingly. They observed viral particles that resemble herpes simplex viruses in appearance but they noted that they vary in size (Epstein, Achong & Barr, 1964). Consequently, EBV was later determined to be a virus associated with malignancies when Henle and Henle (1976) detected high IgA titers in patients suffering from malignancies such as BL, nasopharyngeal carcinomas and patients who have infectious mononucleosis (IM). These IgA titers were directed against the EBV viral capsid antigen (VCA) and early antigen (EA) complex. The VCA IgA antibodies were noted to decrease as treatment progressed (Henle & Henle, 1976). However, these antibodies remained elevated in more advanced or challenging cases.

##### ***2. Structure and Genome***

EBV, a gamma herpesvirus belonging to the *lymphocryptovirus* genus of the *Herpesviridae* family, has an outer surface envelope with embedded spike glycoproteins and a nucleocapsid, which encases its linear, double-stranded DNA genome (Choi et al., 2018; Vetsika & Kallan, 2004). Its genome contains six nuclear antigen genes known as EBV nuclear antigens (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C), which in turn encode six nuclear antigen proteins. It also consists of three latent membrane

protein genes (LMP1, LMP2A, LMP2B). All virally infected cells express EBNA1 whose primary purpose is to conserve and replicate the episomal EBV genome. Episomal DNA molecules may integrate into chromosomal DNA in prokaryotes or establish circular DNA molecules in prokaryotes and eukaryotes. When it comes to EBV, the latter episomal state is maintained throughout latency. Thereby, the circular DNA molecules are capable of initiating replication during latency and later viral reactivation (De Leo et al., 2020; Young & Rickinson, 2004).

### ***3. EBV Life Cycle and Infection***

EBV is a highly prevalent virus thought to infect more than 90% of the human population. Its major mode of transmission is mainly via salivary fluid exchange; it spreads to a much lesser extent through blood transfusions, organ transplants, and via semen during sexual intercourse (Chesnokova & Hutt-Fletcher, 2014). Following viral exposure, EBV establishes an active, oropharyngeal infection and initiates its replicative phase. During this stage, the lytic cycle proteins are expressed. They are known as the immediate early, early, and late lytic proteins. Early proteins aid in the expression of late genes that in turn encode the viral structural proteins mainly the nucleocapsid and envelope proteins. The main function that these proteins serve is the step of viral assembly (Choi et al., 2018; Grinde, 2013).

EBV utilizes the viral glycoprotein gp350 to bind to cell-surface complement receptor type 2/cluster of differentiation 21 (CR2/CD21) of naïve B-cells. Subsequently, envelope fusion occurs and the virus is endocytosed into the cell (Chesnokova & Hutt-Fletcher, 2014; Spear & Longnecker, 2003). Post endocytosis, the virus expresses the initial full latency protein spectrum (EBNA1, EBNA2, LP, LMP1, and LMP2A) known

as the latency III program (Bhattacharjee et al., 2016). EBNA2 plays a crucial role in B-cell immortalization and sustained cell proliferation (Sample & Sample, 1999); it binds to cellular sequence-specific DNA-binding proteins. The P3HR-1 EBV strain with an EBNA2 gene deletion has been shown to be unable to transform B-cells *in vitro* indicating its vital role in the transformation process (Fields et al., 2007).

The virus proceeds with its infectious cycle by activating and proliferating in these naïve B-cells as they form germinal centers after they migrate to lymphoid follicles. This is where the second stage of latency develops as LMP1 and LMP2 are now expressed. Of importance, LMP1 upregulates anti-apoptotic protein production and surface adhesion molecules indicating its pivotal role in oncogenesis (Young & Rickinson, 2004). In addition, EBNA3 family proteins are also expressed during latency. In genetic studies, EBNA-3A and -3C have been indicated to be responsible for B-cell transformation *in vitro* (Bhattacharjee et al., 2016). These proteins do not directly bind to DNA; instead, they interact with multitudinous cellular DNA binding proteins and accessory transcription factors leading to malignancy. LMP2 guides B-cells into mucosal follicles where germinal centers form and upregulates LMP2A and LMP1, which respectively mimic the B-cell receptor (BCR) and CD-40 (Thorley-Lawson, 2005). LMP1 apparently increases the expression of anti-apoptotic proteins and surface adhesion molecules, which are thought to aid in the transformation process (Young & Rickinson, 2004).

Furthermore, EBV contains Epstein-Barr Virus-encoded RNA 1 and RNA 2/ (EBER1 and EBER2) ubiquitously. Although their function is not completely understood, EBER1 and EBER2 are thought to play a role in the transformation and proliferation of B-lymphocytes (Wu et al., 2007). These small nonpolyadenylated,

noncoding (nc) RNAs are the most abundant viral transcripts in latently infected B-lymphocytes (Iwakiri & Takada, 2010; Rymo, 1979). EBERs have been found to play a role in maintaining malignant phenotypes in BL cells (Komano et al., 1999); they seem to prevent protein kinase RNA-dependent (PKR)-mediated apoptosis in BL and epithelial cells (Nanbo et al., 2002, 2005). EBERs have also been indicated to induce the transcription of cytokines such as interleukin (IL)-10 in BL cells, insulin-like growth factor (IGF)-1 in epithelial cells, and IL-9 in T cells all the former act as autocrine growth factors of the EBV-infected cancer cells (Iwakiri et al., 2003, 2004; Kitagawa et al., 2000; Yang et al., 2004). During latency, EBV protein expression is extremely downregulated, which renders the virus undetectable to the host immune system; this contributes to its persistence in B-cells. In this stage, during cellular mitosis and DNA replication, host DNA polymerase replicates the chromosomes and the viral DNA without the need for the EBV viral polymerase. When the B-cells are redistributed to the oropharyngeal area, the EBV active replication cycle may be again initiated whereby the viral DNA polymerase takes over in viral genome replication. Afterwards, viral protein synthesis is initiated followed by EBV shedding in the saliva (Grinde, 2013).

#### ***4. Infectious Mononucleosis***

EBV is known to be a causative agent of IM as it infects B-cells and epithelial cells. In childhood, the infection is usually self-limited and asymptomatic; however, in early adolescence and in adulthood IM may occur with symptoms that are more apparent. IM is not associated with significant complications; however, sometimes the disease may progress into a chronic illness referred to as chronic, active Epstein-Barr

virus disease (CAEBV). In this case, there is a notably elevated viral load in both the oral cavity and the blood. IM symptoms such as fatigue, pharyngitis, tonsillitis, muscle aches, fever and chills, lymphadenopathy, hepatosplenomegaly, and CD8+ T lymphocytes may be markedly expanded in response to the infection (Balfour et al., 2015; Kimura & Kohen, 2017; Young & Rickinson, 2004). An increasingly activated EBV lytic cycle results in continuous shedding of high EBV titers in the saliva, which increases its ease of spread (Balfour et al., 2015; Young & Rickinson, 2004). To a less common extent, IM patients may experience cardiovascular abnormalities, central nervous system (CNS) involvement, hematological abnormalities, pneumonitis, and problems in kidney filtration (Balfour et al., 2015; Luzuriaga & Sullivan, 2010). During a primary EBV infection, EBV replicates in both epithelial cells and B cells. EBV-infected B cells that evade the immune response downregulate their viral gene expression and differentiate into memory B cells rendering them invisible from the immune system (Fournier & Latour, 2021). It is believed that upon exposure to their cognate antigen, infected B cells undergo plasma cell differentiation. The former reactivates the viral lytic cycle, which then allows further infection of neighboring epithelial and B cells (Chiu & Sugden, 2016; Fournier & Latour, 2021). In immunocompetent individuals, primary and long-term responses to EBV have been well characterized. The primary immune response involves the innate immune system and NK cells via their p46 receptor. Humans who have X-linked lymphoproliferative disease 1 (XLP1), a defect related to NK and T cells inability to kill or control EBV-infected B cells, may suffer from fulminant infectious mononucleosis (FIM) and hyperinflammation (Pende et al., 2019). FIM is a rare and life-threatening condition that is distinguished by fever, massive hepatosplenomegaly, and atypical lymphocytosis

(Pende et al., 2019). The humoral immune response is also involved when it comes to IM. Specific EBV IgM and IgG antibodies targeted against VCA, EA, EBNA1, and EBNA2 proteins may neutralize EBV, activate the complement system, and opsonize EBV for phagocytosis (Lamy et al., 1982; Sternbæk et al., 2019). IgM antibodies are typically undetectable a few weeks post infection. Although in rare cases, they may persist for a few months. VCA IgG appears at the time of the onset of acute infection, and then usually remains positive for life (Lamy et al., 1982; Sternbæk et al., 2019). The presence of the VCA IgM and VCA IgG isotypes in the absence of EBNA1 IgG indicates that it is likely an early or an acute primary infection; if IgM is absent and VCA plus EBNA1 IgG are present, this would typically indicate a latent infection (Sternbæk et al., 2019). It appears that an adequate immune response, in immunocompetent hosts, mostly involves CD8+ T cells as they serve to eliminate cells that are infected with EBV. After the resolution of the infection, cytotoxic T-lymphocyte counts decrease until they return to normal. Other T cell subsets such as CD4+ T cells, NK and gamma delta ( $\gamma\delta$ ) T-cells likely do not play a major role and are involved to a much lesser extent (Djaoud et al., 2017; Fournier & Latour, 2021; Taylor et al., 2015).

### ***5. EBV-Related Diseases and Malignancies***

As aforementioned, EBV is a highly prevalent virus thought to infect more than 90% of the human population. EBV succeeds in doing so due to its ability to evade the host immune response, its persistence and establishment of latency in memory B cells (Chesnokova & Hutt-Fletcher, 2014). Significantly, EBV is postulated to be the cause of more than 200000 new cancer cases, worldwide, annually (Cohen et al., 2011), which

is why in addition to a broad variety of evidence in the literature, it is grouped as a class I carcinogen (Xu et al., 2019). EBV-related B cell lymphoproliferative diseases and cancers include Hodgkin's lymphoma (HL), B-lymphoproliferative disease (B-LPD), diffuse large B-cell lymphoma (DLBCL), Burkitt's lymphoma (BL), plasma cell proliferative lymphoma (PBL) and primary exudative lymphoma (PEL) (Li & Zhang, 2021; Shannon-Lowe & Rickinson, 2019). Non-B cell EBV-related tumors can be lymphoproliferative lesions or lymphomas that emerge from T/NK cells and epithelial cells (Shannon-Lowe & Rickinson, 2019). Undifferentiated nasopharyngeal carcinoma of the T/NK cells remains to be the most associated with EBV since it accounts for more than 95% of nasopharyngeal carcinoma (NPC) in epidemic areas (Bray et al., 2018). It is a type of squamous cell carcinoma; it frequently occurs in the upper and lateral walls of the nasopharyngeal cavity (Palmer et al., 2011). NPC is endemic in South Asia and North Africa with estimates of around 130000 cases reported yearly (Bray et al., 2018). Recently, reports have portrayed that nearly 10% of gastric cancers have shown a positive EBV infection and this gastric cancer has been referred to as EBV associated gastric cancer (EBVaGC) (Gao et al., 2021). In a recent study by Gao et al. (2021), a possible cause may have been identified. EBV-encoded LMP1 inhibited the expression of the ras association domain family member 10 (RASSF10) protein, and promoted tumorigenesis by recruiting DNA methyltransferase 1 (DNMT1) and inducing the DNA methylation of *RASSF10*.

In the immunocompromised, EBV is associated with oral hairy leukoplakia and PTLD. The latter disorder may be especially lethal to those who have undergone organ transplants or patients who have Acquired Immunodeficiency Syndrome (AIDS) (Fournier & Latour, 2021; Hopwood, 2000). HIV infected patients are more prone to

develop B-cell lymphomas when compared with the general population (Grulich & Vajdic, 2015). The most common lymphomas arising in HIV patients include BL and DLBCL. They also often involve the central nervous system, primary effusion lymphoma, and plasmablastic lymphoma. In recent studies, analyses of HIV-positive patients who have lymphomas indicated that around 40 % or more of the cases are associated with EBV infections and its related disorders (Hagiwara et al., 2019; Manipadam et al., 2019; Marques-Piubelli et al., 2020). This indicates the importance of a competent immune system, especially functional cytotoxic T-lymphocytes, in clearing actively replicating EBV-infected cells (Fournier & Latour, 2021).

EBV has also been linked to autoimmune disorders. Marcucci and Obeidat (2020) indicate a possible link between EBV proteins EBNA1, EBNA2, and EBNA3, low levels of vitamin D, and the pathogenesis of multiple sclerosis. It is also noteworthy to mention that the link between EBV and autoimmune disorders was first observed in individuals who suffer from systemic lupus erythematosus (SLE). EBNA1 seems to be correlated with autoimmune disease; autoimmunity may occur partly due to molecular mimicry and the formation of autoantibodies (McLain et al., 2004; Marcucci & Obeidat, 2020). Our lab group has also studied the effects of interleukin 17A, a proinflammatory cytokine and its link between EBV DNA and rheumatoid arthritis (Fadlallah et al., 2021). It appears that endosomal Toll-like receptors (TLR3, 7, and 9) serve a role when they interact with EBV DNA and increase the production of IL-17A Salloum et al., 2018; Shehab et al., 2019).

## ***6. EBV Treatment Options***

EBV infections are usually mild and self-limited. Infection during childhood often resolves without the need for additional therapy. In adolescents and adults, EBV might result in symptomatic IM. Typically, IM resolves without the need for additional interventions; in some instances, it may require some mild supportive therapy. Moreover, as discussed above, EBV is associated with malignancies as well as autoimmune diseases, and is especially harmful to those who have a compromised immune system, which is why there is a need to explore therapeutic options. The following are experimental approaches and agents that have been examined for their possible use in treating EBV-associated diseases:

### ***a. Synthetic Antivirals***

Commonly used synthetic antivirals that have been tested against EBV include nucleoside and nucleotide analogues. The former include ACV, ganciclovir (GCV), penciclovir (PCV), and their prodrugs while the latter include cidofovir (CDV) and adefovir (ADV), and the pyrophosphate analogue foscarnet.

These above-mentioned antivirals have been used in acute and symptomatic EBV infections with ACV and GCV being the most commonly employed (Crumpacker, 1996; Resnick, 1988). Their mechanism of action begins with the virally-encoded thymidine kinase enzyme as it converts these analogs to their monophosphate forms. They are then converted into their triphosphate form by host kinases. Afterwards, they are incorporated in newly synthesized DNA, leading to premature termination of DNA synthesis and killing of the infected cell by apoptosis.

Generally, nucleoside and nucleotide analogues may serve as competitive inhibitors for naturally occurring nucleosides or nucleotides utilized by the viral DNA polymerases to transcribe the viral DNA chain. Ultimately, they are incorporated into the replicating viral DNA chain and this leads to chain termination or significantly impedes the DNA polymerase activity (Poole & James, 2018). In other words, they interfere with the viral DNA polymerase. The disadvantage of these drugs is that they do not function in the case of latently infected cells; as an example, the EBV thymidine kinase is specifically expressed during the lytic/active replication of the virus (Dong et al., 2021; Ghosh et al., 2007; Poole & James, 2018). Thus, these agents are unable to completely halt an active infection, or in preventing EBV reactivation and reinfection. Ghosh et al (2007), attempted to counteract this disadvantage by adding butyrate with nucleoside analogues such as ACV and GCV *in vitro* to EBV positive PR3HR-1 cells. Butyrate appears to increase the expression of the viral thymidine kinase even in latently infected cells, which is how the nucleoside analogues were able to reduce the EBV viral load significantly. Clinically, these drugs remain to be ineffective with development of resistance, and their long-term use is not advised (Dong et al., 2021; Pagano et al., 2018; Toner & Bollard, 2021). In addition, the use of butyrate with nucleoside or nucleotide analogues warrants further investigation especially in an *in vivo* experimental model.

The effect of GCV and valgancyclovir (VGCV) prophylaxis on the EBV load was evaluated in a group of EBV-naïve pediatric renal transplant recipients who were at risk of primary infection with EBV because they had an EBV-positive donor. During the first year post-transplant, antiviral prophylaxis with VGCV or GCV indicated a significantly decreased chance of EBV acute infection: 9/20 (45%) in the prophylaxis

group versus 8/8 (100%) in the non-prophylaxis group. In addition, the authors highlighted that there was a notable decrease in the EBV load (Höcker et al., 2012).

Acyclic adenosine analogue (S)-9-(3-hydroxy-2-phosphonyl methoxypropyl) adenine {(S)-HPMPA}, better known as cidofovir (CDV), a nucleoside analogue, with significant activity against cytomegalovirus (CMV) and other herpesviruses. CDV is indicated for the treatment of CMV retinitis, a sight-threatening condition, in patients with AIDS (Plosker & Noble, 1999). Additionally, cidofovir was previously used for the treatment of papillomatosis in the hypopharynx and esophagus due to the human papillomavirus (HPV) (Andrei et al., 2001). Cidofovir is recognized for its antiviral and for its anti-proliferative properties via an unclear mechanism. In studies that were performed with EBV-positive NPC xenografts in nude mice, direct injection of cidofovir in tumor tissue was able to suppress growth of the NPC tissue (Wakisaka et al., 2005; Yoshizaki et al., 2001). The ribonucleotide reductase (RR) inhibitors hydroxyurea and didox (3,4-dihydroxybenzohydroxamic acid) increased the cidofovir-based apoptosis in EBV-transformed epithelial cells and in EBV-positive nasopharyngeal carcinoma xenografts (Wakisaka et al., 2005). Therefore, indicating a potential use in patients suffering from EBV-positive NPC.

Maribavir (MBV), an oral benzimidazole L-riboside, was indicated to have anti-EBV and anti-CMV activity (Avery et al, 2021; Trofe et al., 2008; Whitehurst et al., 2013). In EBV positive BL cell lines, MBV had a half-maximum inhibitory concentration (IC<sub>50</sub>) of 0.15 to 1.1 μM, with at least 10-fold more potency than ACV. The drug functions via an alternate mechanism of action compared to other nucleosides/nucleotides; it is an inhibitor of phosphorylation of viral DNA processivity factor Beta-glucosidase 4 (BGL4). There was a significant decrease in EBV transcript

levels; however, MBV's inhibition of EBV warrants further investigation (Whitehurst et al., 2013).

An antiviral activity against EBV has been indicated in P3HR-1 and Raji cell-lines using the following drugs: (a) CDV and another acyclic adenosine analogue (b) 9-(2-phosphonylmethoxyethyl) adenine {PMEA}, known as adefovir (ADV). These drugs were demonstrated to have a broad-spectrum of antiviral activity. In viruses such as the HIV, HCMV, and the herpes simplex virus type 1 (HSV-1) (Mulato & Cherrington, 2011; Hostetler et al., 2006; Zakharova et al., 2011). In a patient who has PTLN, the administration of CDV plus rituximab resulted in a complete remission from an EBV-associated lymphoma involving neurological symptoms. Moreover, EBV DNA remained negative as verified by Polymerase Chain Reaction (PCR) (Hänel et al., 2001). Unfortunately, it is important to note that CDV is nephrotoxic and hydroxyurea results in myelosuppression, which is why the search for drugs that are more effective with fewer adverse effects is ongoing (Wakisaka et al., 2005). Although, in the previous section, multiple anti-EBV drugs were identified and discussed, as of yet, safe and clinically effective antiviral EBV drugs are absent.

### ***b. Non-Antiviral Treatment of EBV-Associated Proliferative Diseases***

In previous findings, EBV was associated with the elevation of programmed cell death protein 1 (PD-1) and its ligands PD-L1 and PD-L2. This correlation indicated a potential pathophysiological pathway that leads to EBV-related lymphomas (Green et al., 2012; Kinch et al., 2018; Schiefer et al., 2019). To counteract the former, checkpoint inhibitors have been used to block the PD-1 pathway. They have shown promise in treating EBV+ Hodgkin lymphoma (HL). Nivolumab and vedotin have been

successfully used post-autologous stem cell transplants in patients suffering from EBV-related HL (Ansell, 2016; Toner & Bollard, 2021). In addition, an overall response rate of 38% was recorded for relapsed/refractory EBV-related NK/T cell lymphomas with relatively acceptable adverse effects, indicating that PD-1 blockade may be successful for different types of EBV-associated lymphoproliferations (Kim et al., 2020).

Another treatment modality involves the generation of EBV-specific T cells (ESTs). ESTs appear to target EBV antigens that are present on the cell surface via the major histocompatibility complex changes related to virally infected cells. This method, in trials, has been used successfully since the mid 1990s (Kankary & Ambinder, 2013; Toner & Bollard, 2021) mainly to counteract EBV-related PTLD. The basis of this technique is exposing B-cells *in vitro* to EBV that leads to the generation of lymphoblastoid cell lines (LCL). In succession, LCLs express EBV latency antigens, which can then be specifically presented to selectively expand EBV-specific T cells from hematopoietic stem-cell transplantation donors. The technique can also be performed similarly in terms of autologous presentation to ameliorate the risk of graft versus host disease in solid organ transplant recipients (Bollard et al., 2012; Rooney et al., 1995; Toner & Bollard, 2021). Nonetheless, this approach remains to be difficult and challenging. ESTs take time to develop and the host will be chronically immunosuppressed due to being a transplant recipient. Thus, its broad application is very arduous and risky (Wistinghausen et al., 2013).

A chimeric anti-CD20 monoclonal antibody, known as rituximab is standardly used in therapeutic regimens for multiple B-cell malignancies since its approval in 1997 (Salles et al., 2017). Rituximab targets malignant CD20+ B cells and potentially eliminates the EBV+ latently infected CD20+ memory B cell population. The

disadvantage however, is that rituximab also targets healthy CD20+ or EBV- B cells, which leads to a serious suppression in the immune system.

In a study by Zheng et al (2012), the bioactivity of lactoferrin, a multifunctional glycoprotein, against EBV infection was assessed. In NPC specimens, lactoferrin was found to be significantly downregulated, possibly indicating its role in the development of NPC post EBV infection (Zheng et al., 2012). Its mechanism of action is unique since it potentially binds to the EBV receptor CD21 and inhibits the EBV entry into the cell.

Currently, a wide variety of small molecule inhibitors are being evaluated for their potential use against EBV-associated lymphoproliferative disorders (Toner & Bollard, 2021). Among these small molecules are EBNA1 inhibitors, histone deacetylase (HDAC) inhibitors, proteasome inhibitors such as bortezomib, ixazomib, cyclin-dependent kinase inhibitors, PI-3K inhibitors, AKT inhibitors, and mTOR inhibitors (Bayraktar et al., 2013; Pei et al., 2020; Sang et al., 2019). These warrant further investigations before their clinical use. A ribonucleotide reductase inhibitor known as hydroxyurea (HU) was capable of eliminating EBV episomes from BL cells and EBV-immortalized lymphoblastoid cell lines *in vitro*, and has shown promising results in a small group of patients with EBV-associated primary central nervous system lymphoma associated with AIDS (Slobod et al., 2000). Zhou et al., (2009) portrayed that HU treatment correlated with hyperacetylation of histone H3 and loss of telomere repeat factor 2 (TRF2) when binding at the EBV origin of plasmid replication. The former appear to play a role in the stability of the EBV episome, which might explain the mechanism of action.

Some studies have also explored the potential effects of epigallocatechin-3-gallate, a histone acetyltransferase inhibitor that is abundant in green tea, and its role in inhibiting EBV lytic cycle and B cell transformation (Chang et al., 2003; Choi et al., 2009). When it comes to the inhibition of the viral lytic cycle, epigallocatechin-3-gallate (EGCG) was added to EBV positive P3HR-1 cells and Raji cells in varying concentrations after the administration of 12-O-tetradecanoylphorbol-13-acetate TPA, which induces viral active replication. Immunoblotting showed that the EBV early antigen-diffuse (EA-D) was significantly reduced in both cases potentially indicating that EGCG plays a role in preventing EBV lytic replication (Chang et al., 2003). Notably, EGCG was also cytotoxic and resulted in 50% P3HR-1 cell death in 100  $\mu$ M concentrations 24 hours post treatment. In another study, p300/CBP-mediated hyperacetylation of RelA (p65), deemed critical for nuclear factor-kappaB (NF- $\kappa$ B) activation was assessed after EGCG administration. The NF- $\kappa$ B pathway is linked to inflammation and tumorigenesis. EGCG at a 50  $\mu$ mol/L dose blocked EBV infection-induced cytokine expression thereby preventing B-lymphocyte transformation. In addition, EGCG appears to have reduced the binding of p300 to the promoter region of the IL-6 gene; excess IL-6 activation was also linked to inflammation and malignancies (Choi et al., 2009).

As of yet, an EBV vaccine is unavailable (Cohen, 2018; Toner & Bollard, 2021). In the search for potential vaccine targets, a study by Coghill et al (2016) examined 2557 individuals from 358 high-risk NPC multiplex families in Taiwan. EBV-neutralizing antibodies (nAbs) blocking B-cell infection, mainly anti-gp350 antibodies, were correlated with disease-free states and a lower incidence of NPC. In an attempt to

develop a more potent EBV vaccine that would hypothetically prevent EBV entry into B-cells, Zhao et al (2018) constructed an Fc-based form of gp350 to serve as a dimeric antigen. Evidently, the Fc-based form indicated enhanced immunogenicity when compared to the wild type gp350. By immunizing mice using gp350-Fc dimers, potent nAbs against EBV were formed, which may be an efficient candidate in preventing clinically significant EBV infections (Zhao et al., 2018). Another vaccine approach is to target different EBV antigens such as LMP2. Wang et al (2011) used three combined vector vaccines expressing the EBV-LMP2. A DNA vector, an adeno-associated virus (AAV) vector, and a replication-defective adenovirus serotype 5 (Ad5) vector were respectively used to immunize female BALB/c mice. EBV-LMP2-specific T cells were determined by Elispot; anti-EBV-LMP2 antibodies were also assessed and the findings were positive as a potential candidate method to prevent EBV-related disease via enhancing cellular-specific immunity (Wang et al., 2011).

Future prospects for novel therapeutic approaches may also involve the clustered regulatory interspaced short palindromic repeat (CRISPR)-associated 9 (Cas9) system, a powerful and reliable genome editing tool that may prove useful against viruses that are capable of latent infections such as EBV (White et al., 2015). Targeted viral DNA sites may be subjected to insertion/deletion mutations (InDel), which would plausibly, negatively impact the virus. For example, EBNA1 may be targeted to disrupt EBV episomal maintenance in host cells (Wang & Quake, 2014; White et al., 2015).

## **7. Antiviral Natural Products**

Natural products are old and great sources of novel drugs against emerging microbial public health threats. Viruses remain to be one of the major causes of illnesses and morbidity. In addition, immunocompromised hosts are at a higher risk of succumbing to

viral diseases. Antivirals from natural sources that have been explored are extracted from medicinal plants, fungi, algae, and bacteria (Sagaya Jansi et al., 2021).

Surfactins from *Bacillus subtilis* displayed significant antiviral activity against HSV (Ongena & Jacques, 2008). Spongouridine, spongothymidine, statins, myriocin, NA255, and cyclosporine were detected to have antiviral activity active against HSV1, herpes simplex virus type 2 (HSV2), hepatitis B virus (HBV), HIV, influenza virus, hepatitis C virus (HCV), and coronaviruses (Nkongolo et al., 2014).

Actinomycetes are a ubiquitous bacterial class found as part of the microbiota of animals, and in terrestrial and marine environments (Sagaya Jansi et al., 2021). Anti-HIV-1 specific activity was shown in Xiamycin and its methyl ester of *Streptomyces* sp. GT2002/1503 (Xu et al., 2014). The compound (4*S*)-4-hydroxy-10-methyl-11-oxo-dodec-2-en-1,4-olide, derived from *Streptomyces* sp. Smu03 was characterized with an antiviral property over a broad range of Influenza A virus strains (Li et al., 2018).

Of interest, there are no specific anti-EBV compounds of bacterial origins that have been identified and properly tested so far *in vivo* (Sagaya Jansi et al., 2021). Takemura et al (1995) demonstrated that 5-Hydroxynoracronycine and acrimarine-F commonly found in citrus fruits have anti-EBV effects when tested *in vitro*. Yet, these findings were not developed any further and have not been clinically applied. Takasaki et al (1990) also explored the potential of eucalyptus extracts to inhibit EBV activation in TPA-treated cells *in vitro*. The preliminary findings indicated that eucalyptus extracts euglobal-G1, -G2, and -G3 inhibited EBV active replication; however, these effects were not tested *in vivo*.

A previous study conducted by our group (Shams Eddin, 2021) concluded that compounds present in subfractions prepared from crude extracts of the QSB-12

bacterial isolate grown in the Vegetative medium, and crude extracts prepared from the BM-12 bacterial isolate, can inhibit EBV replication *in vitro* in the P3HR-1 cell lines with no significant cytotoxicity. In the current study, we isolated and identified the particular compound responsible for the inhibition via HPLC then LC-MS and assessed its anti-EBV activity in the P3HR-1 cell-line. The molecular mass and formula of the compound were hence determined. The IC<sub>50</sub> was also assessed to characterize the potency of this compound for future applications. The need exists to discover a drug that can be used against EBV and natural producers may serve as an ideal source of such an agent.

## CHAPTER 3

### MATERIALS AND METHODS

#### 1. Cell lines

P3HR-1 is a BL cell line that grows in suspension, and which is latently infected with an EBV type 2 strain (The American Type Culture Collection, 2021) (ATCC). This cell line is an EBV producer and was obtained from the (ATCC), Rockville, Maryland. Cells were maintained in complete RPMI medium (Roswell Park Memorial Institute) 1640 (Lonza, Basel, Switzerland) with 20% Fetal Bovine Serum (FBS) (Sigma-Aldrich), and 1% penicillin-streptomycin (PS) (Lonza) incubated at 37°C in presence of 5% CO<sub>2</sub> in a humidified incubator (Thermo Scientific). Cell passaging was performed every 2-4 days maintaining cells at a confluency of  $4 \times 10^5$  -  $1 \times 10^6$  cells/mL.

#### 2. Induction of EBV from P3HR-1 Cells

For induction of cells and release of EBV,  $0.1 \times 10^6$  P3HR-1 cells were cultured for 5 days at 37°C and 5% CO<sub>2</sub> in complete RPMI 1640 medium containing 65 ng/mL Phorbol 12-myristate 13-acetate (PMA) purchased from Sigma-Aldrich. PMA was dissolved in 100% DMSO at a concentration of 1 mg/mL. Then, it was aliquoted to avoid light exposure and recurrent thawing and freezing. After 5 days, PMA-treated cells were centrifuged at 800 rpm for 8 minutes at room temperature (RT) to pellet the cells. The EBV-containing supernatants were then collected and centrifuged in a Thermo Scientific centrifuge at 16,000 g for 90 minutes at 4°C in a fixed angle-rotor to pellet the virus. The viral pellet was then resuspended in 100µL of 1% phosphate-buffered saline (PBS) (Sigma-Aldrich) and stored at -20°C.

### **3. High-performance Liquid Chromatography**

Following bio-guided fractionation, the most effective and least cytotoxic QSB-12 fractions that were previously collected and assessed in the initial, preliminary research, were mixed together to perform HPLC and purify them further. Four fractions were effective in inhibiting EBV and are not cytotoxic as researched and portrayed in the thesis by Shams Eddin (2021). As follows, they are (a) QSB-12 Veg Ethyl Acetate C, (b) QSB-12 Veg Chloroform B, (c) QSB-12 Veg Chloroform C and (d) QSB-12 Veg Chloroform D. HPLC is a chromatography method that separates a mixture of compounds using a stationary phase mainly silica based column and a mobile phase. A sample is injected with solvent in a mobile phase and it flows through an adsorbent column containing a medium suitable for separation in a stationary phase. The aforementioned fractions were initially mixed together before undergoing HPLC to potentially produce a greater yield. Afterwards, the fraction was mixed with water and acetonitrile (ACN) in a stepwise gradient allowing the separation of the compounds based on their different polarities. In summary, A Phenomenex Luna® 5 µm C18 column 100 Å 250 x 10 mm was used and a gradient from 10 % to 90% B in 20 minutes with (A) H<sub>2</sub>O + 0.1% FA and (B) ACN + 0.1% FA at a flow rate of 5 mL/minute at room temperature. Elution was monitored at 220 and 280 nm.

Consequently, the compounds migrated through the column at different rates. The eluted fractions were collected based on their different time points (X-axis) and milli-absorbance units (mAU) (Y-axis) that were detected on the data system graph (as shown below in section IV-1). The mAU is a numerical value that indicates the capacity

of a substance to absorb light of a specific wavelength and this is done via the HPLC machine's ultraviolet detector.

#### **4. Effect of HPLC-Isolated Compounds from Soil-Dwelling Bacteria on EBV**

Six compounds that were collected following HPLC (as described below in section IV-1) and then stored in DMSO at -20°C were tested for their antiviral activities against EBV in induced (lytic) P3HR-1 cells. Per tested preparation,  $0.1 \times 10^6$  P3HR-1 cells were incubated per well in a 96-well culture plate (Corning®, NY) in 250 µL of complete RPMI 1640 (Sigma-Aldrich) with 65 ng/mL of PMA (Sigma-Aldrich) and a concentration of 0.08 mg/mL of the of the 6 collected HPLC compounds. PMA was added to induce the lytic replication cycle of the virus. Controls included cells with 2% DMSO in addition to cells treated with 65 ng/mL PMA and 2% DMSO; the latter two controls were included to account for the DMSO concentration present in the HPLC compound preparations. Applying a 0.08 mg/mL of extracts results a concentration of 2% DMSO per treatment, respectively. After five days of incubation, viral particles were collected from the culture supernatants and suspended in 100 µL of PBS (Sigma-Aldrich) as described above in section III-2. These were then subjected to DNA extraction followed by real-time PCR, as described below, to determine the EBV particle concentration and hence the effect of the collected HPLC compounds on viral production and release into the culture medium. Testing was conducted in triplicates.

#### **5. DNA Extraction**

To extract DNA from the concentrated viral pellet, lysis of the virus was achieved by adding 100 µL of phenol, which is equal to the volume of PBS added to the viral pellet,

then mixed using a homogenizer. The phenolic solution was prepared with Tris HCl to reach a pH between 6.7 and 7.9. The preparation was then centrifuged at 13000 rpm for 15 minutes at 4°C. The upper aqueous layer containing the genomic DNA was collected and mixed with 3M sodium acetate (1/10th of the collected aqueous layer volume), and 70% ethanol (prepared from absolute ethanol (Sigma-Aldrich) and autoclaved distilled water) at a volume equivalent to 3× that of the collected aqueous layer. Samples were then stored at -80°C overnight for DNA precipitation. Afterwards, the mixture was centrifuged at 13000 rpm for 15 minutes at 4°C, and then the DNA pellet was washed 2 times with 1000 µL of 70% ethanol and centrifuged at 13000 rpm for 15 minutes at 4°C. The final DNA pellet was then left to air-dry and then was resuspended in 30 µL of nuclease-free water (sterile distilled water). Samples were then stored at -20°C for further use. Then, the DNA concentration was measured using a NanoDrop (DeNovix) spectrophotometer. After quantification, the DNA extracts were stored at -20°C.

## **6. Real-Time PCR**

EBV DNA copies were determined using quantitative real-time polymerase chain reaction in a Bio-Rad CFX96™ Real-Time PCR Detection System using Taq Universal SYBR Green Supermix (Bio-Rad, Berkeley, California). Primers for the EBV-encoded small RNA (EBER-2) DNA sequence were purchased from Macrogen. The forward primer sequences was 5'-CCCTAGTGGTTTCGGACACA-3' and the reverse primer sequence was 5'-ACTTGCAAATGCTCTAGGC G-3' (Bonnet et al., 1999). Real-time qPCR was performed in a reaction volume of 10µL containing: 4µL SYBR, 3µL water, 1µL of isolated DNA, 1µL of the forward primer and 1 µL of the reverse primer (7.5 pmol/µL each). The thermal cycling program consisted of an initial step of activation at

95°C for 5 minutes, then 40 cycles at 95° and 58°C (annealing) for 15 seconds and 30 seconds, respectively. The qPCR standard curve was generated using different copies of the EBV DNA control per reaction mixture (1000, 2000, 5000, 10000 and 54000 copies) (Amplirun Epstein-Barr Virus DNA Control, Vircell S.L., Granada, Spain). The standard curve was considered acceptable with a slope between -3.0 and -3.6 if the correlation coefficient was at least 0.98. Afterwards, the concentration of EBV DNA in each culture supernatant was calculated from the standard curve using the quantification cycle (Cq) value for each sample and employing the following formula:

$$EBV\ DNA\ copies/\mu\ L = \frac{(Extracted\ DNA\ resuspension\ volume\ x\ F\ x\ X)}{Total\ volume\ per\ culture\ well}$$

Where X is the number of EBV genome copies derived from the standard curve and F is the dilution factor used for setting up the DNA utilized per PCR reaction.

## **7. Cytotoxicity Assay**

To verify that inhibition of viral production is due to the antiviral activity of the compounds acquired post HPLC rather than a cytotoxic effect; cells were tested for their viability. An amount of  $0.1 \times 10^6$  P3HR-1 cells were incubated per well in a 96-well culture plate (Corning®) with the compounds at the same concentration described in section III-3. However, PMA was excluded to assess for the effect of the compounds alone. Controls included cells alone, and cells with 2 % DMSO. Cytotoxicity was assayed after 1, 3, and 5 days of incubation at 37°C in presence of 5% CO<sub>2</sub> in a humidified incubator (Thermo Scientific). Cell viability was assessed by the trypan blue exclusion test. It included adding 10 µL of the cells in suspension to 10 µL of 0.4% Trypan Blue in PBS (Sigma-Aldrich). This mixture was then applied to a hemocytometer and was assessed in experimental triplicates per each of the 6 tested

fractions to enhance accuracy. Viable and nonviable cells were counted separately. To acquire the total number of viable cells, viable cells were multiplied by 2 (to account for the dilution factor with the trypan blue dye). To obtain the total number of cells, viable and nonviable cells were added and multiplied by 2 to account for the dilution factor (Strober, 2015). The following formula (Strober, 2015) was then used to calculate the percentage of viable cells:

$$\% \text{ Viability of cells} = \frac{\text{Total number of viable cells per aliquot}}{\text{Total number of cells per aliquot}} \times 100$$

## **8. Half-Maximal Inhibitory Concentration**

Half-maximal inhibitory concentration (IC<sub>50</sub>) is a technique widely used to assess the efficacy of a drug; it is a pharmacological method that examines the inhibition of the intended biological target of the drug. In the case of this study, it indicates how much of the HPLC fraction of interest is required to inhibit the EBV viral load by 50%. Thereby, providing a measure of potency of the anti-EBV effect. Per tested preparation,  $0.1 \times 10^6$  P3HR-1 cells were incubated per well in a 96-well culture plate (Corning®, NY) in 250  $\mu$ L of complete RPMI 1640 (Sigma-Aldrich) with 65 ng/mL of PMA (Sigma-Aldrich). All plates had a concentration of 65 ng/ml of PMA to induce viral replication. What differs is that the fraction of interest as discussed below, which is QSB-C, was serially diluted to concentrations of 0.04 mg/mL, 0.02 mg/ml, and 0.01 mg/ml. Testing was conducted in triplicates. GraphPad Prism software was used to calculate the % inhibition and to generate the IC<sub>50</sub> curve based on concentration and % inhibition of the treated and non-treated control.

## 9. Liquid Chromatography-Mass Spectrometry

The fraction that was least cytotoxic and most effective was chosen for further characterization via liquid chromatography-mass spectrometry (LC-MS). LC-MS is a powerful tool for the detection of chemical compounds and identifying organic molecules. The fractions were mixed with water and ACN in varying concentrations as described above in section III-3. MS ionizes molecules to facilitate their separation and detection based on their molecular masses and charges via a detector that determines the species and quantity of the ions. This allows for the determination of chemical formulas of molecules. Measurements were performed using an AB Sciex X500R QTOF ESI mass spectrometer. LC flow was split to 500 nL/minute before entering the ion source. Mass spectra were acquired in centroid mode ranging from 150-1000 m/z, resolution  $R = 30000$ . A Luna Omega C18, 150 x 2.1 mm, 1.6  $\mu\text{m}$  column was used, injection volume = 1  $\mu\text{L}$ . A gradient of A) H<sub>2</sub>O + 0.1% FA and B) MeCN + 0.1% FA at a flow rate of 0.55 mL/minute was used to achieve separation. Gradient conditions start at 5% B, increase to 10% B in 1 minute, then to 35% B from minute 1→15, then to 50% B from minute 15→22, and finally to 80% B from minute 22→25. After a one-minute hold at 80% B, the system was re-equilibrated for 5 minute with the initial conditions. UV data was acquired using a PDA (wavelength 200-800 nm  $\pm$  8 nm), MS detection was performed simultaneously.

## **10. Statistical Analysis**

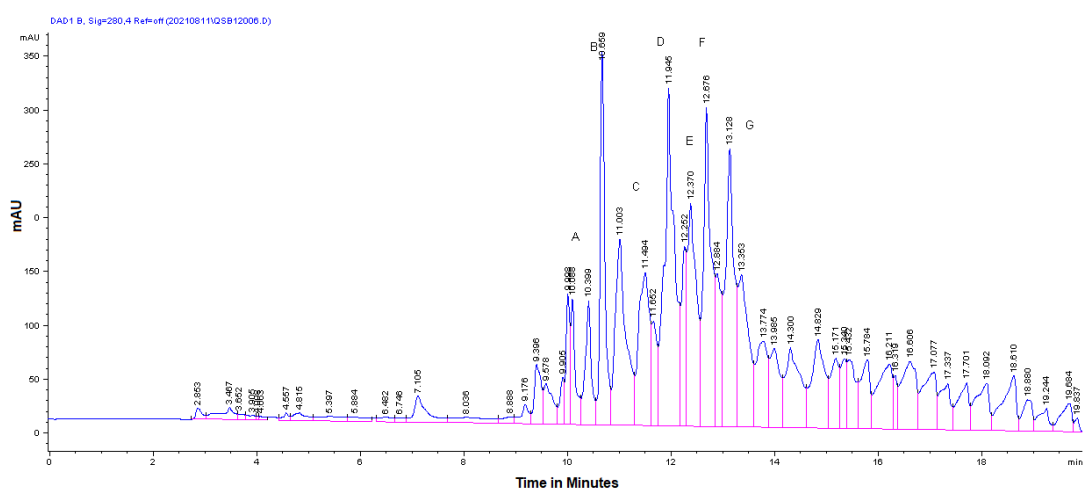
Data are presented as means with standard deviation (SD) derived from triplicates of experimental samples. The data were analyzed using the two-tailed unpaired student t-test using GraphPad prism. A p-value of  $<0.05$  was considered statistically significant.

# CHAPTER 4

## RESULTS

### 1. HPLC Findings

We previously assessed biologically active extracts from soil-dwelling bacterial isolates for their anti-EBV activity. Fractions from some of these extracts were effective and were promising due to their low levels of cytotoxicity. Therefore, we proceeded with purifying and identifying the anti-EBV activity in fractions obtained post high-performance liquid chromatography (HPLC). Four QSB-12 fractions from the previous study were chosen for further characterization based on their limited cytotoxicity and high EBV inhibition in the culture medium. These 4 fractions were combined together and underwent HPLC for separation and purification as well as potential identification of their respective organic molecule constituents (**Figure 1**).



**Figure 1. HPLC chromatogram and the eluted fractions as per time (X-axis) and (milli-Absorbance Units) mAU (Y-axis).** HPLC was conducted on fractions of metabolites from the QSB-12 bacterial isolate.

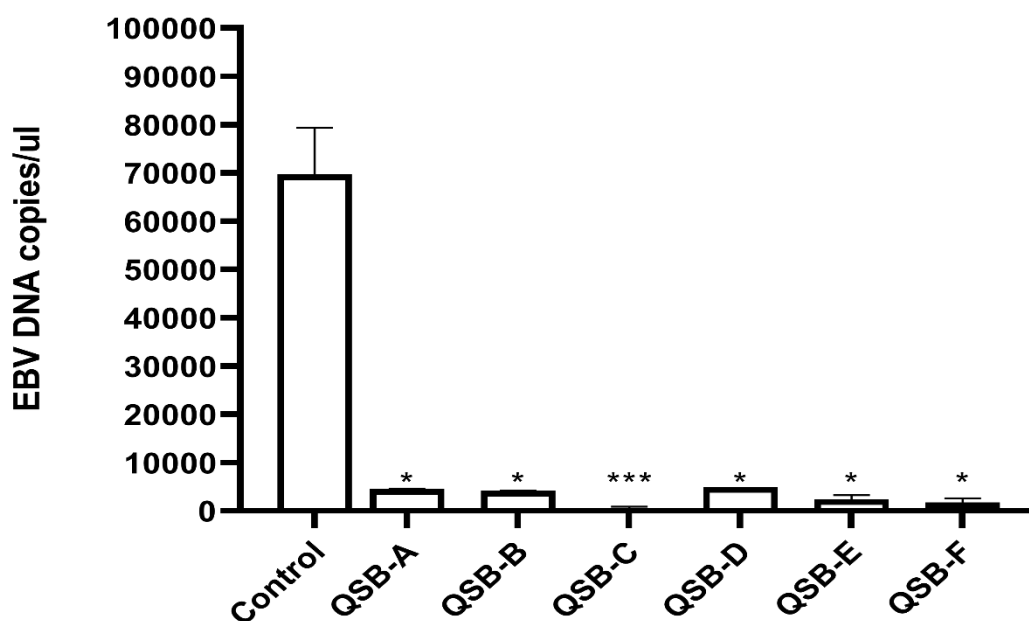
Following HPLC, 7 fractions were selected on the graph based on their distinct peaks/mAU and were then collected based on their time of elution. They were accordingly named QSB-A, B, C, D, E, F and G respective of their time of collection. The acquired samples were then lyophilized. The freeze-dried samples were then collected and weighed on a scale. The resulting yield was as follows, QSB-A = 1 mg, QSB-B = 2 mg, QSB-C = 1 mg, QSB-D = 1 mg, QSB-E = 2 mg, and QSB-F = 1 mg. QSB-G resulted in a low yield of <1 mg, so it was not chosen for further assessment and characterization. The other fractions were dissolved in 250  $\mu$ l of dimethyl sulfoxide (DMSO) per 1 mg to result in a concentration of 4 mg/ml of each fraction.

## **2. Assessment of HPLC-Collected Fractions for Their Anti-EBV Activity**

In alignment with our previous research, a concentration of 0.08 mg/ml of each of the collected 6 samples post-HPLC were assessed for their anti-EBV effect. To assess for this effect, the fractions were incubated with the EBV-producing P3HR-1 cells for 5 days and then the EBV DNA copies in cell-free supernatants (viral load) were quantified by real-time quantitative PCR. Since DMSO was used to solubilize the HPLC fractions, its effects were tested as well in a concentration (v/v = 2%) in correspondence to the concentration of extract that was used (0.08 mg/mL).

Approximately, a 12-fold increase in EBV DNA copies was observed in PMA-induced DMSO-treated cells when compared to non-induced DMSO-treated cells (negative

control). All fractions excluding the fraction QSB-C resulted in approximately a 17-fold decrease ( $p < 0.05$ ) in EBV DNA copies when compared to their respective PMA-induced DMSO-treated control. QSB-C resulted in approximately a 115-fold ( $p < 0.01$ ) decrease per the amount of EBV DNA copies (**Figure 2**).



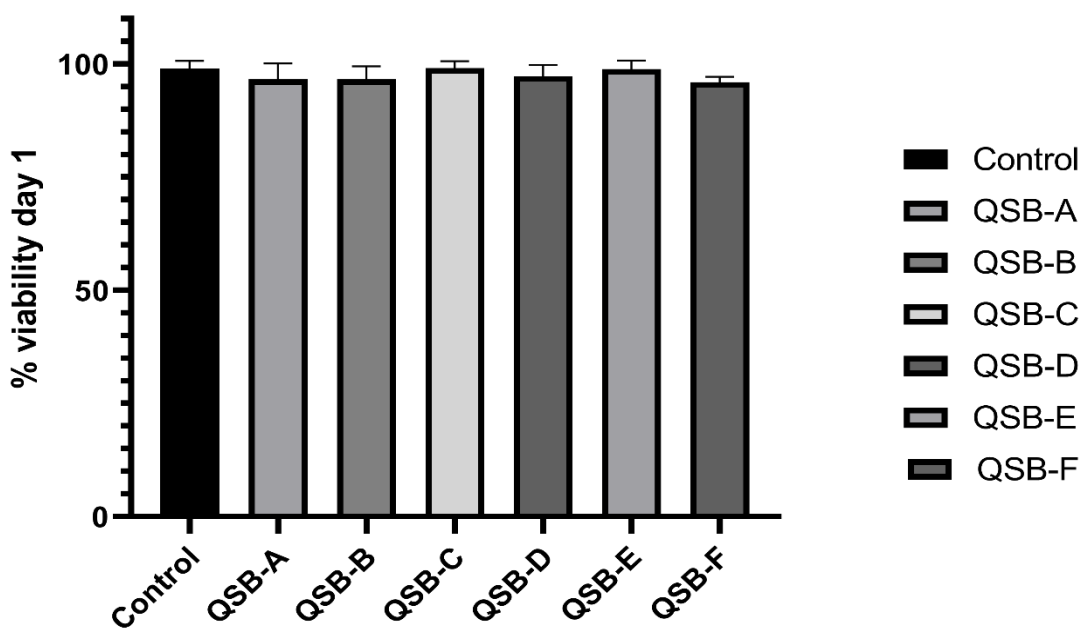
**Figure 2.** The effect of QSB-HPLC fractions on the EBV load from P3HR-1 cells.

The number of EBV DNA copies/ $\mu$ L was quantified using real time PCR in culture supernatants from PMA-induced P3HR-1 cells treated with HPLC-acquired fractions of metabolites from the QSB-12 bacterial isolate. The control included 65 ng/ml PMA and 2% DMSO-treated cells. \*\*\* indicates  $p < 0.001$ ; \* indicates  $p < 0.05$ .

### 3. Assessment of HPLC-Collected Fractions for Their Effect on Cell Viability

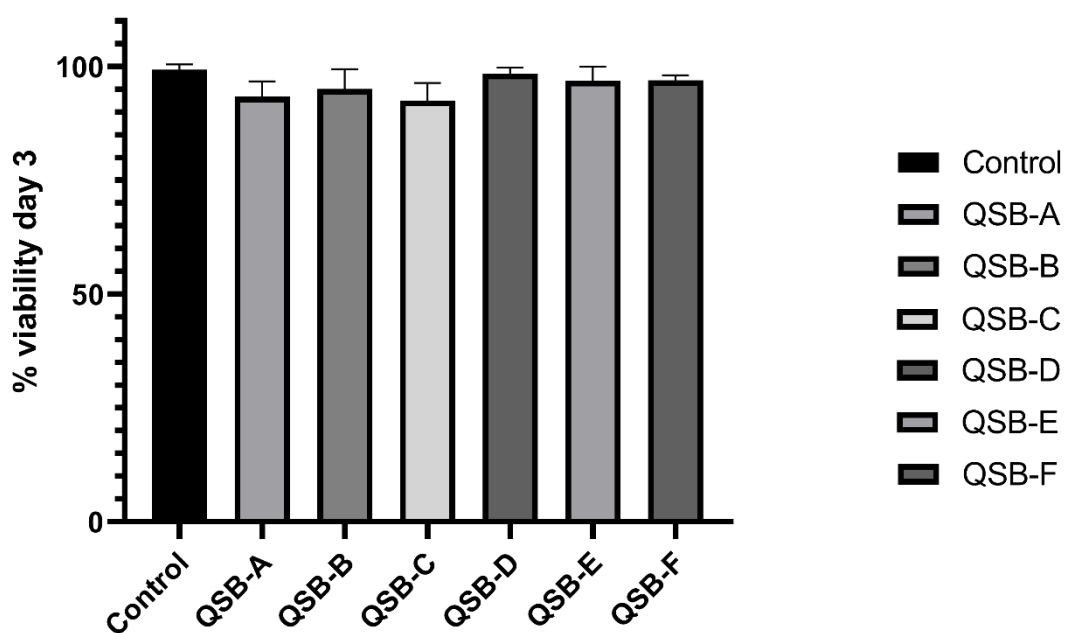
At a concentration of 0.08 mg/mL, the 6 HPLC-acquired QSB fractions (QSB-A, B, C, D, E, F) showed no significant cell death. The % cell viability was  $>90\%$  on days 1 and

3 post-incubation for all the tested fractions when compared to 2% DMSO-treated controls excluding PMA to account for DMSO present in the tested fractions. Cells were incubated for 5 days and were assessed on days 1 (**Figure 3**), 3 (**Figure 4**) and 5 (**Figure 5**) post-incubation using the trypan blue exclusion assay. Percent viability was assessed and calculated based on the description above in section III-7.



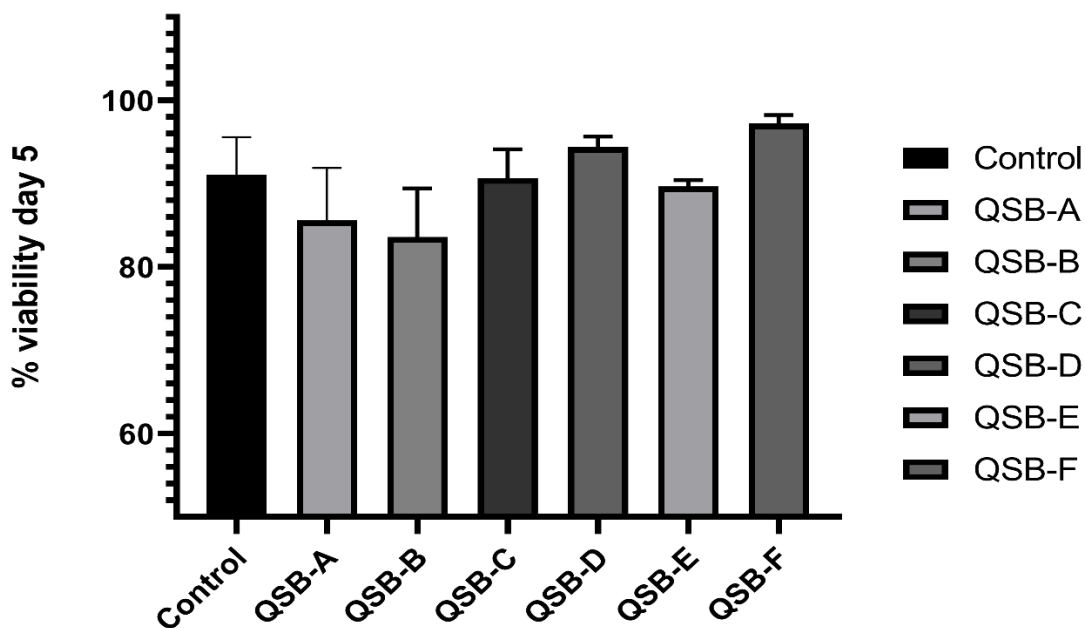
**Figure 3. The effect of HPLC fractions on % viability of P3HR-1 cells on day 1 post-incubation.**

Cell viability was determined using trypan blue staining of P3HR-1 cells treated with HPLC-acquired fractions of metabolites from the QSB-12 bacterial isolate on day 1 post-incubation.



**Figure 4. The effect of HPLC fractions on % viability of P3HR-1 cells on day 3 post-incubation.**

Cell viability was determined using trypan blue staining of P3HR-1 cells treated with HPLC-acquired fractions of metabolites from the QSB-12 bacterial isolate on day 3.



**Figure 5. The effect of HPLC fractions on % viability of P3HR-1 cells on day 5 post-incubation.**

Cell viability was determined using trypan blue staining of P3HR-1 cells treated with HPLC-acquired fractions of metabolites from the QSB-12 bacterial isolate on day 5 post-incubation.

Four fractions on day 5 had a % viability >90%. Most importantly, the fraction of interest QSB-C had a % viability of 90.6%, which is comparable to the control at 91.7%. QSB-A and QSB-B respectively had a % viability of 85.6 and 83.6, which is slightly undesirable indicating that they may not be suitable for a long-term treatment.

#### 4. LC-MS

The fractions indicated above were assessed with LC-MS. Findings indicated that all fractions contained compounds of similar mass and molecular formula; hence, the

compounds detected were likely derivatives of each other. Of interest, QSB-C had a high purity of the compound with exact mass of 245.1271 for an [M+H]<sup>+</sup> ion. The molecular formula was determined to be C<sub>15</sub>H<sub>12</sub>N<sub>5</sub>O, a compound that has not been previously reported to have an anti-EBV activity in the literature (**Figure 6**). The presence of 5 nitrogen atoms with the described molecular formula may indicate a similar molecular structure and function to nucleoside analogues such as ACV.



**Figure 6. LC-MS assessment of the QSB-C fraction.**

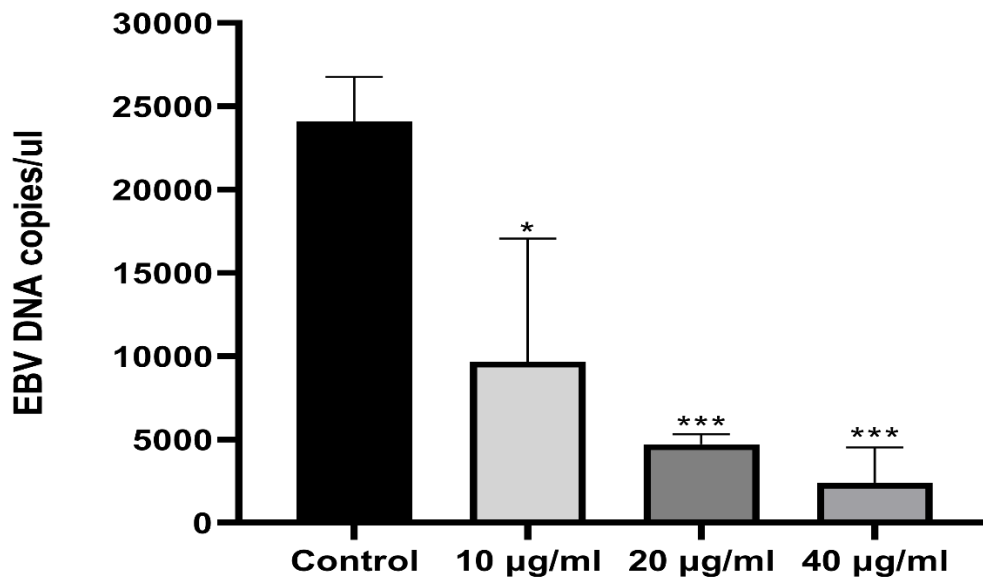
The QSB-C fraction obtained post-HPLC conducted on metabolites produced by the QSB-12 bacterial strain was subjected to LC-MS.

## 5. Assessment of the QSB-C Fraction and Determining Its IC<sub>50</sub> value

Since QSB-C had a very significant inhibition of EBV (\*\*p<0.01) and >90% cell viability, it was selected for further assessment. As mentioned above, LC-MS findings indicated that the compound present in QSB-C may be chemically similar to nucleoside analogues and it is present in 98% purity in this fraction when compared to the other QSB fractions. Per tested preparation, 0.1×10<sup>6</sup> P3HR-1 cells were incubated per well in

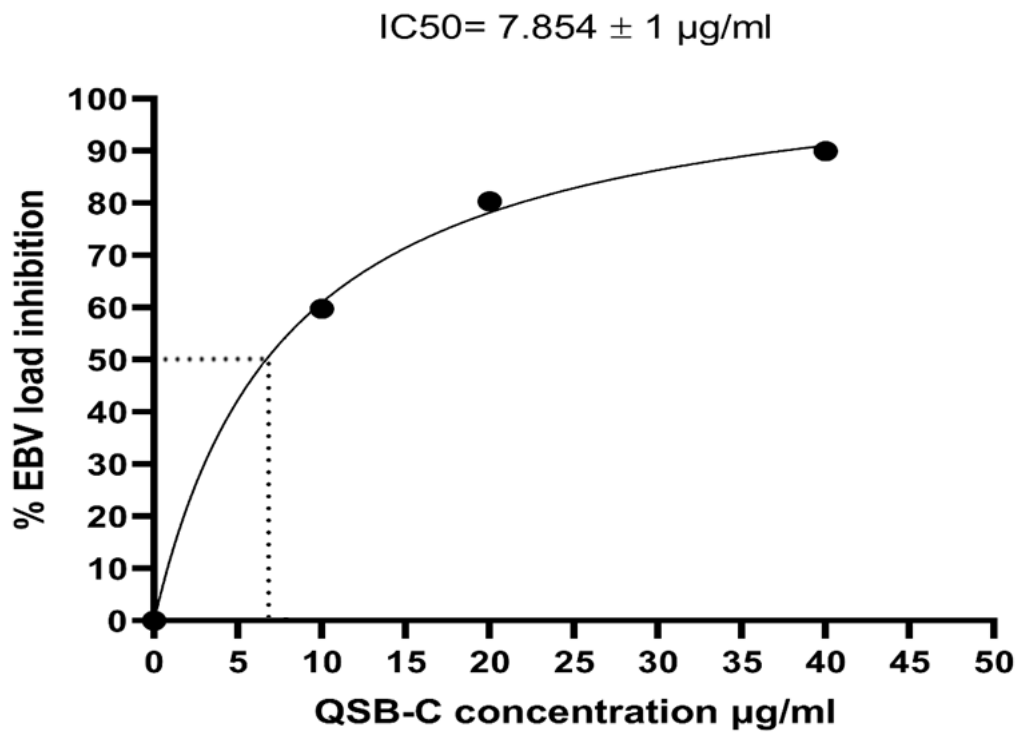
a 96-well culture plate (Corning®, NY) in 250 µL of complete RPMI 1640 (Sigma-Aldrich) with 65 ng/mL of PMA (Sigma-Aldrich). All plates had a concentration of 65 ng/ml of PMA to induce viral replication. QSB-C was serially diluted at a concentration of 0.04 mg/mL, 0.02 mg/ml, and 0.01 mg/ml. DNA extraction followed by RT-PCR were performed to assess for the number of DNA EBV copies in culture supernatants. The findings were compared to a control with  $0.1 \times 10^6$  P3HR-1 induced with 65ng/ml PMA and treated with 1% DMSO similar to the concentration of DMSO found in the diluted, QSB-C treated groups (**Figure 7**).

The IC50 was determined to be a concentration of approximately 7.854 µg/ml. At 0.04 mg/ml, the % inhibition was at 89.98, at 0.02 mg/ml the % inhibition was at 80.38, at 0.01 mg/ml the % inhibition was at 59.79 as indicated in **Figure 8**.



**Figure 7. The anti-EBV effect of the QSB-C fraction in diluted concentrations.**

The number of EBV DNA copies/ $\mu$ L was quantified using real time PCR in culture supernatants from PMA-induced P3HR-1 cells treated with the QSB-C fraction. Cells were incubated with the fraction used at one of three concentrations (0.04 mg/mL, 0.02 mg/mL, or 0.01 mg/ml) for five days. The control included PMA-treated cells with a concentration of 1% DMSO similar to the DMSO concentration found in the QSB-C-treated cells. \*\*\* $p < 0.001$  for QSB-C-treated groups at 0.04 mg/ml and 0.02 mg/ml compared to their respective DMSO and PMA-treated control. \* $p < 0.05$  for QSB-C-treated group at 0.01 mg/ml when compared to its respective DMSO and PMA-treated control.



**Figure 8. IC50 of the QSB-C fraction.**

The number of EBV DNA copies/µL was quantified using real time PCR in culture supernatants from PMA-induced P3HR-1 cells treated with dilutions of the QSB-C fraction. Cells were incubated with the fraction for five days. The % inhibition was determined in comparisons to a control of PMA-treated P3HR-1 cells with a concentration of 1% DMSO similar to the DMSO concentration found in the QSB-C-treated cells.

## CHAPTER 5

### DISCUSSION

EBV is a ubiquitous virus infecting more than 90% of the human population at some stage in their lives. EBV is capable of infecting epithelial cells via a yet to be identified mechanism, and the virus initially proliferates in the oropharyngeal area. However, it preferentially targets naïve B-lymphocytes following the initial primary infection where it proliferates and can establish latency in resting memory B cells. The presence of EBV may increase the risk for recurrent infections, viral reactivation and shedding in saliva where it can be transmitted to new hosts. This increases the risk of EBV associated diseases. The principal route of transmission of EBV is oral, via saliva exchange, but it can also be transmitted, to a lesser extent, via blood or semen (Gerber et al., 1969; Young & Rickinson, 2004). During childhood, primary infection with EBV usually results in an asymptomatic infection or in mild flu-like symptoms; nonetheless, the infected person is now likely a carrier throughout their lifetime. On the contrary, in adolescence or young adults, primary infection likely results in IM where symptoms may be moderate to severe (Young & Rickinson, 2004). EBV is also well associated with some malignancies such as nasopharyngeal carcinoma (undifferentiated), Burkitt's lymphoma, Hodgkin's and non-Hodgkin's lymphomas among others (Fatima et al., 2019; Shair et al., 2008). In addition, EBV is also associated with several autoimmune and inflammatory diseases such as systemic lupus erythematosus and Sjögren's syndrome, rheumatoid arthritis, multiple sclerosis and other diseases (Houen & Trier, 2021). Those who are immunocompromised and/or are undergoing transplants or chemotherapy are particularly susceptible to EBV and its repercussions. The

aforementioned details necessitate finding novel and safe drugs in order to treat EBV and possibly prevent/decrease its associated diseases.

When it comes to synthetic drugs with anti-EBV activity, several drugs have been explored and some were tested clinically. Of importance, nucleoside and nucleotide analogues have been traditionally used to treat herpesviruses. Generally, nucleoside and nucleotide analogues may serve as competitive inhibitors for naturally occurring nucleosides or nucleotides utilized by the viral DNA polymerases to transcribe the viral DNA chain. Ultimately, they are incorporated into the replicating viral DNA chain and this leads to chain termination or significantly impedes the DNA polymerase activity (Poole & James, 2018). Some of the usually used drugs include ACV, GCV, PCV, ADV etc... In the case of EBV, the latter have been found however to be clinically ineffective with development of resistance (Dong et al., 2021).

Natural products are small particles produced/acquired from a biological source. In this sense, a natural product also known as a secondary metabolite is a biochemically dynamic substance that is naturally present in nature and is produced by a living creature (Fatima et al., 2019; Sagaya Jansi et al., 2021). Natural products may be a source or resort to develop a safe and effective drug against EBV. Some of the natural products that have been explored in the literature as potential anti-EBV drugs include, 5-hydroxynoracronycine and Acrimarine F and flavonoids found in citrus plants/fruits (Sagaya Jansi et al., 2021; Takemura et al., 1995). Afromosin and Formononetin found by Konoshima et al., (1989) to be effective against EBV in *in vitro* assessments. Extracts produced by *Eucalyptus* species such as Euglobal 1, Euglobal 2, and Euglobal 3 and Euglobal T1 (Kokumai et al., 1991; Takasaki et al., 1990). A relatively recent study indicated that Lindernia Crustacea plant extracts have an anti-EBV effect by

inhibiting EBV Rta protein during the viral lytic cycle and thereby potentially decreasing viral load *in vitro* (Tsai et al., 2020). Of interest, a clinically effective EBV drug acquired from natural producers has not been developed yet or tested *in vivo*. The current study is unique since it employs the use of environmental bacteria, which has not been previously explored when it comes to a secondary metabolite with anti-EBV activity (Sagaya Jansi et al., 2021).

The need exists to discover a novel treatment against EBV and its associated diseases. The fractions that were acquired post HPLC in our study likely work in a similar fashion to the extracts that were tested in our preliminary research (Shams Eddin, 2021). The findings indicate that a common compound and its possible derivatives are responsible for the anti-EBV activity. The six fractions QSB-A to F were assessed on P3HR-1 cells that are already latently infected with EBV, the compound is suspected to work by inhibiting the late steps of the viral life cycle particularly DNA synthesis and replication. All tested fractions other than QSB-C were able to inhibit the EBV viral load in culture supernatants by approximately 17-folds. QSB-C decreased the EBV viral load by approximately 150-folds and indicated a very significant and potent viral inhibition. At a concentration of 10  $\mu\text{g}$ , the percent inhibition was approximately 60%. The IC<sub>50</sub> value indicated that QSB-C is indeed very potent at approximately a concentration of 7.854  $\mu\text{g/ml}$ . When compared to a drug such as ACV, in a study by Romain et al., (2010), the computed IC<sub>50</sub> of ACV appeared to be 765  $\mu\text{g/ml}$ , which is significantly higher. This was assessed in PMA-induced P3HR-1 cells as well. Yet, unlike the study at hand, the drug was incubated with the cells and PMA for 7 days instead of 5 and different concentrations of PMA and DMSO were used. In future research, ACV and GCV IC<sub>50</sub> can be compared to our drug *in vitro* following a similar

format to what was conducted in this study. In addition, an EBV-negative B cell line or healthy B cells may be used to assess for a potential mechanism of action by using time of addition assays. Briefly, the viral lifecycle can be divided into three stages, which are entry, DNA replication and protein synthesis and finally exit (Ryu, 2017). By incubating the cells with the anti-EBV drug at specific time points then stopping the treatment and comparing that with non-treated controls via DNA extraction and RT-PCR, a specific, inhibited phase of the viral lifecycle may be determined.

LC-MS was also conducted on the fraction of interest QSB-C the findings indicated that a particular compound is responsible for the anti-EBV activity. It has an exact molar mass of 245.1271 g/mol and a formula of  $C_{12}H_{15}N_5O$ . Its structure may likely be similar to other nucleoside analogues. However, to determine stereochemistry and further understand the compound's biochemical properties, nuclear magnetic resonance (NMR) spectroscopy is required.

To conclude, the current study demonstrates that the 6 fractions acquired post-HPLC from the initial QSB-12 bacterial isolate reported previously in preliminary research, can inhibit EBV replication *in vitro* in the P3HR-1 cell line with a high percentage of cell viability. QSB-C in particular had a very potent inhibitory effect and a cell viability that was >90% at day 5 post incubation. A compound of interest with a molar mass of 245.1271 g/mol was detected post LC-MS with high purity in the QSB-C fraction, which is likely correlated with its strong anti-EBV effect. Further research efforts are required to determine its CC50, calculate the selectivity index, which is a measure of a drug's safety and efficacy, compare the drug to other antivirals such as ACV and GCV and determine the safe concentrations to be used *in vivo*. The drug can eventually also be tested on a humanized mouse model and *in vivo* pharmacological

parameters and biologic effects may be assessed. EBV is a widespread virus that currently lacks any effective clinical treatments and is associated with a plethora of conditions such as IM, cancers, autoimmune and chronic inflammatory diseases. Natural products have historically been shown to provide novel drugs with antimicrobial properties. Therefore, the study above has indicated that the compound isolated from soil-dwelling bacteria, present in the fraction QSB-C, may be a resort to treat EBV and its associated diseases.

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