AMERICAN UNIVERSITY OF BEIRUT

# IDENTIFICATION OF ANTI-EPSTEIN-BARR VIRUS COMPOUNDS IN EXTRACTS FROM SOIL-DWELLING MICROORGANISMS

by
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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
of the Faculty of Medicine
at the American University of Beirut

Beirut, Lebanon
April 2021

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# ACKNOWLEDGEMENTS

Foremost, I would like to express my deepest gratitude to my advisor, Dr. Elias A. Rahal for his continuous support, patience, encouragement, thoughtful comments, mentoring, and guidance throughout my time in the lab, and for his assistance and motivation for my future plans. Also, I am extremely grateful to my co-advisor Dr. Antoine Abou Fayad for his considerable support, insightful comments, and guidance throughout this research.

Besides, I would like to thank the rest of my thesis committee: Prof. Ghassan M. Matar, Dr. Hassan Zaraket, and Dr. Zeina Kanafani for their encouragement and considerate comments.

A very special thanks to my fellow lab members, particularly Mr. Hadi Hussein, Mrs. Sukayna Fadlallah, and Dr. Mary-Ann Jallad, and for Dr Abou Fayad's lab members particularly Mr. Bassel Awada and Ms. Ghada Derbaj for their invaluable support and feedback.

Lastly, my sincere thanks go to my support system, my parents, my brother, and my soulmate Rima for supporting me in every way possible. You were a source of endless support, inspiration, and strength.

# ABSTRACT
# OF THE THESIS OF

Marcel Safwan Shams Eddin for Master of Science
Major: Microbiology and Immunology

Title: Identification of Anti- Epstein-Barr Virus Compounds in Extracts from Soil-dwelling Microorganisms

**Introduction:** Epstein-Barr virus infects more than 90% of the human population and establishes latency in resting memory B-cells. It is associated with infectious mononucleosis (IM) and many other malignant and autoimmune diseases. There is currently no available effective and safe therapy for the treatment of EBV. Given that natural products possess antimicrobial properties, our group previously examined secondary metabolites secreted by several soil-dwelling bacterial isolates for their efficacy in inhibiting EBV replication *in vitro.* Natural products from the QSB-12 strain grown in the Vegetative medium and BM-12 strain grown in the A medium had shown an effective anti-EBV activity with no significant cytotoxicity. The complexity and nature of these crude extracts, and their mechanism of action remain to be examined. Therefore, the main objective of this study was to fractionate and purify these crude extracts to isolate effective anti-EBV compounds with minimal cytotoxicity.

**Methods:** Soil samples were taken from Qsaybeh (QSB) and Beit-Meri (BM) regions in Lebanon. Bacterial isolates were inoculated into different productive culture media for secondary metabolite extraction. To assess the antiviral effects of the crude extracts, 0.1 x $10^6$ P3HR-1 producer cells were incubated per well in a 96-well culture plate with 35 ng/mL Phorbol 12-myristate 13-acetate (PMA) and either of two concentrations (0.008 mg/mL and 0.08 mg/mL) of the crude extracts 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 cytotoxic effect of the extracts was assayed using either 0.4% trypan blue applied to cell counting slides and read with an automated cell counter or adding 0.1µg/mL of the DAPI stain and subsequent reading with a BD FACSAria flow cytometer. Cytotoxicity was assessed after an incubation with the extracts under the same conditions mentioned above but excluding PMA, the inducer of EBV production. To fractionate the crude extracts, liquid-liquid partitioning, thin layer chromatography (TLC), and column chromatography were performed, and the resulting fractions were tested for their anti-EBV abilities and cytotoxicities as described for the crude extracts.

**Results:** Crude extracts from the QSB-12 bacterial isolate grown in the Vegetative (Veg) medium showed a very high reduction in the EBV load in cell-free supernatants with minimal cytotoxicity. QSB-12 16S rRNA sequencing showed that the genus of the QSB-12 bacterium is *Pseudomonas*. Liquid-liquid partitioning of the crude extracts prepared from the QSB-12 Veg scale-up led to the preparation of 4 fractions: water, hexane, ethyl acetate, and chloroform. The ethyl acetate and chloroform fractions were able to decrease the EBV load, at the two concentrations tested, by 55-folds ($p=0.001$), with no cytotoxicity observed for the lower concentration (0.008 mg/mL) tested. TLC and column chromatography resulted in the preparation of three subfractions from the ethyl acetate fraction and four from the chloroform fraction. The three ethyl acetate subfractions decreased the EBV load at a concentration of 0.08 mg/mL ($p=0.002$). Two of the ethyl acetate subfractions used at a concentration of 0.08 mg/mL exerted a significant cytotoxicity, indicating that one ethyl acetate subfraction is safe and effective in decreasing the EBV load *in vitro*. On the other hand, three of the four chloroform subfractions resulted in a significant decrease in the EBV load at a concentration of 0.08 mg/mL ($p=0.01$). Only one of the four chloroform subfractions exerted a significant cytotoxicity at a concentration of 0.08 mg/mL, indicating that three chloroform subfractions are safe and two out of them are effective in decreasing the EBV load *in vitro*.

**Conclusion:** Our findings suggest that one or more compounds with an anti-EBV activity are present in fractions from secondary metabolites released by soil-dwelling bacteria. The nature and structure of these compounds, and their respective modes of action remain to be assessed.

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# CHAPTER I

# INTRODUCTION

Epstein Bar virus (EBV) is a human herpes virus that infects more than 90% of the human population. EBV may result in an asymptomatic infection, but it is also the agent of infectious mononucleosis (IM). EBV is also associated with autoimmune and chronic inflammatory diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) as well as with several types of cancers, such as Burkitt's lymphoma (BL), Nasopharyngeal carcinoma (NPC), Post-transplant lymphoproliferative disease (PTLD), in addition to other head and neck cancers. EBV is mainly transmitted orally, it infects epithelial cells and B-lymphocytes and establishes latency in memory B-lymphocytes. EBV persists in infected individuals with the ability to reactivate at any time and be shed in the saliva. Several therapeutic approaches were studied and tested against EBV including many antiviral drugs, but none were able to permanently clear the infection or to ameliorate the symptoms of the diseases caused by or associated with EBV. There are currently no approved or recommended treatments for EBV. Hence, exploring safe and effective novel anti-EBV drugs is a necessity.

Natural products have been essential to drug discovery, as their producers continue to offer new sources of drugs with antimicrobial properties. Secondary metabolites and bio-active molecules released by environmental bacteria, fungi, marine organisms, and plants have been successfully used against infectious and non-infectious disease entities. Crude extracts from the Lebanese soil-dwelling bacterial isolates QSB-12 and BM-12 were previously tested by our group and showed an effective inhibition in EBV production from a viral-producer cell-line. The overall objective of this study

was to isolate anti-EBV compounds with minimal cytotoxic potential from the secondary metabolites present in these extracts.

The specific aims of this study were to:

1. Use bio-guided fractionation to purify secondary metabolites from Lebanese soil-dwelling organisms and to assess their ability to inhibit EBV replication.
2. To assess the cytotoxicity of the fractionated crude extracts and purified secondary metabolites.

# CHAPTER II

# LITERATURE REVIEW

## A. Epstein-Barr Virus

### *1. Discovery*

The Epstein-Barr virus (EBV) was first identified by Anthony Epstein and Yvonne Barr while studying tumor cells from Burkitt's malignant lymphoma. Using electron microscopy, they were able to spot viral particles within these tumor cells; these particles resembled those of Herpes Simplex viruses in structure, but did not have the same size [1]. Henle et al. [2], later indicated EBV as a tumor virus upon detecting high IgA titers for the EBV viral capsid antigen (VCA) and early antigen (EA) complex which decreased after therapy in patients with different types of cancers, such as NPC, BL, different head and neck cancers, with increasing titers in patients with more advanced disease cases. EBV IgA titers were quantified before and after treatment. These antibodies decreased after treatment when compared with the titers before treatment, while they became undetectable in some patients. These antibodies were also detected in patients with IM before and after treatment.

### *2. Structure and Genome*

EBV, also referred to as the *Human herpesvirus 4*, is a γ-herpesvirus that belongs to the *lymphocryptovirus* genus of the *Herpesviridae* family [3]. Like other herpesviruses, EBV has an outermost surface envelope surrounded by glycoprotein spikes. This envelope encloses the nucleocapsid which houses the viral DNA [4]. EBV has a linear double-stranded DNA genome consisting of six EBV nuclear antigen genes

(EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C) encoding for six EBV nuclear antigen proteins, and three latent membrane genes (LMP1, LMP2A, LMP2B) encoding for three latent membrane proteins. EBNA1 is expressed in all virally infected cells and contributes to the conservation and replication of the episomal EBV genome. Episomal DNA is a term used to describe mobile DNA molecules that can integrate into host DNA or form circular DNA molecules. In the case of EBV, after primary infection, the genome remains in an 'episomal' state during latency; it is hence circular, unintegrated within the host genome and competent for replication during latency [5, 6].

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

Epstein-Barr virus is one of the highly prevalent viruses in humans infecting more than 90 percent of the world's population. The major mode of transmission of EBV among individuals is oral through saliva. The viral particles enter the oral cavity and establish a lytic infection in the oropharynx. During the replicative phase, the lytic cycle proteins are expressed, and these include "immediate early", "early", and "late" lytic proteins. The major function of the early proteins is to enhance the expression of the late genes; while the late genes encode structural proteins such as the nucleocapsid proteins and the envelope proteins that aid in viral assembly [3, 7].

The virus infects naïve B-cells by binding to the complement receptor type 2/ cluster of differentiation 21 (CR2/CD21) on the cell surface using the viral glycoprotein gp350. The virus then fuses with the membrane and gets endocytosed into the host cell[8, 9]. After fusion, the virus expresses the full spectrum of latent proteins (latency III program) activating the expression of EBNA1, EBNA2, LP, LMP1, and LMP2A. EBNA2 is mainly involved in the transformation process in which it acts as an adapter

molecule that binds to cellular sequence-specific DNA-binding proteins. The inability of the P3HR-1 EBV strain, which carries a deletion in the EBNA2 gene, to transform B-cells *in vitro* highlights the important role of EBNA2 in the transformation process [10]. The virus is then able to drive the activation and proliferation of these naïve B-cells which can form germinal centers after their migration into lymphoid follicles; this, in turn, starts the second state of transient latency, expressing the 2 major latency proteins LMP1 and LMP2. LMP1 is the major protein responsible for transformation. Upon its expression, LMP1 upregulates the production of surface adhesion molecules and anti-apoptotic proteins [5]. EBNA3 comprises a family of proteins that are expressed in latent EBV infection. EBNA3A and EBNA3C are involved in malignant transformation by interacting with transcription factors. LMP2 is not directly involved in B-cell transformation; it rather guides the B-cells to migrate into mucosal follicles, then form germinal centers, and activate signaling pathways as LMP2A and LMP1 mimic both the B cell receptor (BCR) and CD-40 receptor, respectively [11, 12]. Upon the expression of LMP2A and LMP1, B-cells will be able to survive in the germinal centers and form resting memory B-cells, thus completing the first latency program [3, 8]. EBV also expresses small non-polyadenylated RNAs (EBER1 and EBER2); while the function of these RNAs is still not fully understood, they are expressed in all forms of EBV latent infections, and they seem to regulate translation of proteins like Protein Kinase RNA dependant (PKR) and some interleukins, in addition to message splicing. Both EBERs inhibit PKR activation which stops the phosphorylation of eIF2a, thus resulting in the blockage of inhibition of protein synthesis at the level of initiation; besides, EBER2 and not EBER1 activate the production of interleukin 6 (IL-6) and contribute to the B-cell growth transformation. EBERs also increase the expression of IL-10, IL-9, and insulin-

like growth factor (IGF-1) [5, 13-16]. During cellular mitosis and DNA replication, the host DNA polymerase replicates the cellular DNA (chromosomes) as well as the EBV DNA without the need for the viral polymerase. At this stage, the EBV protein expression is down-regulated, allowing the virus to persist inside B-cells. If these cells re-circulate to the oropharynx, the EBV lytic cycle may be reactivated and the viral DNA polymerase will then synthesize the EBV genome resulting in viral particle production, and shedding again in the saliva [7].

### *4. Infectious Mononucleosis*

EBV infects B-cells as well as epithelial cells after its entry through the oropharynx. It is the major causative agent of IM. The primary infection happens usually in childhood and is most commonly asymptomatic; however, mild symptoms may appear. Infection may occur during adolescence or adulthood and may then manifests as IM; this disease is rarely associated with complications such as organ damage but might end in a chronic infection referred to as chronic active Epstein-Barr virus disease (CAEBV) with elevated EBV DNA, and IM symptoms [5, 17]. Upon primary infection, latency is established in memory B cells. During symptomatic infection, EBV is mainly found in the germinal centers and memory B-cells, however, the viral particles could also be found in some naïve B-cells. IM patients shed high titres of EBV through saliva, due to the increased activation of the EBV lytic cycle [4, 18].

The clinical features that accompany IM range from mild to severe, and they could be generalized/systemic, including fever and malaise or they could be organ specific. The first organ to be affected by the infection is the oropharynx, in which the patients will experience pharyngitis or tonsilitis. The reticuloendothelial system and the

lymphoid system may also be affected, and the patient might experience lymphadenopathy, hepatosplenomegaly, hepatitis, and jaundice. A morbilliform rash might appear if the patient is taking ampicillin or amoxicillin, and in some cases, a systemic pruritic maculopapular rash might appear early in infection, but to a lesser extent. Other organs might be affected, and patients might experience cardiac abnormalities, pneumonitis, kidney filtering problems, central nervous system (CNS) involvement, blood abnormalities, and sometimes secondary infections [19, 20].

The immune response to EBV infection starts with the innate immunity which depends on natural killer cells to clear EBV-infected or transformed B-cells via the NK receptor p46. As such, patients with X-linked lymphoproliferative disease having a defective NK cell immunity are not able to control the primary EBV infection [21]. Humoral immunity also plays a role in the response against EBV in IM patients; EBV antigens trigger the release of IgMs and IgGs heterophile antibodies. Additionally, specific EBV IgM and IgG antibodies targeted against the VCA, EA, EBNA1 and EBNA2 proteins neutralize EBV, facilitate its phagocytosis, and activate the compliment cascade. IgM antibodies decrease within a week of infection, while those of the IgG isotype are long-lasting [22, 23]. T cell immunity also has a role in controlling EBV infection. It is known that IM is associated with an increase in peripheral lymphocyte numbers, with a massive proliferation of activated CD8+ T-cells that kill EBV-infected cells; while clones of cytotoxic effector T-lymphocytes return to normal after resolving the infection, cytotoxic memory T-lymphocytes persist at high circulating frequency. Furthermore, CD4+ T-cells are not highly increased in response to EBV infection, rather, they might still appear in the peripheral blood but show low T-Cell Receptor (TCR) specific clonal expansion [24, 25].

### *5. Other EBV Related Diseases*

EBV persists in memory B cells in all infected individuals. It succeeds to infect more than 90% of the human population and remains inactive in many infected individuals [26]. EBV appears to be found in patients suffering from solid tumors as well as lymphomas. These cancers include but are not limited to BL, Hodgkin's disease (HD), NPC, and lymphoproliferative diseases. EBV is associated with lymphoproliferative diseases such as oral hairy leukoplakia and post-transplant lymphoproliferative diseases (PTLD) [12, 27]. In addition, the expression of EBV latent proteins differs between EBV-associated cancers. Immunocompromised patients such as those undergoing organ transplantation, or patients suffering from the Acquired Immunodeficiency Syndrome (AIDS) are more prone to EBV-associated cancers that might be more aggressive and often fatal [28]. This highlights the importance of the immune system integrity in preventing EBV-driven tumorous transformation.

Lymphoproliferative diseases like immunoblastic lymphomas and PTLDs are more common in EBV-infected immunocompromised patients, particularly those with cytotoxic T lymphocyte deficiencies; cytotoxic T lymphocytes are, after all, well-known to play a pertinent role in blocking the growth of EBV-infected cells [12]. LMP1 acts as a constitutively active receptor, recruiting cellular signaling molecules like the tumor necrosis factor receptor-associated factor (TRAF) to mimic the signals of the active CD40 receptor in EBV-infected B lymphocytes. LMP1 continual expression in EBV infected patients, is responsible for the transformation of EBV infected cells to Hodgkin's lymphoma [29, 30]. EBV is also associated with BL. EBV gene products such as EBNA-1 and the EBERs increase the likelihood of generating MYC-activating translocations that are directly associated with BL. They also aid BL cells in survival

and immune evasion through reducing the expression of MHC class I, transporter associated with antigen processing (TAP), and the proteosome subunit LMP7 in tumor cells [31, 32]. All of these cancers, among many others, appear in patients latently infected with EBV, with varying expression of EBV genes [33]. EBV is also associated with autoimmunity, and this association was first identified in patients suffering from SLE; these patients had high levels of anti-EBV antibodies [34]. Interleukin 17A (IL-17A), a proinflammatory cytokine that was shown to be associated with autoimmune diseases, was studied by our group in patients suffering from rheumatoid arthritis (RA). The average IL-17A serum levels and EBV DNA copy numbers showed a propensity for linearity among RA patients when compared to non-RA controls. Our group also demonstrated a role for endosomal Toll-like receptors (TLR3, 7, and 9) in the EBV DNA-mediated triggering of IL-17A production in mice [35]. Furthermore, we also showed that EBV DNA enhances the production of IL-17A from T-helper 17 (Th17) cells to produce IL-17A via TLR9 in mice [36].

### *6. EBV Therapeutic Approaches*

As stated earlier, EBV infection in children is usually asymptomatic; however, in adolescents and adults, it may result in a symptomatic infection termed IM. In most cases, IM resolves in patients without the need for therapy; however, in some cases, the disease might be accompanied by complications that demand therapeutic intervention.

a. Antiviral Therapy of EBV Infections

Supportive therapy is helpful in cases of mild IM. On the other hand, severe and complicated IM is mainly treated with corticosteroids and to a lesser extent with some antivirals like acyclovir (ACV) among others, depending on the severity of the

infection. However, no antiviral drugs are approved for the treatment of EBV infections [37, 38].

Drug candidates studied against EBV could be synthetic or natural. Synthetic drugs are discussed in this section, while anti-EBV drug candidates derived from natural products are discussed below in section B-5.

Synthetic EBV drugs fall into two groups, the first group includes nucleoside analogues such as ACV, ganciclovir (GCV), penciclovir (PCV), and their prodrugs, as well as nucleotide analogues like cidofovir (CDV) and adefovir (ADV), in addition to the pyrophosphate analogue foscarnet. While the second group includes compounds like maribavir (MBV) and indolocarbazole, unlike the nucleoside and nucleotide analogues, both MBV and indolocarbazole do not have an activity on the viral DNA polymerase [39].

Many studies have been conducted on ACV. Pagano et al. [40, 41], demonstrated the inhibitory effect of ACV against EBV in lymphoblastoid cells with no cytotoxicity reported. This drug was also tested *in vitro* in Vero B-cells and was found to be effective against Murine Gamma herpesvirus 68 which is very similar to EBV, and *in vivo* in a Severe Combined Immunodeficiency (SCID) mouse model [42]. The proposed mechanism of action of ACV is the interaction of the EBV DNA polymerase with the triphosphate moiety of ACV. This nucleoside analogue is added into the nascent EBV DNA strand thus forming a dead-end complex that prevents the DNA polymerase from continuing the elongation process. ACV was used in clinical trials in patients with IM and managed to decrease oropharyngeal viral secretion, but it did not show any improvement of clinical symptoms [43]. Oral ACV was used to treat AIDS individuals suffering from oral hairy leukoplakia. While after treatment, ACV regressed

oral hairy leukoplakia occurrences, some recurrences were manifested when the treatment was stopped. In contrast, in 1989, it was reported that there was a fatal case of lymphoproliferative disorder with no response to ACV [44, 45].

Even though ACV was found to be effective against EBV, it did not affect the duration of the clinical symptoms among IM patients. Moreover, according to a study by Rafaidilis et al. [46], patients receiving antiviral monotherapy consisting mainly of ACV or other antivirals had a higher mortality rate compared to patients receiving immunosuppressive therapy either with ACV alone, or with a combination of antivirals. It is worth mentioning that another study by Hoshino Y. et al. [47], showed that the use of valacyclovir (VACV) in healthy volunteers positive for EBV for a long period of time decreased the population of EBV-infected B-cells while keeping the EBV DNA copies per B-cell the same. This suggests that the usage of VACV for a long period of time might be able to eradicate EBV from the body only if reinfection does not occur. Moreover, ACV does not exert any inhibitory effect on latent EBV infection. There are many reasons that underlie the failure of ACV to clear the infection and lessen the IM symptoms. EBV is shed in the saliva, while ACV oral administration does not achieve the needed concentration of the drug that is able to clear EBV infected cells in the oropharynx [48-50]. It is suggested that another reason behind the failure of this antiviral therapy in minimizing the symptoms of IM is that these symptoms are due to the immunopathologic effects of EBV-infected B-lymphocytes rather than the viral cytopathology in infected tissues [39]. Even though ACV is still one of the most tested and used drugs among many available treatments for IM in immunocompetent patients with severe symptoms, neither ACV nor VACV are yet recommended by the Center of Disease Control and Prevention (CDC) and have not been shown to be beneficial in

immunocompromised patients in the studied clinical trials. These drugs are also not yet approved by the Food and Drug Administration (FDA) as a treatment for EBV. Hence, further regimen testing or drug modifications may result in better efficacy and safety [46, 47, 51, 52].

On the other hand, GCV which is also a nucleoside analogue, has also been tested and used in the treatment of acute IM; it succeeded in decreasing the incidence of primary EBV acute infection by 45% in pediatric renal transplant patients after receiving grafts from EBV positive donors, in addition to decreasing the EBV viral load among infected individuals. GCV however, appears to be more toxic than ACV, which might prevent its use in non-severely affected patients [53].

Additionally, the prodrug valganciclovir (VGCV) was tested on twenty-six men in a randomized, double-blind, placebo-controlled study, aiming to examine its effect on EBV oral shedding. VGCV significantly decreased the proportion of days with EBV shed in the saliva and the quantity of the virus present [54]. Moreover, the efficacy and safety of VGCV were tested in 47 children previously infected with EBV that had undergone liver transplantation. PTLD occurred in 1 child (2.1%), while 47% of the children had undetectable EBV-DNA under prolonged treatment, suggesting that VGCV could lead to a viral load drop [55].

Another drug assessed for the treatment of IM is valomaciclovir (VALM). This is a prodrug derived from H2G – a guanosine nucleoside. It led to a better outcome when studied in acute IM patients compared to the placebo group. Concerning the oral viral load, VALM-treated individuals had a lower median EBV load when compared to the placebo group, indicating an antiviral effect of VALM on EBV *in vivo*. Epiphany Biosciences announced a phase 2 clinical trial with VALM that appears to have broad

antiviral activity against herpes zoster and EBV-IM. Yet, further research and trials are still needed to evaluate the efficacy and safety of VALM in EBV infected individuals [47, 56].

One of the newly examined drugs is MBV; it is an oral benzimidazole L-riboside, that was previously tested and shown to be effective against the human cytomegalovirus (HCMV). Whitehurst C. et al. [57], demonstrated its inhibitory effect against EBV DNA replication in BL cell lines. Its half maximum inhibitory concentration (IC50) was 0.15 to 1.1 μM, with at least 10-fold more potency than ACV. Additionally, unlike the other nucleoside analogues, MBV inhibits the phosphorylation of the viral DNA processivity factor (BGL4). They have identified a decrease in the EBV transcript levels suggesting a relation between the BGL4 inhibition and a decrease in the expression of multiple viral RNAs. EBV BGL4 kinase has many viral targets, hence, the MBV's inhibitory effect on the EBV BGL4 must be further investigated [57-59].

Yao G. et al. [60], also evaluated the antiviral effect of a novel L-nucleoside, 2'-fluoro-5-methyl-p-L-arabinofuranosyluracil (L-FMAU), on EBV replication *in vitro*. L-FMAU belongs to the family of nucleoside analogues, yet it has a unique mode of inhibition of the EBV DNA polymerase. The 5'-triphosphate of L-FMAU did not appear to be a substrate for the EBV or the cellular DNA polymerase, yet it could inhibit the elongation process, 3' to 5' exonuclease activity and nucleotide turnover [59, 61, 62].

Acyclic adenosine analogues including (S)-9-(3-hydroxy-2-phosphonyl methoxypropyl) adenine {(S)-HPMPA}, known as CDV, and 9-(2-phosphonyl-methoxyethyl) adenine {PMEA}, known as ADV, were also demonstrated as having an

antiviral activity against EBV in P3HR-1 cells and Raji cells. These drugs have a broad antiviral activity including Human Immunodeficiency virus (HIV), HCMV, and herpes simplex virus type 1 (HSV-1) [63-66]. The use of CDV with Rituximab in a patient with PTLD led to a complete remission from an EBV-associated lymphoma with neurological symptoms, while keeping the EBV DNA negative when tested by Polymerase Chain Reaction (PCR) [67]. Another study demonstrated the enhanced apoptotic activity of CDV when used with ribonucleotide inhibitors such as hydroxyurea and didox against EBV infected NPC cells. It was also shown that the use of this combination was effective in athymic mice, indicating that ribonucleotide analogues should enhance the anti-tumor activity of nucleoside phosphonate analogues. However, given that CDV is nephrotoxic, and hydroxyurea leads to myelosuppression in human beings; in the indicated study, mice lost 10% of their weight, suggesting that less cytotoxic and more effective combinations should be developed [68]. Hence, many prospective antiviral treatments for Epstein-Barr virus are being tested for their efficacy in clearing EBV-infected lymphocytes and diminishing the symptoms of IM, but very few are recommended to be used in critical cases and immunocompromised patients, with none of these treatments having been approved for their ability to clear an EBV infection.

b. Other Novel Anti-EBV Therapeutic Approaches

The treatment of infectious mononucleosis and EBV-associated diseases is challenging to healthcare professionals as the efficacy of available medications was shown to be low whether in treating IM or other diseases associated with EBV infection. Therapeutic approaches include but are not limited to antivirals, immunotherapy, and gene therapy [69].

In addition to the antivirals described above, gene therapy was also used to treat EBV infections and EBV-associated cancers. EBV-positive NPC cells were used to study the efficacy of one of the gene therapies used in the treatment of EBV *in vitro* with a novel replication-deficient adenovirus vector (ad5.oriP). This vector targets the oriP region of EBV DNA which is a region that supports replication and stability of the episomes. The expression of the transgene is under the transcriptional regulation of the family of repeats (FR) domain of the oriP region of EBV. Endogenous EBNA1 which binds to the FM activates the expression of down-stream genes, including the transgene (p53 tumor suppressor gene) and consequently expressing apoptotic proteins yielding significant cytotoxicity and EBV-positive cell death. These results indicate that targeting the oriP sequence could increase gene expression yielding apoptosis specifically in EBV-positive NPC cells in the context of the adeno virus vector [70]. The induction of the EBV lytic cycle by certain pharmacological agents that activate viral-induced kinases which phosphorylate the nucleoside analogue GCV makes GCV cytotoxically more effective. Hydroxyurea was also tested and found to be effective in reducing EBV episomes *in vitro* but was shown to have some toxicity and a limited clinical efficacy in EBV-positive AIDS-related CNS lymphomas [70-72].

Immunotherapy has also been used to target EBV-positive cancers by the infusion of cytotoxic T cells that recognize EBV antigens. EBV-associated NPC and HD have been treated with autologous or donor-derived cytotoxic T lymphocytes directed against EBV antigens. This treatment was effective, but tumor-escape should be taken into consideration because of the low immunogenicity of some subdominant epitopes [73]. Additionally, EBV associated PTLD has also been treated with cytotoxic T lymphocytes. This therapy was used at first to treat EBV positive post-transplant

lymphomas, by using EBV latent-antigen-specific effector T cells. Even though, the number of people suffering from PTLD is low (1-5%) and finding HLA-matching donors then preparing safe and EBV-specific CTLs might be a limiting factor, this therapy was used in prophylaxis and showed high potency in treating existing diseases like immunoblastic lymphoma after 4 CTL infusions, for instance [74].

Even though the EBV genome being fully sequenced improved the therapeutic approaches against this virus, none of the tested therapies has been approved. Hence, discovering novel drugs that are effective in improving the symptoms of IM and eliminating latently EBV infected cells or assessing new treatment modalities for agents that are available is of pertinence [5].

## B. Natural Products as Anti-infectives

Natural products have been used to treat cancers and infections among other human diseases. These products could be secondary metabolites secreted by soil or sea-dwelling bacteria or fungi. Alternatively, they could be plant and environmental products or biomolecules. The wide use of natural products in the treatment of diseases has shown a decline in recent years [75]. However, natural producers continue to provide new sources of compounds that have low molecular weights and diverse structures. These compounds serve as potential novel therapies for emerging infections [76].

### *1. Secondary Metabolites*

Secondary metabolites, in other words, natural products, are compounds secreted by an organism that are non-essential for the growth, development, or

reproduction of the organism. They are often secreted by organisms as a result of an environmental change or against natural predators as self-defense mechanism. Secondary metabolites are extracted from fungi, plants, marine environments, marine algae, marine sponges, and environmental bacteria among other sources [77, 78]. One of the optimal ways to enhance the diverse production of secondary metabolites is to optimize the chemical and physical conditions, in other words, the environmental conditions that the natural producers are living in. For instance, Rajnisz, A., et al. [79], tested and optimized the conditions needed to allow *Streptomyces sp.* 8812 isolated from Brazilian soil to produce biologically active secondary metabolites. The 2 compounds isoquinoline alkaloid, 7-hydroxy-6-oxo-2,3,4,6-tetrahydroisoquinoline-3-carboxyl acid (C10H9NO4) and protoalkaloid, N-acetyl-3,4-dihydroxy-l-phenylalanine (C11H13NO5) were found to exhibit antimicrobial activities. Several additives that enhance the production of secondary metabolites were tested. These variables include the bacterial utilization of nitrogen and carbon sources, bacterial biomass growth, pH differences, age of the seed culture, amino acids supplementation, and resin addition to the production media [79].

### *2. Natural Products from Environmental Bacteria*

Several bacterial species, such as *Streptomyces spp., Actinomycetes spp.* and *Pseudomonas spp.*, are found in the environment and are used as sources of secondary metabolites. Actinomycetes are environmental bacteria that were discovered to produce secondary metabolites with anti-infective properties. The fact that the pathogenic bacteria can only survive for a short period of time in soil suggested that soil microbes can successfully inhibit disease-causing bacteria. These secondary metabolites comprise

a major portion of the antibiotics present in the market [80]. These include aminoglycosides and tetracyclines secreted by *Actinomycetes spp.*, and ivermectins and rapamycins secreted by *Streptomyces spp.* [76]. One of the most identified bacterial and fungal species in soil is *Streptomyces*, which implies that *Streptomyces* possess a high capability of resisting environmental conditions including the inhibition of other organisms. The natural product Actinomycin which was secreted after the culture of 244 *Actinomycetes* possessing antimicrobial properties gave Waksman's group the first promising lead for the presence of an antibiotic released from soil microorganisms, for example [81].

### *3. Natural Products as Antivirals*

Natural products were tested for their antiviral activity against a variety of DNA and RNA viruses. The immune system is in most cases responsible for clearing viral infections without the need of treatment. However, some viruses acquired the ability to cause serious infections, resist available drugs, and evade the immune system resulting in latent or long-life infections [82]. For this reason, it is of great importance to look for antivirals that target and clear viral infections with latent properties, primarily the ones associated with later complications. Kim et al. [83], in 2015, examined extracts of halophilic actinomycetes for anti-viral activity against the Porcine Epidemic Diarrhea virus (PEDV). Through extensive chromatographic separation techniques, they were able to purify Xiamycin C and Xiamycin E and to determine their molecular formula using high-resolution fast atom bombardment mass spectrometry. The inhibitory activities of Xiamycin C-E and the previously reported Xiamycin A were determined and their EC50 ranged between 0.93μM and 10μM. Additionally, Kim et al, studied the

modes of action of the identified Xiamycins, and they appeared to significantly reduce viral RNA levels and spike protein synthesis [83]. Other drugs identified from natural products include, for instance, actinohivin (AH) which is an anti-HIV protein derived from actinomycete genus *Longispora albida*; AH binds to the HIV gp120 aiming to inhibit its entry into cells [84]. Additionally, butenolide was isolated from *Actinobacteria* and found to be effective against Influenza A virus. Its mode of action was also studied, and Heme Agglutinin (HA) appeared to be one of the targets of butanolide [85].

Other compounds present in extracts released by extremophilic actinomycetes appear to exert an antiviral activity against influenza viruses (strains H7N1, H5N3, H1N1, H3N2) and paramyxoviruses (strains Sendai virus and Newcastle Disease virus). Their activity was tested against hemagglutinin and neuraminidase proteins of the studied viruses. They also compared the activity of the studied extracts with the most commonly used influenza drugs like Rimantadine, Oseltamivir, and Flavovir, yielding similar inhibitory activity [86]. These highlight the potential broad antiviral activities that natural products might possess.

### *4. Anti-Herpesviridae Natural Products*

Many previously used and newly discovered natural products have been studied for their inhibitory activity against the *Herpesviridae* family. Natural products from fungi, plants, and environmental bacteria were studied against herpes viruses, and many of them yielded promising results.

An acidic protein bound polysaccharide (APBP) was isolated from the fungus *Ganoderma lucidum* and found to have an anti-HSV activity by plaque reduction assay

*in vitro*. The $EC_{50}$ was 300 μg/mL with no cytotoxicity[87]. Sarkar S. et al. [88], tested *Lentinula edodes* extracts for their anti-HSV-1 activity. The extracts were able to block HSV-1 replication at a late stage in the virus replication cycle. Many other antimicrobials derived from fungal secondary metabolites are still under investigation [88-91]. Ojha D. et al. [92], in 2013, identified the anti-infective ability of natural products from *Odina wodier Roxb* (OWB) against several agents, including the HSV-1 and 2. Subsequent fractionation was performed, and the methanol extract exerted more than 99% inhibition for HSV-1 and 2. To determine the mode of action of the compound, the extract was added at different time points (pre-entry, entry, and post-entry) of the viral life cycle, and they determined the efficacy and cytotoxicity by plaque reduction assay and MTT assay, respectively. The active compound appeared to bind to the viral particles and inhibit the attachment and penetration into Vero cells, with no cytotoxicity. Lin T. et al. [93], have demonstrated the antiviral activity of chebulagic acid (CHLA) and punicalagin (PUG) on viruses that use glycosaminoglycans for entry. These plant extracts were tested against HCMV, hepatitis C virus (HCV), dengue virus (DENV), measles virus (MV), and respiratory syncytial virus (RSV), *in vitro*. CHLA and PUG significantly reduced viral replication, without causing significant cytotoxicity. These extracts had blocked all steps of infection for the HCMV, HCV, and MV. Extracts from another plant, *Terminalia chebula Retz,* were tested by Kesharwani, A., et al. [94], for any potential anti-HSV-2 activity. The extracts were tested in Vero cells for their cytotoxicity, and they yielded 95% cell viability at 200 μg/mL. Then, the potential antiviral activity was examined at various concentrations; the plant extracts showed a dose-dependent *in vitro* anti-HSV-2 activity. Unlike ACV, these extracts successfully inhibited viral attachment and entry into the

Vero cells. Nevertheless, in the post-infection plaque assay, the *T. chebula Retz* extracts showed lower IC50 values than ACV.

*Eucheuma* is one of the most commonly used plants for medicinal purposes. Natural products secreted by *Eucheuma* have been tested for their anti-tumor, anti-bacterial, immunoregulatory, and antiviral activities. Polysaccharides (EGP) from *Eucheuma gelatinae* were tested for their anti-HSV 1 and anti-HSV 2 activities in Vero cells. EGP showed the highest potency when it was added directly after HSV infection, suggesting that EGP's antiviral activity is exerted at the early stages of infection, through directly inactivating viral particles and suppressing viral attachment to cells [60].

Digitoxin, which is a natural product secreted by *Digitalis lanata Ehrh* appears to also inhibit HSV-1 *in vitro* at an EC50 of 0.05μM. The highest potent antiviral activity of the drug was exerted at early and late stages of the infection, indicating that Digitoxin might be inhibiting viral attachment and release [95, 96].

On the other hand, Sacramento D. et al. [97], identified the antimicrobial effects of secondary metabolites secreted from *Streptomyces spp.* isolated from Brazilian tropical soil against several infectious agents, including HSV-1.

These examples among many other natural products were tested for their anti-herpesviridae activity. While some of them showed high potency in inhibiting viral replication *in vitro* with minimal cytotoxicity, further studies are to be made regarding the potency of inhibition and cytotoxicity *in vivo* models and in clinical trials, before being approved as antivirals.

### *5. Anti-EBV Natural Products*

Several natural products were studied for their anti-EBV activity. Triterpenoids extracted from the fungus *Ganoderma lucidum* were assayed for their inhibitory effects on EBV activation. The inhibitory effect was tested on the induction of EBV-EA TPA-induced Raji cells. All of the 16 *G. lucidum* triterpenoid constituents that were tested showed high potency (96-100%) in inhibiting the EBV-EA induction by TPA, while preserving a high cell viability (around 70%) [98]. Another study by Zheng D. et al. [99], further tested the inhibitory effect of five *G. lucidum* triterpenoids on EBV, through inhibiting EBV-EA, EBV-CA, and telomerase activity. Triterpenoids did not affect cell viability, while decreasing the EBV-EA, and EBV-CA significantly. Additionally, the telomerase activity was decreased by the 5 compounds tested, with no significant change in the inhibition among the 5 compounds. The molecular docking was also studied and compound 1 appeared to be capable of completely entering and blocking the binding pocket of the enzyme.

Two extracts, an ethanolic extract and andrographolide, from the plant *Andrographis paniculata Nees* were tested against EBV and found to be effective in inhibiting the expression of the EBV lytic proteins in P3HR-1 cells. To understand the mode of action of these extracts, transient transfection using a plasmid containing the BRLF1 and BZLF1 genes was performed. The lack of the expression of the replication and transcriptional activators (Rta) and (Zta) appeared to be due to the inhibition of the expression of two immediate early genes BRLF1 and BZLF1 that encode for Rta and Zta, respectively. The two extracts did not exert any cytotoxic effects on the cells, showing that these extracts might be used as potential anti-EBV drugs [100].

The bioactive constituents of the plant *Lindernia crustacea* were found to exert an inhibitory effect against EBV *in vitro* through hindering the expression of the Rta in the EBV lytic cycle. Thirty-three compounds were isolated and tested; most of them exerted a significant antiviral effect when compared to controls [101].

*Polygonum cuspidatum* is a medicinal plant often used in Asia; Yiu C. et al. [102], tested the inhibitory effect of the compounds in the ethyl acetate fraction of the *P. cuspidatum* roots on the EBV lytic cycle. Emodin, a major compound in the ethyl acetate fraction inhibited the transcription of the EBV immediate early genes, EBV lytic proteins, EBV-EA, and EBV DNA replication in P3HR-1 cell lines, without being toxic to cells at concentrations below 6.3 μg/mL. Emodin was further tested for its inhibitory effect on EBV reactivation and for its repression of NPC tumorigenesis. Emodin inhibited the expression of EBV lytic proteins along with blocking virion production in EBV-positive epithelial cell lines. Emodin also appeared to inhibit Zta and Rta promoters preventing EBV reactivation in addition to inhibiting the tumorigenic properties accompanied by EBV reactivation. This compound repressed the micronucleus formation, cell proliferation, migration, and Matrigel invasiveness, thus preventing tumorigenesis. Emodin was also found to repress the tumorous growth induced by EBV in mice after being injected with EBV-infected NPC cells. These results show that emodin could be a potential drug that can inhibit EBV reactivation and NPC recurrences [103].

Anti-EBV natural products showing a high potency in inhibiting EBV replication *in vitro* should be further studied and tested *in vivo* for their efficacy in clearing EBV infected cells, preventing EBV-induced transformation, and lessening the symptoms of IM, without causing cell and tissue damage.

# CHAPTER III

# MATERIALS AND METHODS

## A. Soil Sample Preparation

Soil samples from the Qsaybeh (QSB) and Beit Meri (BM) regions in Lebanon were collected, placed in a beaker, and covered with an aluminum foil. The beaker was then placed in an incubator to dry for 7 days at 37°C. Three grams of each soil sample were taken, and 100 mL of distilled water was added. Then, the obtained mixture was heated on a stirrer at 55°C for 30 minutes. The mixture was then left for 3 minutes at room temperature for sedimentation. Then a volume of the supernatant was taken from the sedimented preparation and was serially diluted with autoclaved distilled water using dilution factors of 5, 10, 100, and 1000, having a total volume of 1 mL for each dilution. The measures were as follows:

1/5: 200 µL non-diluted mixture + 800 µL distilled water

1/10: 500 µL of the 1/5 mixture + 500 µL distilled water

1/100: 100 µL of the 1/10 mixture + 900 µL distilled water

1/1000: 100 µL of the 1/100 mixture + 900 µL distilled water

The prepared dilutions as well as the starting heated solution were each inoculated onto International Streptomyces Project medium (ISP3) agar- which is used to isolate and grow *Streptomyces* species, and soil agar plates. The ISP3 agar constituents are 20 g/L of oats (Commercial), 18 g/L of agar (Lab M, Neogen Heywood, UK), and 2.5 ml/L of ISP3 trace elements ($FeSO_4$, $MnSO_4$, and $ZnSO_4$) prepared in house, while the Soil agar constituents are 30 g/L of dried soil, 10 g/L of

starch, and 18 g/L of agar (Lab M, Neogen). The soil used in the Soil agar is autoclaved soil obtained from the same source that was sampled.

### B. Primary Inoculation

Samples were inoculated onto sterile ISP3 agar and soil agar plates prepared as indicated above and incubated at 28°C for 14 days in an incubator (Amerex Instruments, Concord, CA). The culture plates were checked regularly for growth.

After 14 days, the detected colonies were subcultured onto sterile ISP3 agar plates. After inoculation, multiple successive passages were performed on ISP3 agar plates until only uniformly shaped colonies indicating likely purity were observed. Subsequently, the colonies were suspended in a 50% glycerol: 50% water aqueous solution purchased from Sigma-Aldrich, Darmstadt, Germany, and stored at -80°C.

### C. Secondary Inoculation

A starter culture was initiated as a first seed by inoculating 35µL of the bacterial stock originally stored in glycerol in 5 mL of ISP3 broth, and then incubating the culture for 48 hours on a shaker at 150 revolutions per minute (rpm) at 28°C. Two mL of the first seed were then removed and inoculated as a second seed into 20 mL of fresh liquid ISP3 media (prepared as indicated in section III-A above, however excluding the agar), also cultured for 48 hours on a shaker at 150 rpm at 28°C. Afterwards, 1 mL of the second seed was removed and used to inoculate 50 mL of 14 different types of production media (**Table 1**) in an Erlenmeyer flask, as a small scale culture. The cultures were kept on a shaker at a speed of 150 rpm at 28°C for 7 days. These 14 types of production media were used to promote bacterial production of secondary

metabolites (natural products) with possible anti-EBV effects. The small-scale culture was conducted to perform general screening of crude extracts for their anti-EBV effect as indicated below in section D. Media resulting in viral replication inhibition in the general screen were then used in scaled-up culture preparations as described in section F below.

**Table 1: Components of the Veg, A, B, C, and RA3 production media.**

| Component (g/L) | Vegetative | A | B | C | RA3 |
|---|---|---|---|---|---|
| Peptone | 5 | 4 | | | 2 |
| Soluble starch | 20 | | | | |
| Dextrose | | | | | |
| Meat extract | 2 | 4 | | | |
| Yeast extract | 3 | 2 | | | 4 |
| Malt extract | | | | | 10 |
| Soy-bean meal | 2 | 2 | | | |
| Glucose | | | | | 10 |
| Triptose | | | | | |
| Maltose | | 20 | | | |
| Dextrin | | 10 | | | |
| CaCO3 | 1 | | 0.1 | 0.1 | |
| Glycerol | | | 20 | | 5 |
| Glycine | | | 2.5 | 2.5 | |
| NaCl | | | 1 | 1 | |
| KH2PO4 | | | 1 | 1 | |
| FeSO4 | | | 0.1 | 0.1 | |
| MgSO4.7H2O | | | 0.1 | 0.1 | |
| MgCl2.6H2O | | | | | 2 |
| Tween 60 | | | | 20 | |
| pH | 7 | 7 | 7 | 7 | 7.4 |

**Table 2: Components of the GPMY, V6, GYM, M8, NL2, and COM production media.**

| Components (g/l) | GPMY | V6 | GYM | M8 | NL2 | COM |
|---|---|---|---|---|---|---|
| Potato starch | 20 | | | | | |
| Peptone | | 5 | | | | |
| Soluble starch | | | | 20 | 30 | |
| Dextrose | | | | | | |
| Meat extract | | 5 | | 2 | | |
| Yeast extract | 5 | 5 | 4 | 2 | 2.5 | |
| Malt extract | 5 | | 10 | | | |
| Soy-bean meal | | | | | | |
| Glucose | | 20 | 4 | 10 | | 25 |
| Dextrin | | | | | | |
| CaCO3 | | | | 3 | 10 | 2 |
| Glycerol | 20 | | | | | |
| Glycine | | | | 4 | | |
| Hydrolyzed casein | | 3 | | | | |
| NaCl | | 1.5 | | | | 2 |
| KH2PO4 | | | | | | 0.15 |
| Molasses | | | | | 20 | |
| Soy flour | | | | | 15 | 25 |
| Dried beer yeast | | | | | | 3 |
| Ammonium Sulfate | | | | | | 2 |
| Soybean Oil | | | | | | 3 |
| pH | 7.02 | 7.05 | 7 | 7 | | 8.4 |

## D. Small Scale Secondary Metabolite Production and Extraction

As described above, the 14-production media were inoculated and then incubated on a shaker at a speed of 150 rpm at 28°C for 7 days. At the end of the

incubation period, 1 mL of sterile Amberlite XAD 16N (Sigma-Aldrich) resin solution, pre-washed in acetone and water, was added into each of the 14 culture flasks containing the 50 ml of media. Several alternating resin washes were performed with acetone and water. Then several washes with water were performed to ensure no acetone remains in the resins. Non-polar XAD is used to adsorb organic substances from aqueous solutions and polar solvents. The Amberlite XAD 16N resins are hydrophobic polyaromatic compounds used to remove hydrophobic compounds up to a Molecular Weight (MW) of 40,000 and hence allow isolation of large organic molecules, particularly proteins [104]. The resin-culture mixture was then left on a shaker at a speed of 150 rpm at 28°C for 3-4 hours. Then, the mixture was centrifuged in a Thermo Scientific (Waltham, MA) centrifuge at 4000 rpm at 4°C for 20 minutes. The supernatant was discarded, and the pellet that contains the secondary metabolites-resin mixture was transferred into a new flask and resuspended in an acetone-methanol solution (30 mL acetone + 10 mL methanol) under a fume hood for 2 hours at room temperature, to extract the secondary metabolites. Subsequently, the resins were filtered using a glass wool, and the liquid phase was then kept in the fume hood at room temperature for the acetone-methanol to evaporate and for the crude extracts to dry. Then, the remaining crude extracts were dissolved in 1 mL of 100% dimethyl-sulfoxide (DMSO) purchased form Sigma-Aldrich, and stored at -20°C.

### E. Bacterial 16S Ribosomal Ribonucleic Acid (rRNA) Sequencing

For Deoxyribonucleic acid (DNA) extraction, the QIAamp DNA Mini kit (50) from Qiagen, Germantown, MD, was used. The manufacturer’s instructions were used

to extract the bacterial DNA. A NanoDrop (DeNovix, Wilmington, DE) spectrophotometer was then used to quantify the DNA concentrations.

PCR was performed on the 16S rRNA gene of the extracted DNA. PCR was performed twice, each time with a different set of forward and reverse primers (Macrogen, Seoul, South Korea). The primer sequences (Macrogen) were:
SSU-bact-27F: 5'-AGAGTTTGATCMTGGCTCAG-3'
SSU-bact-519R: 5'-GWATTACCGCGGCKGCTG-3'

The FIREPol Master Mix (Solis BioDyne)- as per manufacturer's instructions, was used and the PCR was run in a Bio-Rad T100 Thermal Cycler. For the used primer three steps were included: an initial step of activation was at 95°C for 2 minutes, then 30 cycles at 94° and 53°C (annealing) for 30 seconds each, and at 72°C for 1 minute. After a final step of 72°C for 10 minutes, samples were subsequently stored at 4°C.

Only the QSB-12 isolate was sent for sequencing, and PCR samples were run using the SSU set of primers.

Gel Electrophoresis was used to visualize the amplified PCR products. The migrated fragments were visualized under an Ultra-violet (UV) illuminator (Ultra-Violet Products, San Gabriel, CA). PCR samples were subsequently sequenced by Macrogen using Sanger sequencing (South Korea). The sequences were then aligned using the Molecular Evolutionary Genetics Analysis Program (MEGA) and the National Center for Biotechnology Information (NCBI)'s Basic Local Alignment Search Tool (BLAST).

## F. QSB-12 Vegetative (Veg) Scale-up and Secondary Metabolite Production and Extraction

The same procedure for culture described in section III (A and B) was repeated; however, for scaling up secondary metabolite preparation, we increased the total volume of the bacterial inoculum to obtain a larger volume of extracts. In summary, pure bacterial colonies prepared and stored as indicated in section III A and B, were inoculated into 2 Erlenmeyer flasks each containing 5 liters of production media. A total volume of 10 L of media was used in the scale-up (200 times) as opposed to the 50 mL of media for the small-scale. In particular, we scaled up cultures of the QSB-12 isolate in the Vegetative (Veg) medium, whose secondary metabolite extracts showed one of the highest reduction in EBV load in our experiments, with very minimal cytotoxicity.

After 7 days of incubation on a shaker at 150 rpm, at 28°C, the culture was removed, and sterile 100 mL of Amberlite XAD 16N resin (Sigma-Aldrich) pre-washed in acetone and water (as stated in section III-D) was added into each flask. Then, the supernatant was discarded, and the secondary metabolite-resin mixture was resuspended in an acetone-methanol solution. The resin was then discarded, and the solution was left 4-5 days in the fume hood for evaporation. The remaining crude extract was then dissolved in 1 mL of 100% DMSO and stored at -20°C. In other words, the same procedures for small-scale secondary metabolite production and extraction as described in section III (C and D) were repeated, but on a larger scale.

## G. Liquid-liquid Partitioning

Liquid-liquid partitioning was performed to separate the crude extracts into fractions. From the scale up preparations dissolved in 100% DMSO, 800 µL were taken

and put on a lyophilizer (Labconco, Kansas City, MO), then subjected to liquid-liquid partitioning. A mixture of water and methanol was added to the dried crude extract of the scale-up (180 mL of methanol and 20 mL of water). Subsequently, three rounds of separation were performed using a separation funnel by 3 different organic solvents with a volume of 200 mL each (hexane, ethyl acetate, and chloroform). Each round of separation included a single organic solvent, starting with hexane, then chloroform and finally with ethyl acetate. These three rounds led to the formation of the 4 fractions, water, hexane, ethyl acetate and chloroform. The fractions were then left to dry in the fume hood, and then dissolved in 1.5 mL of 100% DMSO and stored at -20°C.

**H. Thin Layer Chromatography (TLC)**

TLC was used to optimize the solvent system needed for separation to be able to perform the column chromatography, and then to determine the number and purity of compounds present in the fractions obtained after column chromatography, which verifies if separation was done successfully. The fractions with the most strong inhibitory activity against EBV were dried using a lyophilizer (Labconco) then dissolved with 1 mL of methanol. A metal TLC silica gel plate purchased from Sigma-Aldrich, Saint Louis, MO, was placed in a chamber filled with different concentrations of organic solvents, to reach the optimum liquid solvent which was in our case: 90% DCM and 10% methanol. It was ensured that the spots on the TLC were not below the solvent level to avoid being washed into the solvent. Then, the plate was removed with a forceps after the solvent front had risen to the top (1 cm below the top of the plate), and a pencil was used to mark the solvent front. Then, the plate was viewed under UV

light (Ultra-Violet Products) and analyzed depending on the migration and separation of the compounds.

## I. Column Chromatography

The impure, similar but non-identical samples from the TLCs were loaded onto a Silica gel adsorbent column (Acros Organics, Fair Lawn, NJ). Then, 100% dichloromethane (DCM), 90% DCM:10% methanol, 80% DCM:20% methanol, 70% DCM:30% methanol, 60% DCM:40% methanol and 50% DCM:50% methanol were sequentially added each at a total volume of 200 mL. Consequently, the compounds were separated according to their different polarities and migrated through the column at different rates. The eluted fractions were then collected and then the tubes were subjected again to TLCs. TLC analysis was repeated using 90%DCM:10% methanol (mobile phase) on all tubes collected, to confirm that the fractions were successfully separated. All tubes showing the same level of migration of compounds were combined and named as a particular fraction. The tubes were then left to dry in the fume hood at room temperature, and then dissolved in 1.5 mL of 100% DMSO and stored at -20°C.

## J. Cell-lines

P3HR-1 is a BL cell line that grows in suspension, and which is latently infected with an EBV type 2 strain [105]. This cell line is an EBV producer and was obtained from the American Type Culture Collection (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% $CO_2$ in a humidified incubator (Thermo Scientific).

Cell passaging was performed every 2-4 days maintaining cells at a confluency of 4 x $10^5$ - 1 x $10^6$ cells/mL.

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

For induction of cells and release of EBV, 0.1 × $10^6$ P3HR-1 cells were cultured for 5 days at 37°C and 5% $CO_2$ in complete RPMI 1640 medium containing 35 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.

## L. Effect of Extracts from Soil-dwelling Bacteria on EBV

Bacterial crude extracts, secondary metabolite fraction and subfraction preparations previously 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×$10^6$ 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 35 ng/mL of PMA (Sigma-Aldrich) and one of two concentrations (0.008 mg/mL and 0.08 mg/mL) of the bacterial crude

extracts, fraction or subfraction preparations. PMA was added to induce the lytic replication cycle of the virus. Controls included cells alone, cells with 35 ng/mL PMA, in addition to cells treated with 35 ng/mL PMA and 0.8% or 0.08% DMSO; the latter two controls were included to account for the DMSO concentration present in the bacterial extract preparations. Applying a 0.008 mg/mL or 0.08 mg/mL of extracts results a concentration of 0.08% or 0.8% 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-K. 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 bacterial extracts on viral production and release into the culture medium.

**M. 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), 0.05μg/μL glycogen (Qiagen), 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, 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 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 -20ºC.

## N. Real-time PCR

EBV DNA copies were determined using quantitative real-time polymerase chain reaction in a Bio-Rad CFX96$^{TM}$ 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' [106]. Real-time qPCR was performed in a reaction volume of 10µL containing: 5µL SYBR, 2µL 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:

$$\text{EBV DNA copies/}\mu\text{L} = \frac{Extracted\ DNA\ Resuspension\ Volume \times F \times X}{Total\ volume\ per\ culture\ well}$$

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.

**O. Cytotoxicity Assay**

To verify that inhibition of viral production is due to the antiviral activity of the bacterial secondary metabolites rather than a cytotoxic effect, cells were tested for their viability. $0.1\times10^6$ P3HR-1 cells were incubated per well in a 96-well culture plate (Corning®) with the extracts at the same concentrations described in section III-L, however, excluding PMA. Controls included cells alone, cells with 0.8% DMSO, and cells with 0.08% DMSO. Cytotoxicity was assayed after 1, 3, and 5 days of incubation at 37°C in presence of 5% $CO_2$ in a humidified incubator (Thermo Scientific). Cell viability was assessed by two methods:

The first method 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 cell counting slides (Bio-Rad) and assessed using a TC20 ™ Automated Cell Counter (Bio-Rad).

The second method employed flow cytometry using a BD FACSAria™ (Biosciences, Franklin Lakes, NJ). The same time intervals and conditions of incubation were applied as indicated in the previous paragraph. DAPI stain was purchased with an original concentration of 5 mg/mL from Molecular probes (Eugen, OR), and used for assessing cell viability in the flow cytometer. A concentration of 0.1 µg/mL of DAPI was used for cell staining as per manufacturer's instructions. After 5 days of incubation with the extracts, cells were centrifuged at 800 rpm for 8 minutes and the supernatants

were discarded. Then, the cellular pellet was resuspended in 200 μL of 0.1μg/mL DAPI stain diluted in PBS and was analyzed via the BD FACSAria for cell viability. Controls for the BD FACSAria ™ included cells resuspended with 1% PBS (Sigma-Aldrich).

% Cytotoxicity was determined using the following formula:

% Cytotoxicity=

$$\frac{(average\ number\ of\ living\ cells\ in\ controls - average\ number\ of\ living\ cells\ per\ treatment)}{average\ of\ living\ cells\ in\ controls} \times 100$$

## P. Statistical Analysis

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

# CHAPTER IV

# RESULTS

## A. Crude Extracts General Screening

### *1. The Effect of QSB-12 and BM-12 Crude Extracts on the EBV Load from P3HR-1 Cells*

16 Crude extracts prepared from the bacterial strains, QSB-12 and BM-12, isolated from Lebanese soil samples and prepared as described in the methods section, were assessed for their anti-EBV effects. To assess this effect, crude extracts 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 crude extract preparations, its effects were tested as well in the two concentrations (v/v% of 0.08 and 0.8) corresponding to the two concentration of the extracts used (0.008 mg/mL and 0.08 mg/mL), respectively. About a 4-fold increase in the number of EBV DNA copies was observed in PMA-induced DMSO-treated cells when compared to induced non-DMSO-treated cells, and non-induced non-DMSO-treated cells (mock treated). Hence, the two employed DMSO-treated concentrations (v/v% of 0.08 and 0.8) showed no inhibitory effect on viral replication. Among the crude extracts prepared from the QSB-12 strain upon culture in different productive culture media, 6 extracts (V6, Com, A, Veg, Ra3, Nl2) in the two concentrations used (0.008 mg/mL and 0.08 mg/mL) were able to significantly reduce the EBV DNA copies by approximately a 40-fold decrease ($p<0.05$), when compared to their respective DMSO-treated controls (**Figure 1**). On the other hand, among the BM-12 crude extracts prepared from the BM-12 strain also cultured in different productive culture media, 5 extracts (A, Veg, B, C, V6)

successfully decreased the EBV DNA copies by about 30 folds ($p<0.05$), compared to the DMSO-treated controls at the concentrations tested (0.008 mg/mL and 0.08 mg/mL) (**Figure 2**).

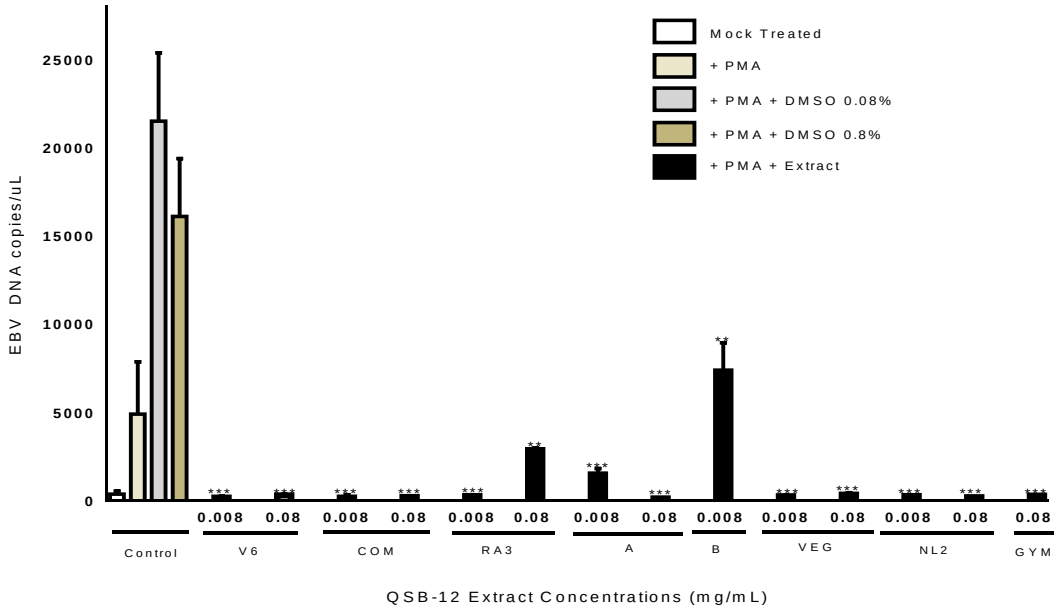


**Figure 1. The effect of QSB-12 crude extracts on the EBV load from P3HR-1 cells.**

The number of EBV DNA copies/μL was quantified in culture supernatants from PMA-induced P3HR-1 cells treated with crude extracts from the QSB-12 bacterial isolate grown in different culture media. Cells were incubated with the extracts used at one of two concentrations (0.008 mg/mL or 0.08 mg/mL) for five days. Controls included untreated cells (mock treated), PMA-treated cells, PMA and 0.08% DMSO-treated cells in addition to PMA and 0.8% DMSO-treated cells. Usage of at an extract at 0.008 mg/mL results in a DMSO concentration of 0.08% while usage of an extract at 0.08 mg/mL results in a DMSO concentration of 0.8% in the culture supernatant. ** $p<0.05$, ***$p<0.01$ for extract-treated groups compared to respective DMSO-treated

control groups.

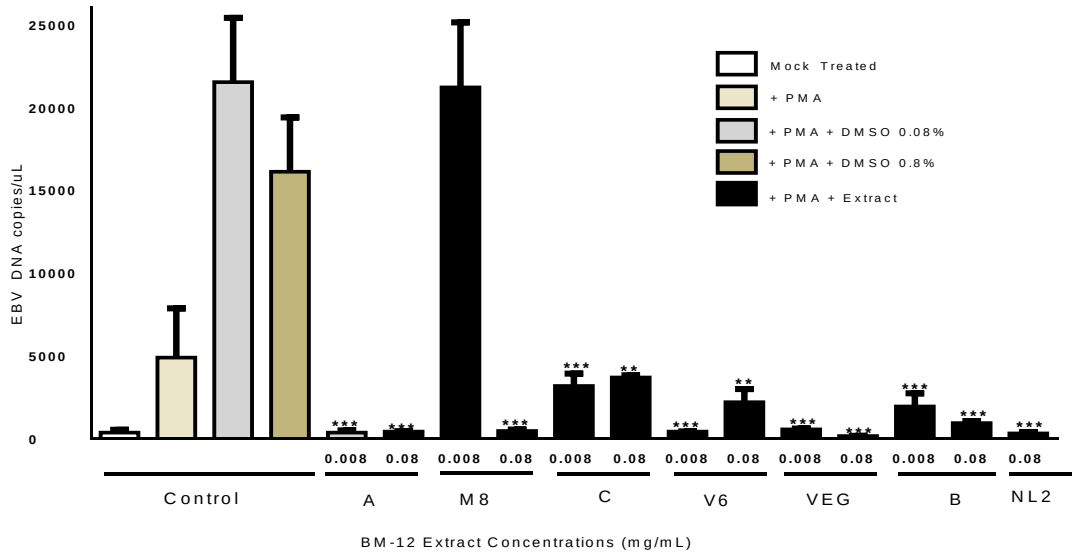


**Figure 2. The effect of BM-12 crude extracts on the EBV load from P3HR-1 cells.**

The number of EBV DNA copies/μL was quantified in culture supernatants from PMA-induced P3HR-1 cells treated with crude extracts from the BM-12 bacterial isolate grown in different culture media. Cells were incubated with the extracts used at one of two concentrations (0.008 mg/mL or 0.08 mg/mL) for five days. Controls included untreated cells (mock treated), PMA-treated cells, PMA and 0.08% DMSO-treated cells in addition to PMA and 0.8% DMSO-treated cells. Usage of at an extract at 0.008 mg/mL results in a DMSO concentration of 0.08% while usage of an extract at 0.08 mg/mL results in a DMSO concentration of 0.8% in the culture supernatant. ** $p<0.05$, ***$p<0.01$ for extract-treated groups compared to respective DMSO-treated control groups.

The viral load reduction by the QSB-12 and BM-12 crude extracts varied but was mostly consistent in both concentrations used (0.008 mg/mL and 0.08 mg/mL). Among the cells treated with the QSB-12 crude extracts, all 6 extracts mentioned above strongly decreased the EBV load. Two concentrations 0.008 mg/mL and 0.08 mg/mL of the crude extracts of the QSB-12 strain in the 6 productive media indicated above showed relatively potent reduction in decreasing the EBV load. The two concentrations used (0.008 mg/mL and 0.08 mg/mL) from the QSB-12 crude extracts grown in the Veg medium had an effective EBV load reduction of 98.94% and 98.03%, respectively (**Table 3)**. Additionally, the cells treated with the BM-12 crude extracts in the 5 productive media indicated above showed a potent reduction in decreasing the EBV load. The 2 concentrations (0.008 mg/mL and 0.08 mg/mL) of the crude extracts of the BM-12 strain grown in the A medium showed the highest viral load reduction of 98.38% and 97.44%, respectively in decreasing the EBV DNA load (**Table 4**).

**Table 3: Percent reduction of the EBV load by QSB-12 crude extracts.**

| *Strain* | *Extracts* | *(mg/mL)* | *% Reduction* |
|---|---|---|---|
| *QSB-12* | V6 | 0.008 | 99.50436 |
| | | 0.08 | 98.50199 |
| | Com | 0.008 | 99.27695 |
| | | 0.08 | 98.84326 |
| | A | 0.008 | 93.18434 |
| | | 0.08 | 99.32581 |
| | Ra3 | 0.008 | 98.86964 |
| | | 0.08 | 82.27737 |
| | Nl2 | 0.008 | 98.89175 |
| | | 0.08 | 98.94976 |
| | Veg | 0.008 | 98.94342 |
| | | 0.08 | 98.03341 |

**Table 4: Percent reduction in the EBV load by BM-12 crude extracts.**

| *Strain* | *Extracts* | *(mg/mL)* | *% Reduction* |
|---|---|---|---|
| *BM-12* | A | 0.008 | 98.38528 |
| | | 0.08 | 97.44044 |
| | Veg | 0.008 | 97.49464 |
| | | 0.08 | 98.14 |
| | M8 | 0.008 | 0 |
| | | 0.08 | 97.21377 |
| | C | 0.008 | 85.27514 |
| | | 0.08 | 77.14769 |
| | V6 | 0.008 | 98.0875 |
| | | 0.08 | 86.47086 |
| | Nl2 | 0.08 | 98.13588 |
| | B | 0.008 | 91.04312 |
| | | 0.08 | 94.27736 |

### *2. Cytotoxicity of QSB-12 Veg and BM-12 A Crude Extracts on P3HR-1 Cells*

Our group had previously tested the cytotoxicities of the crude extracts mentioned above. Since the extracts from the QSB-12 Veg and BM-12 A did not show a significant cytotoxicity, we repeated only those crude extracts. After determining the % reduction of the crude extracts from the QSB-12 strain grown in the Veg medium, and the BM-12 strain grown in the A medium, a cytotoxicity assay was performed to rule out a cytotoxic effect for the crude extracts. 0.08% DMSO-treated cells showed 12.6, 2.7, and 1.7% cytotoxicity on days 1, 3, and 5, respectively. The 0.8% DMSO-treated cells were also tested and showed 6.8, 3.8, and 2.4% cytotoxicity on days 1, 3, and 5, respectively. These were not significant when compared to the DMSO negative controls, indicating that 0.08% and 0.8% DMSO does not lead to a significant cell death. The QSB-12 Veg crude extracts did not show any significant cytotoxicity on days 1, 3, and 5. 0.008 mg/mL of QSB-12 Veg crude extracts yielded 0, 2.1, and 3.2%

cytotoxicity on days 1, 3, and 5, respectively. Similarly, 0.08 mg/mL of QSB-12 Veg crude extracts yielded 11.6, 2.1, and 5.6% cytotoxicity on days 1, 3, and 5, respectively. The BM-12 A crude extracts did not result in a significant cytotoxicity either. 0.008 mg/mL of the BM-12 A crude extracts yielded 0, 0.7, and 3.9% cytotoxicity on days 1, 3, and 5, respectively. Likewise, 0.08 mg/mL of the BM-12 A crude extracts generated 4.6, 3.2, and 0.3% cytotoxicity on days 1, 3, and 5, respectively (**Table 5**). Hence, the decrease observed in the viral load from P3HR1 cells with these extracts was due to an antiviral effect rather than a toxic effect exerted on the cells.

**Table 5: Percent Cytotoxicity of the QSB-12 Veg and BM-12 A crude extracts on P3HR-1 cells**

| | *% Cytotoxicity* | | |
|---|---|---|---|
| | *Day 1* | *Day 3* | *Day 5* |
| *0.08% DMSO* | 12.6 ± 0.7 | 2.7 ± 2.3 | 1.7 ± 1.7 |
| *0.8% DMSO* | 6.8 ± 6.2 | 3.8 ± 1.2 | 2.41 ± 1 |
| *QSB-12 Veg 0.008 mg/mL* | 0 ± 1.7 | 2.1 ± 1.1 | 3.2 ± 2.5 |
| *QSB-12 Veg 0.08 mg/mL* | 11.6 ± 0.7 | 2.1 ± 1.5 | 5.6 ± 1 |
| *BM-12 A 0.008 mg/mL* | 0 ± 1.5 | 0.7 ± 1 | 3.9 ± 1 |
| *BM-12 A 0.08 mg/mL* | 10.4 ±5.6 | 3.2 ±3.2 | 0.3 ±1.5 |

## B. QSB-12 16S rRNA Sequencing

Due to the high inhibitory activity of the QSB-12 Veg on EBV replication with a minimal cytotoxicity on P3HR-1 cells, this strain was submitted for 16S rRNA sequencing. The QSB-12 bacterial genus appeared to be *Pseudomonas***.** However, to identify the species and strain of the QSB-12 bacterium, Whole Genome Sequencing (WGS) will be later performed.

## C. QSB-12 Veg Crude Extract Scale Up

### *1. The Effect of QSB-12 Veg Scaled Up Crude Extracts on EBV Load from P3HR-1 cells*

To increase the amount of the secondary metabolites produced, the QSB-12 Veg was scaled up, and the secondary metabolites produced were retested. The BM-12 A is reserved for further possible scale up and testing. Crude extracts from the QSB-12 Veg scale up were able to significantly decrease the EBV load in the cell-free supernatants in the two concentrations used (0.008 mg/mL and 0.08 mg/mL) when compared to their respective DMSO-treated controls, with the lower concentration (0.008 mg/mL) yielding a 25-fold decrease (p=0.001) (**Figure 3**).

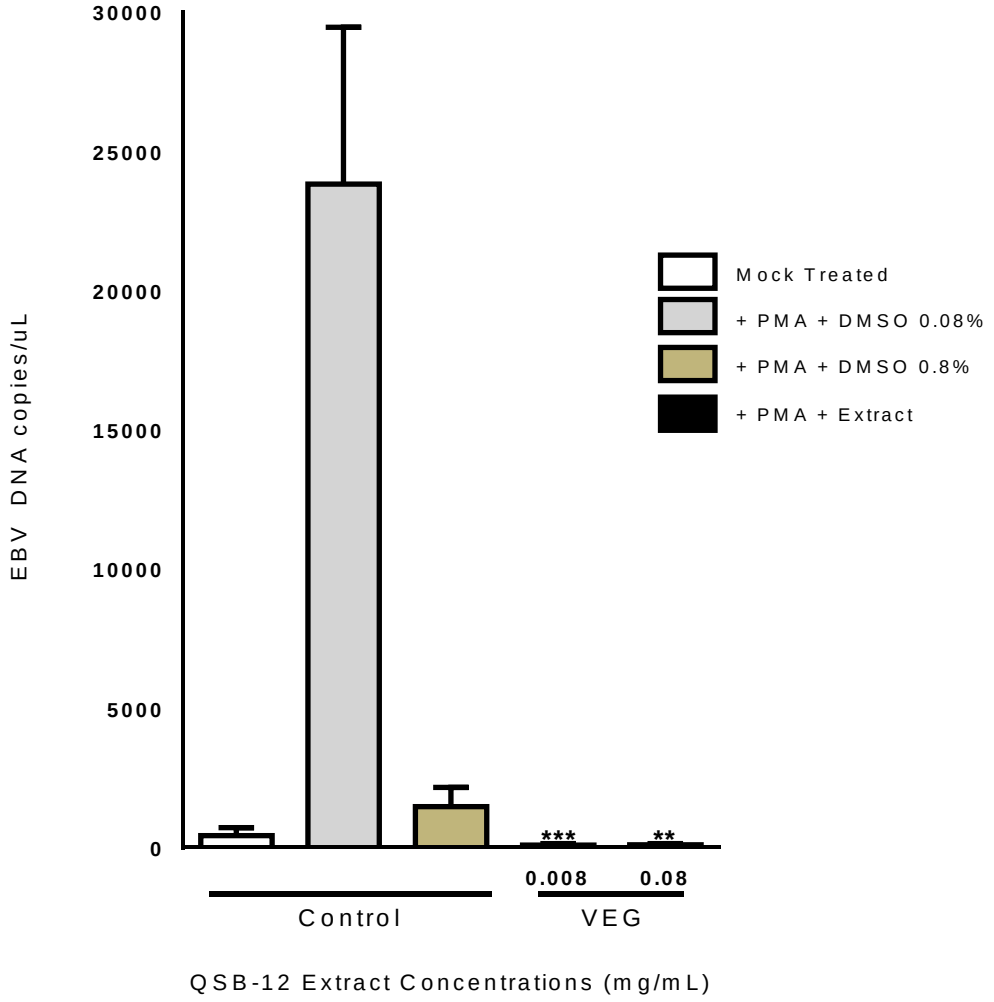


**Figure 3. The effect of QSB-12 Veg scaled-up crude extracts on the EBV load from P3HR-1 cells.**

The number of EBV DNA copies/μL was quantified in culture supernatants from PMA-induced P3HR-1 cells treated with crude extracts scaled up from the QSB-12 bacterial isolate grown in the Vegetative medium. Cells were incubated with the extracts used at one of two concentrations (0.008 mg/mL or 0.08 mg/mL) for five days. Controls included untreated cells (mock treated), PMA and 0.08% DMSO-treated cells in addition to PMA and 0.8% DMSO-treated cells. Usage of at an extract at 0.008 mg/mL results in a DMSO concentration of 0.8% while usage of an extract at 0.08

mg/mL results in a DMSO concentration of 0.8% in the culture supernatant. ** $p<0.05$, ***$p<0.01$ for extract-treated groups compared to respective DMSO-treated control groups.

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The two concentrations (0.008 mg/mL and 0.08 mg/mL) of the crude extracts produced after the scale up showed high reduction in decreasing the EBV load. A 98.42% reduction was observed in the 0.008 mg/mL of QSB-12 Veg scaled-up crude extracts against EBV. 0.08 mg/mL of the QSB-12 Veg scaled-up crude extract showed a lower reduction of 74.69% in decreasing the EBV load (**Table 6**).

**Table 6: Percent reduction in the EBV load by the QSB-12 Veg scaled-up crude extract.**

| *Strain* | *Extracts* | *(mg/mL)* | *% Reduction* |
|---|---|---|---|
| *QSB-12* *Scale Up* | Veg | 0.008 | 98.42 ± 0.8 |
| | | 0.08 | 74.69 ± 23 |

### *2. Cytotoxicity of QSB-12 Veg Scaled-Up Crude Extracts on P3HR-1 cells*

The QSB-12 Veg crude extract produced after the scale up was tested for its cytotoxic effect on P3HR-1 cells. 0.08% DMSO-treated cells showed 0.7, 0, and 0% cytotoxicity on days 1, 3, and 5, respectively. Similarly, 0.8% DMSO-treated cells showed relatively no (0, 0, 7.32%) cytotoxicity on days 1, 3, and 5, respectively. These percentages were not significant when compared to DMSO negative controls. Additionally, 0.008 mg/mL of the QSB-12 Veg scaled-up crude extracts showed no

significant cytotoxic effect on P3HR-1 cells, with 0, 0, and 3.53% cytotoxicity on days 1, 3, and 5, respectively when compared to non-treated controls. In contrast, the 0.08 mg/mL of the QSB-12 Veg scaled-up crude extracts showed a significant cytotoxic effect on P3HR-1 cells with 23.72, 56.84, and 53.75% cytotoxicity on days 1, 3, and 5, respectively when compared to non-treated controls (**Table 7**).

**Table 7: Percent Cytotoxicity of the QSB-12 Veg scaled-up crude extracts on P3HR-1 cells.**

| | *% Cytotoxicity* | | |
|---|---|---|---|
| | *Day 1* | *Day 3* | *Day 5* |
| *0.08% DMSO* | 0.75 ± 2 | 0 ± 0 | 0 ± 1.5 |
| *0.8% DMSO* | 0 ± 0.5 | 0 ± 0.5 | 7.32 ± 3 |
| *QSB-12 Veg 0.008 mg/mL* | 0 ± 0 | 0 ± 0 | 3.53 ± 5.1 |
| *QSB-12 Veg 0.08 mg/mL* | 23.72 ± 8.7 | 56.84 ± 7 | 53.75 ± 9.5 |

## D. QSB-12 Veg Crude Extracts Scale Up Fractionation by Liquid-Liquid Partitioning

The crude extracts from the QSB-12 Veg small scale and scale up were both subjected to fractionation by liquid-liquid partitioning. The three fractions (water, ethyl acetate, and chloroform) of the QSB-12 Veg small scale were previously tested by our group. However, because the water fraction did not exert any inhibitory effect in the small-scale preparation, it was not retested after the scale-up. The hexane fraction was

also not tested by our group after the small-scale and scale up preparations, since the ethyl acetate and chloroform fractions possessed an anti-EBV activity. The hexane fraction is yet reserved for further possible testing.

The chloroform, and ethyl acetate fractions prepared after the scale up were tested for their anti-EBV activity, their % reduction was determined, and their cytotoxicity tested. Even though using the scaled-up crude extract from QSB-12 Veg at a concentration of 0.08 mg/mL showed a relatively high cytotoxicity on P3HR-1 cells (as mentioned above), we continued to assess fractions of this extract at this concentration as well as at 0.008 mg/mL as indicated below. The experiments were conducted as such since the compound exerting cytotoxicity may not necessarily be the same compound exerting the antiviral effect.

### *1. The Effect of the QSB-12 Veg Scaled-Up Fractions on EBV Load from P3HR-1 Cells*

After the scale-up and liquid-liquid partitioning, the chloroform, and ethyl acetate fractions were tested for an anti-EBV activity; the water and hexane fractions were reserved for possible later testing. The ethyl acetate fraction significantly decreased the EBV load with about a 55-fold decrease for the 0.008 mg/mL extracts, when compared to non-treated controls. The extracts of the QSB-12 Veg ethyl acetate fraction used at concentrations of 0.008 mg/mL and 0.08 mg/mL, decreased the EBV DNA copies drastically with a $p=0.001$ for the smaller concentration, when compared to the non-treated controls. Similarly, the QSB-12 Veg chloroform fraction used at concentrations of a 0.008 mg/mL and 0.08 mg/mL significantly decreased the EBV

DNA copies by 20 folds when compared to the non-treated controls (p=0.01 and p=0.05 for the 2 concentrations used, respectively) (**Figure 4**).

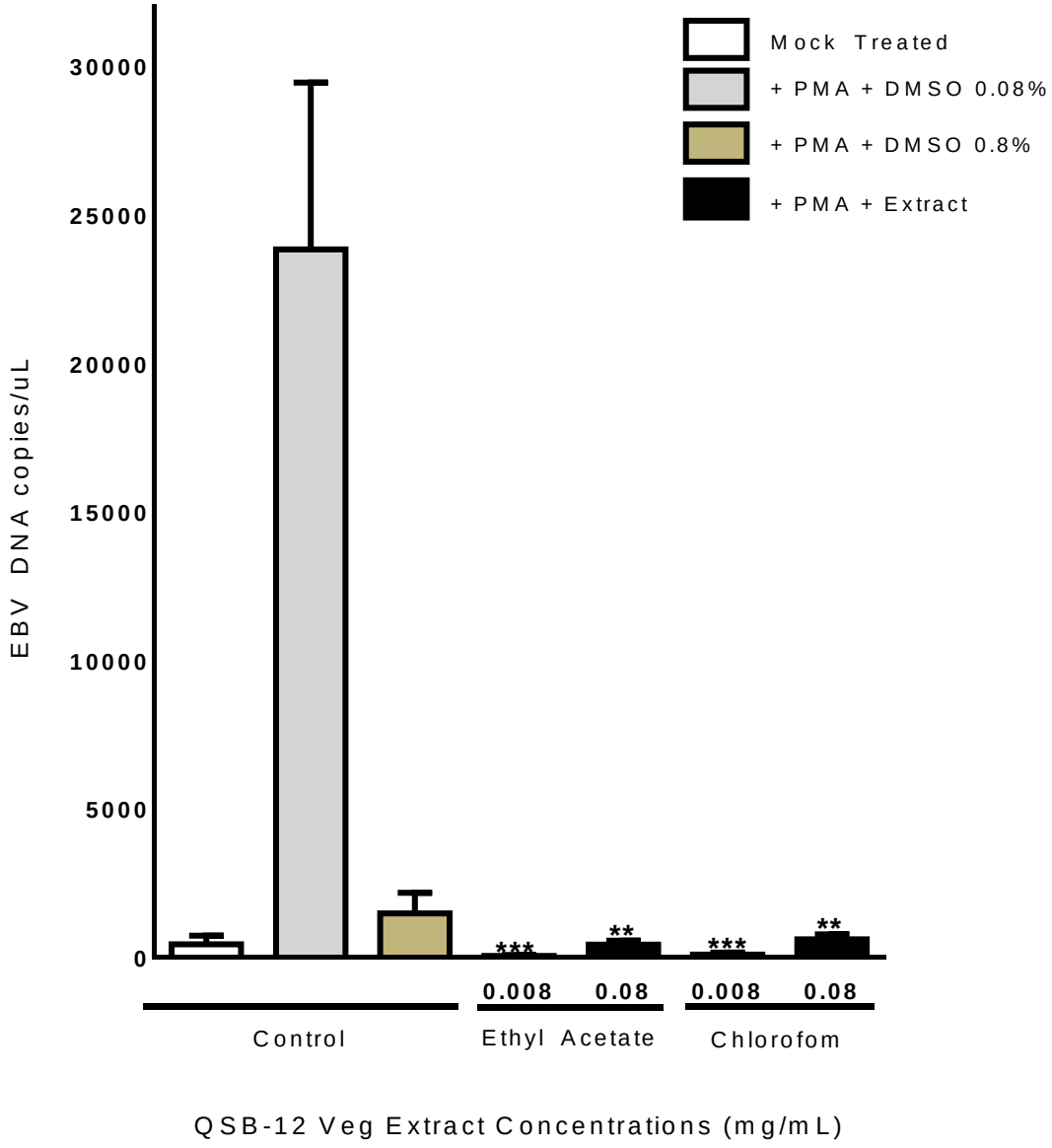


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**Figure 4. The effect of QSB-12 Veg scaled-up chloroform and ethyl acetate fractions on the EBV load from P3HR-1 cells.**

The number of EBV DNA copies/µL was quantified in culture supernatants from PMA-induced P3HR-1 cells treated with ethyl acetate and chloroform fractions of liquid-liquid partitioned QSB-12 Veg scaled-up extracts. Cells were incubated with the

extracts used at one of two concentrations (0.008 mg/mL or 0.08 mg/mL) for five days. Controls included untreated cells (mock treated), PMA and 0.08% DMSO-treated cells in addition to PMA and 0.8% DMSO-treated cells. Usage of at an extract at 0.008 mg/mL results in a DMSO concentration of 0.08% while usage of an extract at 0.08 mg/mL results in a DMSO concentration of 0.8% in the culture supernatant. ** $p<0.05$, ***$p<0.01$ for extract-treated groups compared to respective DMSO-treated control groups.

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The ethyl acetate scaled-up fraction of QSB-12 Veg used at 0.008 mg/mL showed a high reduction of 99.81% in the EBV load while the higher concentration of 0.08 mg/mL showed a lower reduction of 71.57% in the EBV load. Likewise, at 0.008 mg/mL, the chloroform scaled-up fraction showed a high reduction of 99.49% in the EBV load, while a lower reduction in the EBV load of 59.02% was achieved with 0.08 mg/mL of this fraction (**Table 8**).

**Table 8: Percent reduction in the EBV load by the ethyl acetate and chloroform fractions of liquid-liquid partitioned QSB-12 Veg scaled-up extracts.**

| *Strain* | *Extracts* | *(mg/mL)* | *% Reduction* |
|---|---|---|---|
| *QSB-12 Veg Scale-Up* | Ethyl Acetate | 0.008 | 99.812 ± 0.1 |
| | | 0.08 | 71.578 ± 9.2 |
| | Chloroform | 0.008 | 99.499 ± 0.1 |
| | | 0.08 | 59.026 ±10.9 |

### *2. Cytotoxicity of the QSB-12 Veg Ethyl Acetate and Chloroform Fractions on P3HR-1 Cells*

The cytotoxic effect of the extracts in the ethyl acetate and chloroform fractions was assayed. The 0.08% DMSO-treated cells showed no significant cell death, with a 0.75, 0, and 0% cytotoxicity on days 1, 3, and 5, respectively. Similarly, the 0.8% DMSO-treated cells showed no significant cell death, with a 0, 0, and 7.32% cytotoxicity on days 1, 3, and 5, respectively. At a concentration of 0.008 mg/mL, preparations from both the ethyl acetate and the chloroform fractions showed no significant cell death, with a 0, 0, and 1.76%, and 0, 0, and 2.1% cytotoxicity on days 1, 3, and 5, respectively. On the other hand, 0.08 mg/mL, preparations from the ethyl acetate fraction showed a significant cell death, with a 21.89, 68.15, and 71.14%, cytotoxicity on days 1, 3, and 5, respectively. A 0.08 mg/mL of the extracts prepared from the chloroform fraction showed a significant cell death, with a 52.91, 86.64, and 87.74% cytotoxicity on days 1, 3, and 5, respectively (**Table 9**).

**Table 9: Percent cytotoxicity of the ethyl acetate and chloroform fractions of liquid-liquid partitioned QSB-12 Veg scaled-up extracts on P3HR-1 cells.**

| | *% Cytotoxicity* | | |
|---|---|---|---|
| | *Day 1* | *Day 3* | *Day 5* |
| *0.08% DMSO* | 0.75 ± 2 | 0 ± 0 | 0 ± 1.5 |
| *0.8% DMSO* | 0 ± 0.5 | 0 ± 0.5 | 7.32 ± 3 |
| *QSB-12 Veg Ethyl Acetate 0.008 mg/ml* | 0 ± 1.5 | 0 ± 1 | 1.76 ± 3.2 |
| *QSB-12 Veg Ethyl Acetate 0.08 mg/mL* | 21.89 ± 2 | 76.88 ± 9.1 | 76.87 ± 4.9 |
| *QSB-12 Veg Chloroform 0.008 mg/mL* | 0 ± 1.5 | 0 ± 2 | 2.12 ± 2.8 |

| | | | |
|---|---|---|---|
| *QSB-12 Veg Chloroform 0.08 mg/mL* | 52.91 ± 5.1 | 86.64 ± 8.5 | 87.74 ± 1.5 |

## E. Effect of Column Chromatography Subfractions of Liquid-Liquid Partitioned Scaled-up QSB-12 Veg Extracts on the EBV Load from P3HR-1 Cells

The ethyl acetate and chloroform fractions that exerted an anti-EBV activity with no significant cytotoxicity were further fractionated by column chromatography. The ethyl acetate fraction was further fractionated into 3 subfractions A, B, and C according to the compound variability and migration by column chromatography and TLC. The chloroform fraction was further fractionated into 4 subfractions A, B, C, and D also according to the compound variability and migration by column chromatography and TLC. The obtained fractions were tested for their anti-EBV activity, their % reduction was determined, and cytotoxicity assayed.

### *1. The Effect of the QSB-12 Veg Scaled-up Ethyl Acetate Subfractions on EBV Load from P3HR-1 Cells*

Among the ethyl acetate subfractions, the A, B, and C subfractions used at a concentration of 0.008 mg/mL did not decrease the EBV load in the cell-free supernatants. In contrast, at a concentration of 0.08 mg/mL, these subfractions (A, B, C) were able to significantly ($p=0.01$, $p=0.002$, and $p=0.009$, respectively) decrease the EBV load in the cell-free supernatants, when compared to the DMSO-treated controls; with the 0.08 mg/mL of the B subfraction showing the highest fold decrease (8-folds) with $p=0.002$ when compared to the DMSO-treated controls (**Figure 5**).

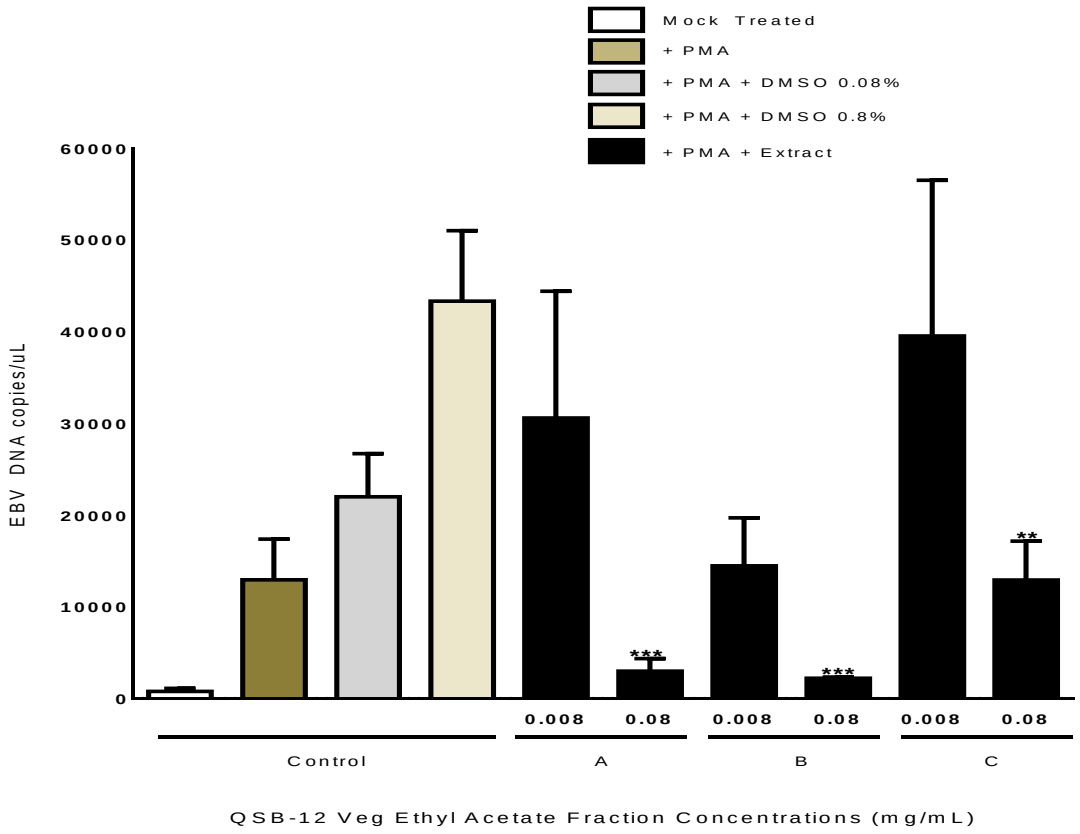


**Figure 5. The effect of QSB-12 Veg A, B, and C ethyl acetate subfractions on the EBV load from P3HR-1 cells.**

The number of EBV DNA copies/μL was quantified in culture supernatants from PMA-induced P3HR-1 cells treated with three ethyl acetate subfractions (A, B, and C) of liquid-liquid partitioned QSB-12 Veg scaled-up extracts. Cells were incubated with the extracts used at one of two concentrations (0.008 mg/mL or 0.08 mg/mL) for five days. Controls included untreated cells (mock treated), PMA- treated cells, PMA and 0.08% DMSO-treated cells in addition to PMA and 0.8% DMSO-treated cells. Usage of at an extract at 0.008 mg/mL results in a DMSO concentration of 0.08% while usage of an extract at 0.08 mg/mL results in a DMSO concentration of 0.8% in the

culture supernatant. ** $p<0.05$, ***$p<0.01$ for extract-treated groups compared to respective DMSO-treated control groups.

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At 0.008 mg/mL, the ethyl acetate subfraction A did not show reduction in the EBV load, while at a concentration of 0.08 mg/mL, this subfraction showed a 93.21% reduction in the EBV load, when compared to controls. Likewise, a 0.008 mg/mL of the ethyl acetate subfraction B showed a low reduction of 34.32%, while a higher reduction of 94.97% in the EBV load was achieved with a 0.08 mg/mL of this subfraction. The ethyl acetate C subfraction showed the lowest reduction in the EBV load among the subfractions. At a concentration of 0.008 mg/mL, the ethyl acetate C subfraction showed no reduction in the EBV load, while there was a reduction of 70.21% in the EBV load using the greater concentration (0.08 mg/mL) of this fraction (**Table 10**).

**Table 10: Percent reduction in the EBV load by the ethyl acetate subfractions A, B, and C of liquid-liquid partitioned QSB-12 Veg scaled-up extracts.**

| *Strain* | *Ethyl Acetate Subfraction* | *(mg/mL)* | *% Reduction* |
|---|---|---|---|
| *QSB-12 Veg* | A | 0.008 | 0 ± 0 |
| | | 0.08 | 93.213 ± 3.2 |
| | B | 0.008 | 34.3273 ± 23.8 |
| | | 0.08 | 94.9737 ± 0.3 |
| | C | 0.008 | 0 ± 0 |
| | | 0.08 | 70.2166 ± 9.7 |

### *2. Cytotoxicity of the QSB-12 Veg Ethyl Acetate Subfractions on P3HR-1 Cells*

The cytotoxic effect of the extracts in the ethyl acetate and chloroform subfractions prepared after TLC and column chromatography was further studied. Both,

the 0.08% and 0.8% DMSO-treated cells showed no cell death, with approximately 0% cytotoxicity on days 1, 3, and 5. At a concentration of 0.008 mg/mL, the three ethyl acetate subfractions (A, B, C) showed no significant cell death, with around 0 to 1% cytotoxicity on days 1, 3, and 5. On the other hand, 0.08 mg/mL from the A and B ethyl acetate subfractions showed a significant cell death on the fifth day (30.71%, and 31.07%, respectively). Unlike the A and B subfractions, the C ethyl acetate subfraction did not yield any significant cell death on days 1, 3, and 5 (**Table 11**). This indicates that the compounds in the C ethyl acetate subfraction with a significant EBV replication inhibition at a concentration of 0.08 mg/mL are safe for cells.

**Table 11: Percent cytotoxicity of the ethyl acetate subfractions of liquid-liquid partitioned QSB-12 Veg scaled-up extracts on P3HR-1 cells**

| | *% Cytotoxicity* | | |
|---|---|---|---|
| | *Day 1* | *Day 3* | *Day 5* |
| *0.08% DMSO* | 0 ± 0.5 | 0 ± 0.5 | 0 ± 0 |
| *0.8% DMSO* | 0 ± 0.5 | 0 ± 0.5 | 1.40 ± 2 |
| *QSB-12 Veg Ethyl Acetate A 0.008 mg/ml* | 0 ± 2.3 | 0.69 ± 1.5 | 0 ± 1.5 |
| *QSB-12 Veg Ethyl Acetate A 0.08 mg/mL* | 0 ± 1 | 8.21 ± 4 | 30.71 ± 5.5 |
| *QSB-12 Veg Ethyl Acetate B 0.008 mg/mL* | 0 ± 0 | 0 ± 0.5 | 0 ± 0.5 |
| *QSB-12 Veg Ethyl Acetate B 0.08 mg/mL* | 0 ± 1.1 | 3.42 ± 1.7 | 31.07 ± 4.9 |

| | | | |
|---|---|---|---|
| *QSB-12 Veg Ethyl Acetate C 0.008 mg/mL* | 0 ± 0 | 4.15 ± 1.1 | 0 ± 0.5 |
| *QSB-12 Veg Ethyl Acetate C 0.08 mg/mL* | 0 ± 1.5 | 2.73 ± 0.5 | 0 ± 0.5 |

### *3. The Effect of the QSB-12 Veg Scaled-up Chloroform Subfractions on EBV Load from P3HR-1 Cells*

Among the chloroform fractions, 0.008 mg/mL of the four subfractions A, B, C, and D, did not yield a significant decrease in the EBV load, when compared to DMSO-treated controls. On the other hand, the three subfractions A, B, and C were able to significantly ($p<0.05$) decrease the EBV load in the cell free supernatants, when compared to the DMSO-treated controls, with the A subfraction showing the highest fold decrease in viral load detection, that of 23-folds ($p=0.01$). Even though a 0.08 mg/mL of the chloroform D subfraction decreased the EBV load, the decrease was not significant. (**Figure 6**).

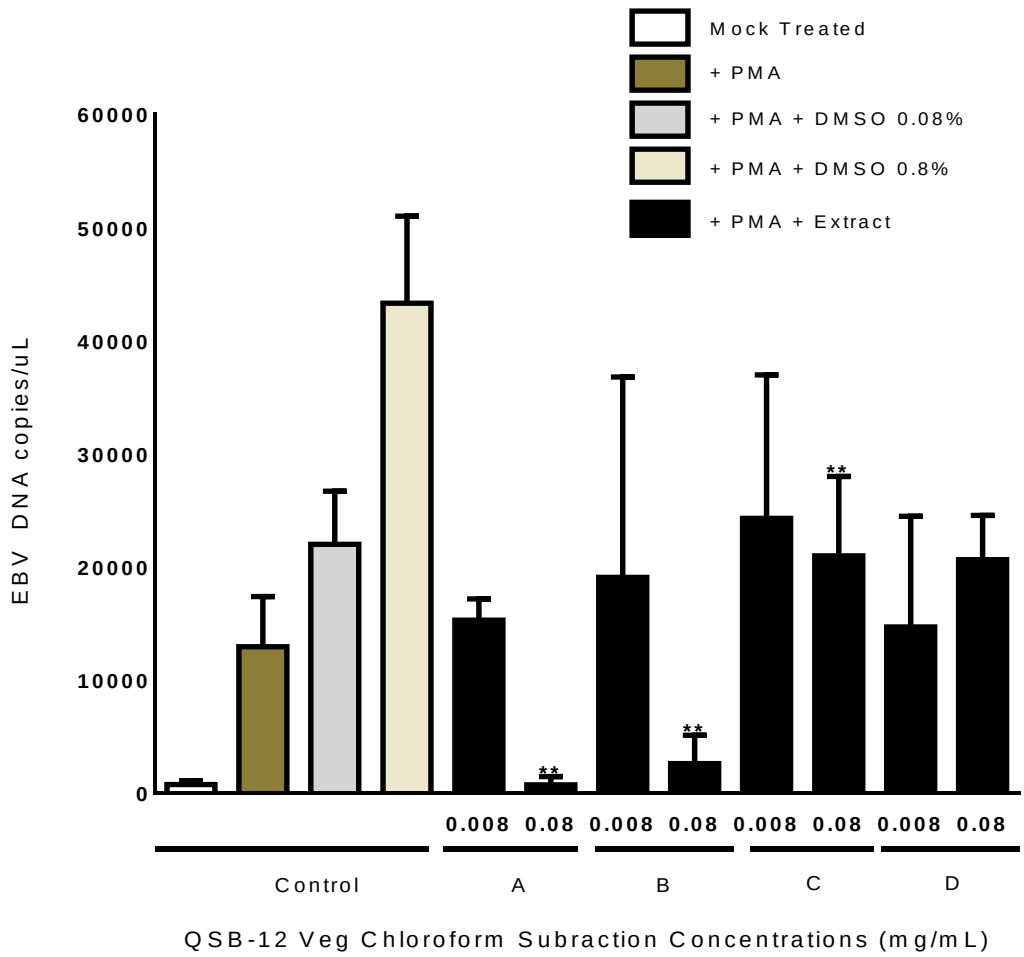


**Figure 6. The effect of QSB-12 Veg A, B, C, and D chloroform subfractions on the EBV load from P3HR-1 cells.**

The number of EBV DNA copies/μL was quantified in culture supernatants from PMA-induced P3HR-1 cells treated with four chloroform subfractions (A, B, C, and D) of liquid-liquid partitioned QSB-12 Veg scaled-up extracts. Cells were incubated with the extracts used at one of two concentrations (0.008 mg/mL or 0.08 mg/mL) for five days. Controls included untreated cells (mock treated), PMA-treated cells, PMA and 0.08% DMSO-treated cells in addition to PMA and 0.8% DMSO-treated cells. Usage of at an extract at 0.008 mg/mL results in a DMSO concentration of

0.08% while usage of an extract at 0.08 mg/mL results in a DMSO concentration of 0.8% in the culture supernatant. ** $p<0.05$, ***$p<0.01$ for extract-treated groups compared to respective DMSO-treated control groups.

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On the other hand, at 0.008 mg/mL, the chloroform subfraction A showed a 30.54% reduction in the EBV load, while at 0.08 mg/mL, this subfraction showed a higher reduction of 98.34% in the EBV load. At 0.008 mg/mL, the chloroform subfraction B showed a low reduction of 13.31% in the EBV load, while a greater reduction of 94% was achieved with 0.08 mg/mL of this subfraction. Similarly, a concentration of 0.008 mg/mL of the chloroform C subfraction showed no reduction in the EBV load, while there was a greater reduction of 51.61% in the EBV load in the greater concentration (0.08 mg/mL) of this fraction. Also, at 0.008 mg/mL, the chloroform D subfraction showed a 33.17% reduction in the EBV load, while a greater reduction of 52.36% was achieved in the 0.08 mg/mL of this fraction (**Table 12**).

**Table 12: Percent reduction in the EBV load by the chloroform subfractions A, B, C, and D of liquid-liquid partitioned QSB-12 Veg scaled-up extracts.**

| *Strain* | *Chloroform Subfraction* | *(mg/mL)* | *% Reduction* |
|---|---|---|---|
| *QSB-12 Veg* | A | 0.008 | 30.548 ± 8.6 |
| | | 0.08 | 98.342 ± 1.6 |
| | B | 0.008 | 13.317 ± 4.1 |
| | | 0.08 | 94.007 ± 29.1 |
| | C | 0.008 | 0 ± 0 |
| | | 0.08 | 51.614 ± 16.2 |
| | D | 0.008 | 33.178 ± 41.1 |
| | | 0.08 | 52.368 ± 9.01 |

### *4. Cytotoxicity of the QSB-12 Veg Ethyl Acetate Subfractions on P3HR-1 Cells*

At a concentration of 0.008 mg/mL, the four chloroform subfractions (A, B, C, D) showed no significant cell death, with a 0% cytotoxicity on days 1, 3, and 5. On the other hand, 0.08 mg/mL from the A chloroform subfraction was the only subfraction that showed a significant cell death after 5 days of incubation (33.21%). On the contrary a 0.08 mg/mL of the three chloroform subtractions (B, C, D) did not yield a significant cytotoxicity ranging between 0 and 7% on days 1, 3, and 5 (**Table 13)**. This indicates that the compounds in the B, C, and D chloroform subfraction at a concentration of 0.08 mg/mL are safe for cells. Hence, the compounds in the C subfraction of the ethyl acetate fraction and both B and C subfractions of the chloroform fraction are safe for cells and effective in inhibiting EBV replication *in vitro*.

**Table 13: Percent cytotoxicity of the chloroform subfractions of liquid-liquid partitioned QSB-12 Veg scaled-up extracts on P3HR-1 cells**

| | *% Cytotoxicity* | | |
|---|---|---|---|
| | *Day 1* | *Day 3* | *Day 5* |
| *0.08% DMSO* | 0 ± 0.5 | 0 ± 0.5 | 0 ± 0 |
| *0.8% DMSO* | 0 ± 0.5 | 0 ± 0.5 | 1.40 ± 2 |
| *QSB-12 Veg Chloroform A 0.008 mg/mL* | 0 ± 0 | 0.34 ± 2 | 0 ± 0.5 |
| *QSB-12 Veg Chloroform A 0.08 mg/mL* | 0 ± 4.5 | 8.90 ± 2.5 | 33.21 ± 5.5 |

| | | | |
|---|---|---|---|
| *QSB-12 Veg Chloroform B 0.008 mg/mL* | 0 ± 0 | 5.19 ± 2.5 | 0 ± 1.7 |
| *QSB-12 Veg Chloroform B 0.08 mg/mL* | 0 ± 0 | 2.05 ± 1.1 | 0 ± 4 |
| *QSB-12 Veg Chloroform C 0.008 mg/mL* | 0 ± 0 | 0 ± 0.5 | 0 ± 1.5 |
| *QSB-12 Veg Chloroform C 0.08 mg/mL* | 0 ± 0 | 3.08 ± 1.1 | 0 ± 4 |
| *QSB-12 Veg Chloroform D 0.008 mg/mL* | 0 ± 0 | 0 ± 0.5 | 0 ± 0 |
| *QSB-12 Veg Chloroform D 0.08 mg/mL* | 0 ± 0 | 7.53 ± 5.5 | 3.92 ± 4 |

# CHAPTER V

# DISCUSSION

Discovering novel therapeutic agents from natural products has been central to drug discovery. Knowing that EBV infects more than 90% of the human population without the presence of an approved effective and safe treatment that can clear EBV infections mandates the need to find safe novel drugs with anti-EBV activity [8]. During the primary infection, EBV is highly shed through saliva, which increases the risk of transmission. Following the primary infection, EBV establishes a latency in resting memory B-cells, which would later increase the risk of recurrent infections, high viral shedding, and EBV-associated diseases. Primary infection with EBV is usually characterized by asymptomatic or flu-like symptoms in children but might be also associated with IM. EBV infection poses a risk factor for several malignant, lymphoproliferative, and autoimmune diseases [4, 5].

Several natural and synthetic drug agents have been under investigation for their efficacy in clearing EBV infections *in vitro* and *in vivo,* and some have reached human clinical trials. ACV is one of the major drugs under investigation for its possible anti-EBV activity. ACV has shown an efficacy in clearing EBV infected cells *in vitro* at concentrations ranging between 2 and 50 μM, which could be compared to compounds present in our subfractions prepared from the QSB-12 Veg crude extracts [41]. Like ACV, one or more compounds present in some of our prepared fractions were able to inhibit EBV replication *in vitro,* at the two concentrations tested (0.08 mg/mL and 0.008 mg/mL). Since the compounds studied in this thesis inhibit EBV replication in induced P3HR-1 producer cells, and ACV is a nucleoside analogue that interferes with the

function of the EBV DNA polymerase, the mechanism of inhibition of the studied compounds could possibly be similar to that of ACV, however, we cannot be certain of this comparison since the structure and nature of the compounds are not yet identified and further studies are needed to be performed to identify the mechanism of action, which will further explain the comparison with other known drugs that inhibit EBV replication *in vitro*. Furthermore, ACV was able to inhibit EBV replication and infectious particle production *in vivo;* however, EBV replication resumed when the treatment was stopped, indicating that ACV could suppress but not eliminate an EBV infection. The studied subfractions in this thesis could be potentially tested for their ability to clear an EBV infection *in vivo,* but they require further investigation before being tested *in vivo.* Moreover, ACV was tested in humans with IM, oral hairy leukoplakia, and other EBV-associated diseases; it was able to decrease the EBV load in IM patients but was not able to alleviate the clinical symptoms of IM and it decreased oral hairy leukoplakia occurrences, but recurrences occurred when treatment was stopped [42-44]. It is also worth mentioning that there was a reported fatal case of EBV-associated lymphoproliferative disorder with no response to ACV, which might contradict the safety of ACV and the ability of ACV to clear an EBV infection [45]. Many other synthetic drugs were tested against EBV as described at length in Chapter II of this thesis, with none being approved as a treatment [26].

Additionally, natural products such as triterpenoids, emodin, and many others were tested against EBV, but none were tested *in vivo,* and thus no drugs are available for EBV treatment [98, 99] [93, 101, 102]. Triterpenoids for instance, extracted from the fungus *G. lucidum* showed a strong activity in repressing EBV replication *in vitro,* by inhibiting the EBV-EA, EBV-CA, and telomerase activity [98, 99]. A similar

decrease in the EBV load *in vitro* was shown in both the compounds extracted from the *G. lucidum* that were tested in induced Raji producer cells [98], and the crude extracts prepared from the QSB-12 strain grown in the Vegetative medium that were tested in this thesis in induced P3HR-1 producer cells. Chung Lin J. et al. [107], demonstrated the anti-EBV activity of glycyrrhizic acid (GL), a component of licorice root (*Glycyrrhiza radix)*, in superinfected Raji cells, *in vitro*. GL appeared to interfere with early steps of the EBV replication most likely to be the penetration step. Compared to our compounds that could inhibit EBV replication processes involved in DNA replication, assembly, or release, GL does not inhibit the late stages of infection. A similar approach to our study was performed by Yih-Yih Kok et al. [108], for the possible presence of anti-EBV compounds prepared from microalgal extracts, and then fractionating the extracts by chromatographic techniques. EBV load was quantified in cell-free supernatants from lymphoblastoid cell lines like P3HR-1 cells after an incubation with methanol extracts from *Ankistrodesmus convolutus*, *Synechococcus elongatus*, and *Spirulina platensis* microalgae. An effective decrease in the EBV load was observed at a concentration of 0.01 μg/mL, with no significant cytotoxicity. It was also reported that after column chromatography, one fraction of *Synechococcus elongatus* extracts (SEF1) reduced the EBV load most effectively, at a concentration of 0.003 mg/mL. Similar to the studied QSB-12 Veg crude extracts and fractions, SEF1 could inhibit EBV replication by affecting DNA replication, viral assembly, or release, since these extracts exert anti-EBV activity in induced P3HR-1 producer cells. While the ethyl acetate and chloroform fractions and their respective subfractions prepared from the QSB-12 Veg crude extracts studied in this thesis resulted in an EBV load drop at a concentration of 0.008 mg/mL and 0.08 mg/mL, respectively, the microalgal

extracts (SEF1) decreased the EBV load significantly at a lower concentration of 0.003 mg/mL. Despite that many studies have examined natural products for the presence of an anti-EBV activity, none of these products have been approved as a treatment, and as such, no anti-viral treatment is currently recommended to treat an EBV infection. Therefore, we intended to explore novel, effective and safe anti-EBV compounds in products from soil-dwelling microorganisms.

To investigate such compounds, about 16 crude extracts were tested. Crude extracts were prepared from two bacterial strains cultured in different productive culture media and tested for their ability to inhibit EBV replication *in vitro* in induced P3HR-1 producer cell lines. Crude extracts prepared from the QSB-12 bacterial isolate were further investigated after showing a high reduction in the EBV load. Knowing that the secondary metabolites from this strain grown in the Vegetative (Veg) medium did not yield a significant cytotoxicity with a high effectiveness in decreasing the EBV load, they were further investigated. Crude extracts from the BM-12 strain grown in the A medium were left for possible later testing. These results signify that the drop in the EBV load is due to an inhibition in the viral replication rather than a cytotoxic effect. Applying a 0.008 mg/mL and 0.08 mg/mL of the extracts would result in a 0.08% and 0.8% DMSO in the treated cells, respectively, which mandated their inclusion as controls. DMSO and PMA appeared to be acting synergistically to increase the induction of EBV in P3HR-1 producer cells. Even though crude extracts prepared in other productive media exerted a significant inhibition, QSB-12 Veg crude extracts were further investigated due to their low cytotoxicity. Consequently, the QSB-12 grown in the Veg medium was further scaled up and tested. The reasons behind scaling-up the crude extracts are that the scale-up would increase the production of certain

secondary metabolites that could possess a strong anti-EBV activity, and it would increase the volume of the crude extracts prepared which aids in the fractionation of the compounds. The crude extracts prepared after the QSB-12 Veg scale-up showed high reduction in the EBV load at the two concentrations tested: 0.008 mg/mL and 0.08 mg/mL. Even though a significant cytotoxicity was observed at the greater concentration tested, further investigation was continued, knowing that the compounds responsible for cytotoxicity might not be the same compounds exerting the antiviral activity.

With the aim of isolating the compounds responsible for the inhibition, liquid-liquid partitioning was performed. This led to the preparation of 4 separate fractions, in which the ethyl acetate and chloroform fractions showed an elevated strong activity in inhibiting EBV replication *in vitro.* No significant cytotoxicity was observed at the lower concentration (0.008 mg/mL) of both fractions. On the other hand, a low reduction and significant cytotoxicity appeared upon testing the higher concentrations (0.08 mg/mL). It is yet possible to change the concentrations tested after the purification of the compounds that might lead to a lower cytotoxicity. These results further explain that certain compounds present in these fractions possess an effective anti-EBV activity with no possible cytotoxic effect. Hence, to answer this question, more separation techniques are required to be performed.

Separation by column chromatography resulted in three ethyl acetate subfractions: A, B, and C, that showed a high reduction in the EBV load only at the greater concentration tested (0.08 mg/mL), with the B subfraction being the most effective. Among these subfractions, only the C subfraction did not yield a significant cytotoxicity after 5 days, which would make it the safest subfraction that is still

effective, to be further investigated *in vitro*. Additionally, three subfractions (A, B, C) out of the four chloroform subfractions showed a high reduction in the EBV load at the greater concentration tested (0.08 mg/mL), with the A subfraction being the most effective. The possible reason that at the liquid-liquid partitioning step, the lower concentration (0.008 mg/mL) showed a significant inhibition, while after sub-fractionation by column chromatography and TLC it did not, is because several anti-EBV compounds were present in the same fraction, and thus a smaller concentration would be sufficient to inhibit EBV replication, also suggesting that these compounds might be inhibiting different steps of replication. However, further fractionation would imply that fewer compounds are present in each separate fraction, thus, a higher concentration might be required to inhibit viral replication. Additionally, future purification steps that will include HPLC might help us separate the compounds and thus determine the origin of cytotoxicity. On the other hand, only the A subfraction resulted in a significant cell death after 5 days of incubation, which suggest that the B and C chloroform subfractions are safe to be further investigated *in vitro*. These results verify that in the subfractions mentioned above, compounds exerting a cytotoxic effect are not the same compounds showing the anti-EBV activity. Since the C ethyl acetate subfraction and both B and C chloroform subfractions did not exert a significant cytotoxicity while inhibiting EBV replication, it is suggested that the cytotoxic compounds resulting in a significant cell death were separated into the A and B ethyl acetate subfractions and the A chloroform subfraction.

Our results taken together suggest that one or more available potential drug candidates against EBV are likely present in our fractions, which are prepared from secondary metabolites secreted by the QSB-12 bacterial isolate (*Pseudomonas spp.*)

grown in the Veg medium. Our future studies will further investigate the effective inhibitory activity of the compounds present mainly in the C subfraction of the ethyl acetate fraction and in the B and C subfractions of the chloroform fraction. This would be conducted by first further fractionating the compounds by HPLC, then identifying the compounds present in these subfractions and elucidating their structures by mass-spectrometry and nuclear magnetic resonance (NMR) spectroscopy. Further exploration would be conducted if these agents have not been previously reported. Elucidating the structure of these compounds will also contribute to our understanding of the nature of these compounds and thus aid in identifying their mechanism of action in inhibiting the EBV replication cycle. Biological assays will also be conducted to identify their anti-viral mechanisms.

Antiviral drugs can inhibit the life cycle of the virus at various stages. They may interfere with early stages of infection by inhibiting viral attachment or entry, such as EGP form *E. gelatinae*, which inhibits HSV-1 attachment to cells [60]. On the contrary, our results do not point out any possible interaction of the compounds with the viral attachment or entry since the cells are already EBV positive. Alternatively, antivirals like andrographolide can inhibit the replication and transcriptional activators (Rta and Zta) which would inhibit the production of EBV lytic proteins, consequently inhibiting viral particle production [100]. Yet, the compounds tested in this thesis could less likely interfere with the induction of the EBV lytic cycle by PMA. Furthermore, some antivirals act on late stages of infection and thus inhibit viral release [108]. Since the P3HR-1 cells used in our experiments are EBV producing, the studied compounds likely inhibit viral life cycle stages that occur after EBV lytic cycle induction; this would include inhibition of processes involved in biosynthesis, viral assembly, and

release similar to the previously tested compound SEF1 from *Synechococcus elongatus* extracts [108]. Therefore, our future studies will include assessing the ability of the compounds examined in this study to inhibit infection of lymphocytes that do not harbor EBV. The possible inhibition of the viral lytic cycle by our compounds may aid in avoiding the spread of EBV and limit the tumor-promoting effect from latently infected cells to neighboring healthy cells.

In conclusion, the present study demonstrates 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 grown in the A medium, can inhibit EBV replication *in vitro* in P3HR-1 cell lines with no significant cytotoxicity. Further research efforts are required to isolate and identify the particular compounds responsible for the inhibition, and to examine their mechanism of action. Structure elucidation and compound purification would also help determine the safe concentrations to be used *in vitro* and *in vivo.* Minimum safe and inhibitory concentrations could be compared with drugs previously shown to inhibit EBV *in vitro* like ACV, for instance [109]. As stated above, natural producers continue to provide new sources of compounds with antimicrobial properties; thus, isolating compounds with anti-EBV activity from natural products secreted by soil-dwelling microorganisms might lead to the discovery of novel drugs that can clear EBV infections and be used in treating EBV-associated diseases.

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