Master's Thesis

Deciphering the Antiviral Mechanism of Endophyte Extract against Coxsackieviruses B3 and A9

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Enteroviruses can cause acute and chronic infections. The only available vaccine is against poliovirus and vaccines cannot help if the infection is already present. As such, antivirals are needed to fight enterovirus infections. A potential source for novel antiviral compounds are endophyte extracts. In this experiment, we studied extracts from fungal endophytes that had previously shown antiviral activity against coxsackieviruses B3 and A9. The aim was to determine the antiviral target of the extracts. Radioactive viruses were created using ^{35}S-labeled amino acids to be used in a binding assay. The binding assay showed that the extracts do not inhibit the binding of the virus on the cell surface and might even increase it in some cases. An ATP-assay was performed to determine the cytotoxicity of the extracts and all were found to be non-cytotoxic. Finally, a pretreatment experiment where cells were incubated with extract before infection was used to determine if host cell factors are affected to produce the antiviral effect. No such results were found and thus it is most likely that the extracts have an immediate effect on the virus capsid itself. Follow-up research on the currently unknown composition of the extracts will shed more light on this subject.

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Enterovirukset voivat aiheuttaa nuhamaisten oireiden lisäksi vakavia, jopa kroonisia, sairauksia. Enteroviruksia vastaan ei ole saatavilla rokotteita, poliovirusta lukuun ottamatta. Antiviraalisia lääkkeitä on kehitettävä virustartuntojen hoitoa varten. Endofyyttiuutteet ovat mahdollinen tutkimuskohde, joista viruksia vastaan tehoavia aineita voisi löytää. Tässä tutkimuksessa tutkimme endofyyttiuutteita, jotka olivat aiemmin osoittautuneet tehokkaiksi coxsackieviruksia B3 ja A9 vastaan. Radioaktiivisia, ^{35}S –merkittyjä viruksia luotiin koetta varten, jossa tutkimme uutteiden vaikutusta virusten kykyyn sitoutua solun pinnalle. Uutteilla ei havaittu olevan tilastollisesti merkittävää sitoutumista häiritsevää vaikutusta, mutta joissain tapauksissa ne lisäsivät sitoutumista. Sytotoksisuuskokeilla määriteltiin uutteiden vaikutus solujen elinkykyyn. Uutteet eivät osoittautuneet sytotoksisiksi. Soluja altistettiin uutteille ennen infektiota, jotta mahdolliset uutteiden vaikutukset solujen sisäisiin mekanismeihin selviäisivät. Tuloksien perusteella uutteilla ei ollut vaikutusta tällä tavalla. Vaikuttaa siis siltä, että tutkitut endofyyttiuutteet vaikuttavat suoraan viruspartikkeleihin ja täten estävät infektiota. Jatkotutkimukset uutteiden sisällöistä ovat suositeltuja ja ne voisivat tarjota lisätietoa niiden antiviraalisista mekanismeista.

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ABBREVIATIONS

A549 cells Adenocarcinomic human alveolar basal epithelial cells

GMK cells Green monkey kidney epithelial cells

EV-B Enterovirus species B

CVA9 Coxsackievirus A9

CVB3 Coxsackievirus B3

VPg genome linked virus-encoded protein

ssRNA single-stranded RNA

FcRn Human neonatal Fc-receptor

MVBs Multivesicluar bodies

CAR Coxsackie and adenovirus receptor

DAF Decay accelerating factor

TJ Tight junction

IRES Independent ribosome entry-site

IPV Inactivated PV vaccine

OPV attenuated oral poliovirus vaccine

VDPV vaccine-derived poliovirus

FDA Food and drug administration

CPE Cytopathic effect

FBS Fetal bovine serum

PBS Phosphate buffer saline

$MgCl_2$ Magnesium chloride

DMEM Dulbecco's modified eagle medium

^{35}S Radioactive Sulphur

UIM Uncoating intermediate particle

CPM Counts per minute

1. INTRODUCTION

1.1 Species B Enteroviruses

Species B Enteroviruses (EV-B) are a group of viruses consisting of coxsackieviruses B1-B6 (CVB1-6) and coxsackievirus A9 (CVA9). In addition to these, there are multiple serotypes of echoviruses and EV-Bs as a part of this group (Marjomäki *et al.* 2015).

1.1.1 Structure

The viruses consist of a positive sense single strand RNA genome (+ssRNA) around 7500 bp long having a genome-linked virus protein (VPg) attached to the 5′ end of genome after a long untranslated region (UTR). This 5′UTR is involved in the onset of protein synthesis and another UTR in the 3′ end is involved in the formation of the negative strand RNA. When translated the genome encodes a polyprotein that is cleaved into 3 precursor proteins (P1-P3) which in turn encode smaller virus proteins (Figure 1). The translated P1 encodes the four structural proteins, VP1-VP4 (Marjomäki *et al.* 2015) . These polypeptides in turn form 60 protomers and 12 pentamers. P2 and P3 are used to form seven different non-structural proteins: 2A, 2B and 2C that work in rearranging the cell's membrane structures and 3A, 3B, 3C and 3D that are used in RNA-synthesis. VP1-3 are capsid proteins present on the surface of the 30 nm diameter non-enveloped virus capsid that is icosahedral in nature (Figure 2). VP4 however is an internal protein of the capsid (Marjomäki *et al.* 2015). Grooves, called canyons, run along the outside of the capsid structure and are believed to aid in binding to specific receptors (Plevka *et al.* 2012). Beneath this canyon, an aliphatic fatty acid is stored in a hydrophobic pocket (Bergelson and Coyne 2013). It functions as a stabilizing agent in all enterovirus types (Marjomäki *et al.* 2015). In the uncoating process, these fatty acids are released and thought to cause increased destability in the capsid structure, resulting in

genome release from the virus (Marjomäki *et al.* 2015).

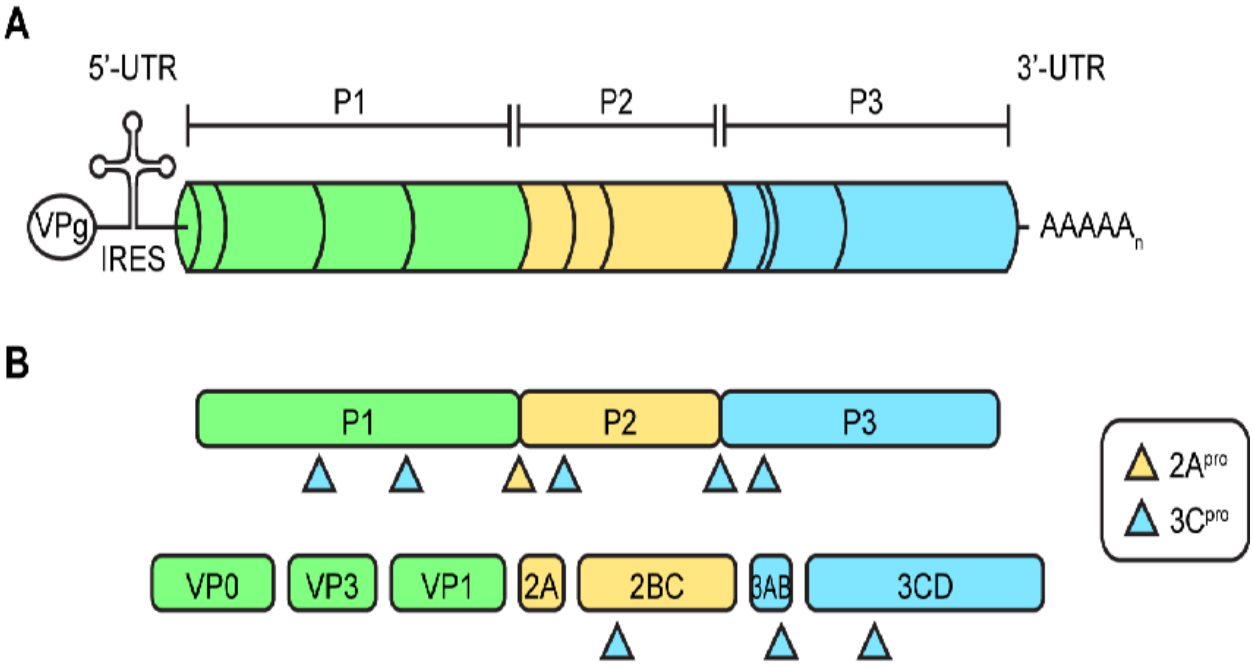

Figure 1. Enterovirus genome. (**A**) The Lead-4-3-4 type genome contains a genome-linked virus protein (VPg) in the 5′ end that acts as a primer in RNA replication, followed by the 5′ untranslated region (UTR) that contains the independent ribosome entry-site (IRES) used for cap-independent translation in the host cell. The encoding part produces a single polyprotein consisting of P1, P2 and P3. (**B**) The polyprotein is processed into smaller parts by the proteases 2A^{pro} and 3C^{pro}/3CD^{pro} (van der Linden *et al.* 2015).

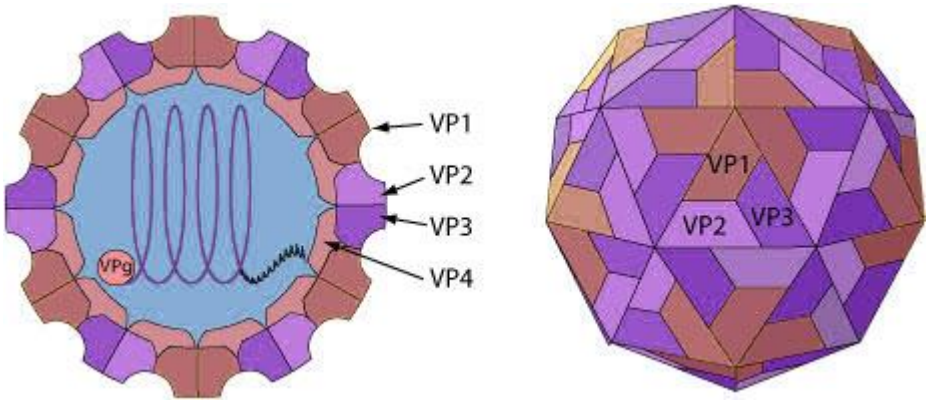

Figure 2. Enterovirus capsid structure. The icosahedral structure is formed by 60 subunits of VP1-3 on the outside, with VP4 residing inside the capsid. On the left, the

inside of the capsid is shown with the RNA genome and genome-linked virus protein.

1.1.2 Illnesses

EV-Bs of all types are known to cause various mild illnesses relatable to a cold, but they have the potential for more severe and possibly chronic infections. For example, it is believed that CVB serotypes increase the risk of myocardial infarction (MI) and are possibly involved in the development of diseases such as atherosclerosis (Roivainen *et al.* 1998). EV-Bs have also been associated with the development of type 1 diabetes (Roivainen and Klingel 2009; Laitinen *et al.* 2014). They are stable in acidic pH and primarily use the fecal-oral route for infection (Marjomäki *et al.* 2015). One reason for the vast variety of different diseases is the availability of their receptors throughout the human body - decay accelerating factor (DAF), Coxsackie and adenovirus receptor (CAR) and integrins to name a few (Marjomäki *et al.* 2015). Newer research shows the human neonatal Fc receptor (FcRn), responsible for developing fetal immune systems, to be used in enterovirus entry (Laajala and Marjomäki 2019).

1.1.3 Coxsackievirus B3 (CVB3)

Six different serotypes of CVBs exist (CVB1-6). The binding and internalization and uncoating of all of them is possible via CAR (Bergelson *et al.* 1997), which is a transmembrane protein of the tight junction (TJ) (Cohen *et al.* 2001). As CAR is found abundantly in the surfaces of cardiomyocytes in developing heart tissue, it is no wonder why CBVs cause myocarditis (Kashimura *et al.* 2004). However, since the TJ is located between cells, the CAR is usually not available to viruses trying to enter from the apical side of cells. Instead, CVB serotypes CVB3 and CVB5 utilize the DAF found in abundance on the apical surface of cells. Binding to DAF causes clustering of receptors and activates tyrosine kinase Abl, which in turn causes Rac1-dependent actin movements that eventually bring the bound virus to the TJ and CAR for entry.

Furthermore, GTPases such as RhoA and Cdc42 seem to play a pivotal role in the entry of CVB3 (Coyne and Bergelson 2006). Another TJ protein, occludin, seems to be important in CVB3 entry as they are internalized simultaneously, but the occludin does not interact with the virus directly (Coyne *et al.* 2007). Essentially, in polarized cells CVB3 binds to DAF and causes a reaction to transport it to the TJ, where it can be internalized by binding to CAR. In non-polarized cells, binding to CAR can happen straight away and DAF is not as important.

1.1.4 Coxsackievirus A9 (CVA9)

CVA9 binds to the cell surface via the integrins αVβ3 and αVβ6. It does so using a functional arginine-glycine-aspartic acid (RGD) motif present in one of its capsid proteins (Williams *et al.* 2004). The binding causes the formation of neutral multivesicular bodies (MVBs) that promote infection (Huttunen *et al.* 2014). In addition to the binding, the internalization process of CVA9 is dependent on β2-microglobulin (β2 m), dynamin, and Arf6, but is independent of clathrin and caveolin-1 (Heikkila *et al.* 2010). β2 m is a subunit of the recently discovered FcRn, which has been proven to be an uncoating receptor for CVA9 (Zhao *et al.* 2019). It has been shown that another key component to CVA9 infection are lipid rafts located along the cell surface as disturbing these has an effect of inhibiting the infection (Triantafilou and Triantafilou 2003).

1.2 Antivirals

1.2.1 Need for Antiviral Drugs

Among enteroviruses, vaccines have been developed only against poliovirus (PV). While the inactivated PV vaccine (IPV) and attenuated oral PV vaccine (OPV) have been mostly successful in controlling the worldwide spread of PV, several problems remain.

Wildtype PV infections and vaccine-derived poliovirus (VDPV), caused by OPV, stand in the way of total elimination of PV infections. Vaccines, while useful to prevent disease, cannot be used if the infection is already present in the system. Furthermore, the sheer number of EV serotypes makes developing vaccines for all of them unfeasible. Broad-spectrum antivirals that treat all EV infections are necessary.

1.2.2 Virucides and Antiviral Drugs

As unenveloped viruses, enteroviruses are more stable and harder to kill than enveloped viruses such as influenza (Linnakoski *et al.* 2018). General hygiene, such as washing hands may not suffice. Virucides are substances that break down virus particles with no specific mechanism of action. They are good for decontaminating surfaces and equipment, but cannot be used to fight infection. Moreover, non-enveloped viruses like enteroviruses are resistant to virucides. Virucides include detergents, chloroform, ultraviolet light, and specially manufactured compounds such as Virkon. Antiviral drugs however, are compounds used specifically to inhibit virus replication and are used to fight infection. Their targets may include any part of the viral life cycle such as receptor binding, entry, uncoating and replication ('Virucides - an overview | ScienceDirect Topics'; De Clercq and Herdewijn 2010). Antivirals against enteroviruses have been in development and some successful results have been made, such as Pleconaril that binds to the canyon structure and prevents uncoating. However, it comes with side effects that prevented the U.S. Food and Drug Administration (FDA) approval (Hayden *et al.* 2003). Currently there are no approved antiviral drugs available against enterovirus infections (Abzug 2014). RNA replication is quite prone to errors so mutation of the virus strain is rapid. This could lead to development of resistance towards any viral-target drugs. A good alternative would be to target host cell factors. Host factors have a low chance of becoming drug-resistant (van der Linden *et al.* 2015). However, it has been shown that viruses can develop resistance against these kind of inhibitors as well (van der Schaar *et al.* 2012). In addition, targeting host cell factors may

lead to cytotoxicity and side effects. Antiviral drug development nowadays uses combinatorial chemistry (Strobel and Daisy 2003). A common problem with traditionally developed antivirals is a compound showing initial antiviral activity but being unable to do so in vivo. The administration can be difficult or side effects can arise (Meijer *et al.* 1992).

1.2.3 Natural Remedies

Natural products are metabolites or by-products derived from plants, animals or microorganisms (Baker *et al.* 2000). We as a species have used natural products in medicine throughout history. One of the most common medicines, aspirin (salicylic acid), is originally found in plants of the Salix genus such as the willow and has been used for thousands of years by multiple civilizations around the world (Norn *et al.* 2009) . Another historical discovery is the antibiotic penicillin. Truly, natural products are still as relevant as ever in medicine as about 40% of prescription drugs and 49% of new chemical products registered by the U.S. Food and Drug Administration are of natural origin (Brewer 2000), including the first billion-dollar anticancer drug Taxol produced from the yew tree (Wani *et al.* 1971). Compared to computational drug discovery methods, nature has an unimaginable amount of compounds with novel structures, which are yet to be discovered. Combinatorial libraries are limited, but the diversity of nature is not (Strobel and Daisy 2003). Furthermore, the use of microorganisms to produce drugs or precursor molecules is a great way to drive down manufacturing costs (Grabley and Thiericke 1999). Natural products as they exist are readily compatible with biology, are relevant to cellular systems and many are inherently stable. In addition, they possess significant value to us and should not be overlooked (Baker *et al.* 2000).

1.2.4 Fungal Endophytes

Endophytes are microorganisms that inhabit plant organisms without causing any immediate negative effects (Bacon and White, 2000). It is also estimated that each of the world's almost 400,000 discovered plant species (Willis 2017) act as hosts to one or more endophytes (Strobel and Daisy 2003). Though several different microorganisms such as bacteria and even viruses can act as endophytes, this study focuses on fungal endophytes (Hilszczańska 2017). Endophytes are largely untapped sources of novel lead molecules for drug discovery (Chandra 2012). There are existing precedents of successful endophyte-based drug discoveries such as the earlier mentioned anticancer drug Taxol, as well as torreyanic acid produced by *Pestalotiopsis microspora* (Selim 2012). Considering all thus far mentioned evidence it is hard to ignore the epic potential that fungal endophytes represent for the medical industry. Indeed, several antiviral compounds have already been found from endophytic fungi (for a review, see Linnakoski et al. 2018). Examples include those used against influenza A (H1N1) and herpes simplex type 1 (HSV-1) viruses (Isaka *et al.* 2007; Zhang *et al.* 2011). To date, no studies have been performed to determine the antiviral activity of fungal endophytes against enteroviruses.

2. AIM OF THE EXPERIMENT

In this experiment a variety of endophytic extracts provided by Professor Ari Pappinen from the University of Eastern Finland (Faculty of Science and Forestry, School of Forest Science) were studied to elucidate their target in enterovirus infection. The three main aims of the experiment were to discover:

1. Do the extracts have an effect on virus binding on the cell surface?

2. Are the extracts cytotoxic?

3. Do the extracts affect host cell mechanisms against virus infection?

3. MATERIALS AND METHODS

The study comprised of 3 experiments. A binding assay, ATP assay, and a pretreatment assay. Each of the experiments with the exception of the ATP assay was performed for each of the viruses CVB3 and CVA9. The endophyte extracts given by Ari Pappinen were issued numbers (4, 5, 6, 7, 9, 13, 15, 17, 21, 22, 23, 25, 27, 28 and 31) to identify different extracts. The fungal endophytes where isolated from a plant belonging to the genus *Salix*. These extracts had previously shown antiviral activity against CVB3 and CVA9 (Reshamwala 2017).

3.1 Cells and Viruses

The cells used were adenocarcinomic human alveolar basal epithelial (A549) cells (from Dr. Petri Susi) and green monkey kidney (GMK) cells (Dr. Timo Hyppä, University of Turku). They were grown in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher) with 10% Fetal Bovine Serum (FBS, Gibco Life Technologies, United Kingdom), 1% L-Glutamax and 1% antibiotics (penicillin/streptomycin) at +37C and 5% CO2. When subculturing, the cells were washed with phosphate buffered saline (PBS) and detached with 2 ml of trypsin (Gibco, Life Technologies, United Kingdom). The virus strains used were CVB3 (batch #44, 6,72 x 10^{10} PFU/ml, Nancy strain) and CVA9 (batch #4, 7,43 x 10^{10} PFU/ml, Griggs strain).

3.2 Radioactive Labeling of CVB3 & CVA9

For the binding assay experiment, radioactively labeled viruses had to be created. Six 75 cm^2 bottles of cells were grown to about 70-80% confluence. GMK cells were used for the radioactive labeling of CVB3, while GMK cells were used with CVA9. 150 µl of crude virus extract and 5 ml 1% minimum essential medium eagle with earle's salts

with 2 g/l sodium bicarbonate and without l-glutamine, l-methionine, l-cysteine and l-cystine (MP Biomedicals) was added. The media was meant to prevent any non-radioactive virus particles from being built. The cells were first kept on ice for 45 minutes to allow the viruses to bind to, but not enter, the cells. Then the cells were moved to +37° C to allow the infection to proceed for 2,5 hours (in a Heraeus HERAcell® 150 CO_2 incubator). Radioactive ^{35}S-L-methionine and ^{35}S-L-cysteine containing media was prepared using PerkinElmer EASYTAG(™)EXPRE^{35}S^{35}S protein labeling solution. 5 ml of the l-methionine and l-cysteine-free media was combined 138 µl of easytag solution to make a 50 µCi/ml solution. The incubated cells had their media changed to the radioactive one and were kept at +37° C overnight. Once the media had all the necessary amino acids the viruses could finally replicate and form radioactively labeled particles.

The bottles were combined and 300 µl of Tween80 (Sigma-Aldrich) detergent was added to the mixture to stabilize the virus. Three freeze-thaw cycles were performed to break apart the cells. The mix was centrifuged at 2500G for 10 minutes in a Thermo Scientific centrifuge (SL 16R) and the supernatant was collected. The supernatant was centrifuged again at 4000 G for 10 min at 4°C and yet again the supernatant was collected. These were done to separate the virus particles from any cell debris. Sucrose cushioned tubes were prepared by adding 2 ml of 40% sucrose (VWR Life Science) to Beckman, thin-walled ultracentrifuge tubes. The supernatant was then divided amongst five tubes and balanced using 2 mM $MgCL_2$/PBS. The tubes were ultracentrifuged for 2,5 hours at 151 263 G (4°C) in a Beckman (Optima TM LE-80K) ultracentrifuge. The centrifugation stacks the virus particles on top of the sucrose cushion. The liquid was carefully removed almost completely and the surface of the cushions was collected and dissolved into 2 mM $MgCl_2$/PBS. This was then ultracentrifuged at 151 263 G for 2 hours. The liquid was removed and the pellet containing the virus particles was dissolved into 500 µl of 2 mM $MgCl_2$/PBS.

A sucrose gradient (5-20%) was created using a Pharmacia Biotech flow pump and the sample was layered on top. This was centrifuged at 151 263 G for 2 hours. The virus particles come in three different forms: empty, uncoating intermediate (UIM) particles and intact virus particles (Myllynen *et al.* 2016). The gradient allows the different viral particles to settle at separate parts in the tube due to their weight difference. The gradient was collected in 500 µl fractions and 10 µl samples were collected from each to scintillation cocktail (PerkinElmer UltimaGold TM) containing tubes. The radioactivity (counts per minute) was measured with a PerkinElmer Tri-Carb ® 2910TR liquid scintillation analyzer and a graph was plotted to determine what fractions contained the radioactive virus. The virus containing fractions were then collected and stored in -80 °C for the experiments.

3.3 Binding Assay

The binding assay was performed to determine the effect of any endophyte extracts on virus binding to the cell surface. The experiments were perofrmed with extracts 4, 7, 13, 21, 22, 27 and 31 for CVB3 and 6, 9, 10, 15, 22, 23 and 28 for CVA9, with 3 replicates of each extract. A549 cells were trypsinized and calculated and 150 000 cells per tube were used for each replicate. The cells within eppendorfs were centrifuged to a pellet at 4435 G for 5 minutes in room temperature. The supernatant was discarded and the pellets were resuspended in 60 µl of 2 mM $MgCl_2$/PBS. Then 60 µl of extract was added to each tube (1:2). The control samples had 2 mM $MgCl_2$/PBS instead of any extract (Table 1). After mixing the cells were incubated for 1h on ice (during which they were further mixed a few times by gently tapping the tubes) to cool the cells down. After Incubation they were centrifuged and washed with 60 µl of 2 mM $MgCl_2$/PBS once and then dissolved in 86 µl + 14 µl of ^{35}S-labeled virus (50000 CPM) in new tubes. They were mixed and incubated for another hour on ice (again while gently mixing them throughout). The incubation on ice allows for virus binding to the cell surface but

prevents them from entering. The cells were then centrifuged (3080 G, 5 min, (4°C) and washed twice with 100 µl and finally resuspended in 50 µl of 2 mM $MgCl_2$/PBS. This was to wash away all the unbound radioactive virus particles. The samples were then mixed in with scintillation cocktail (4 ml) and radioactivity (counts per minute) was measured . Three repeats were performed for both CVB3 and CVA9.

Table 1. Binding Assay. The samples contained an extract and the radioactively labeled virus. The control sample had $MgCl_2$/PBS instead of the extract.

	Extract	**Virus**
Samples	+	+
Control	-	+

3.4 ATP Assay

The ATP assay was performed to determine if the extracts are cytotoxic. In the assay the extracts used were 4, 6, 7, 9, 10, 13, 15, 21, 22, 23, 28 and 31. 54 µl of extract was mixed with 6 µl 2 mM $MgCl_2$/PBS buffer. Control samples had 60 µl of 2 mM $MgCl_2$/PBS and no extract (Table 2). The mixtures were incubated for 15 minutes at +37° C. After the incubation 540 µl of 10% DMEM was added to create a final dilution of 1:10 and another incubation was done for 15 minutes. The samples were then added to confluent 96-well plates containing A549 cells, 100 µl per sample per well and the plate was incubated overnight at +37° C. 100 µl of CellTiter-Glo®-solution (Luminescent Cell Viability Assay, Promega) was added to each well on top of the existing medium. The solution contains luciferin, luciferase and a chemical that lyses the cells. The luciferin reacts with the ATP in the cell and causes luminescence. After a 15 minute incubation the luminescence was measured using a PerkinElmer VICTOR TM X4 multilabel reader. Two repetitions of this study were performed.

Table 2. ATP Assay. The samples included extract. Control samples had $MgCl_2$/PBS instead.

	Extract
Samples	+
Control	-

3.5 Pretreatment of Cells with Extract before Virus Infection Using CPE Assay

The pretreatment experiment was performed to assess the role of cellular factors in the extract's antiviral activity. The experiment was performed on a 96-well plate with 15 000 cells in each well incubated for 24 hours in 37°C. It was assumed that the cell count doubles in this time to a total of 30 000 cells per well. The endophyte extracts in DMEM (1:10) were added on the cells and incubated for two hours prior to virus infection. Before adding the virus, the extracts were washed away by first removing the medium and then washing once with medium. It was thought that if the extracts have an effect on cellular factors that contributes to their antiviral activity, the effect could still be noticed immediately after the extracts had been removed. The virus was then added as a 1:2 000 dilution (multiplicity of infection CVB3: $3{,}36 \times 10^7$, CVA9: $3{,}715 \times 10^7$) and the plate was incubated for 24 hours at 37°C. Two controls were used. One where no extract was added (only DMEM) and infected normally. The other one with no extract and no infection (Table 3). The plate was then stained with cytopathic effect (CPE) DYE (0.03% crystal violet, 2% ethanol and 36.5% formaldehyde), then lysed with lysis buffer (0.8979 g of sodium citrate and 1N HCl in 47.5% ethanol) The CPE dye stains the cells with color which is then released as they are lysed into a homogenous mix. The absorbance was measured at 570 nm using PerkinElmer VICTOR TM X4 multilabel reader to determine the amount of viable cells remaining in the wells (Schmidtke *et al.* 2001).

Table 3. Pretreatment experiment. The test samples had been treated with extract and

infected with the virus. The virus control was not treated with extract but was infected. The cell control was neither treated nor infected.

	Extract	**Virus**
Samples	+	+
Virus Control	-	+
Cell Control	-	-

4. RESULTS

4.1 Radioactive Virus Labeling

The radioactively labeled CVB3 and CVA9 were collected successfully, even though there was a problem in the first CVB3 ultracentrifugation part where the vials collapsed due to not being filled to the brim properly. After the CPM of each fraction was measured a graph was plotted and used to determine which fractions to collect for the experiments. For CVB3, fractions 14-17 were collected and pooled due to them containing the intact virus particles as well as the UIM particles (Myllynen *et al.* 2016) (Figure 3). For CVA9, fractions 16-18, containing the UIM and intact particles, were collected (Figure 4). Empty virus particles form the first major peak due to them being lighter. This is usually around fractions 8-10, but was at 10-11 in both of these experiments.

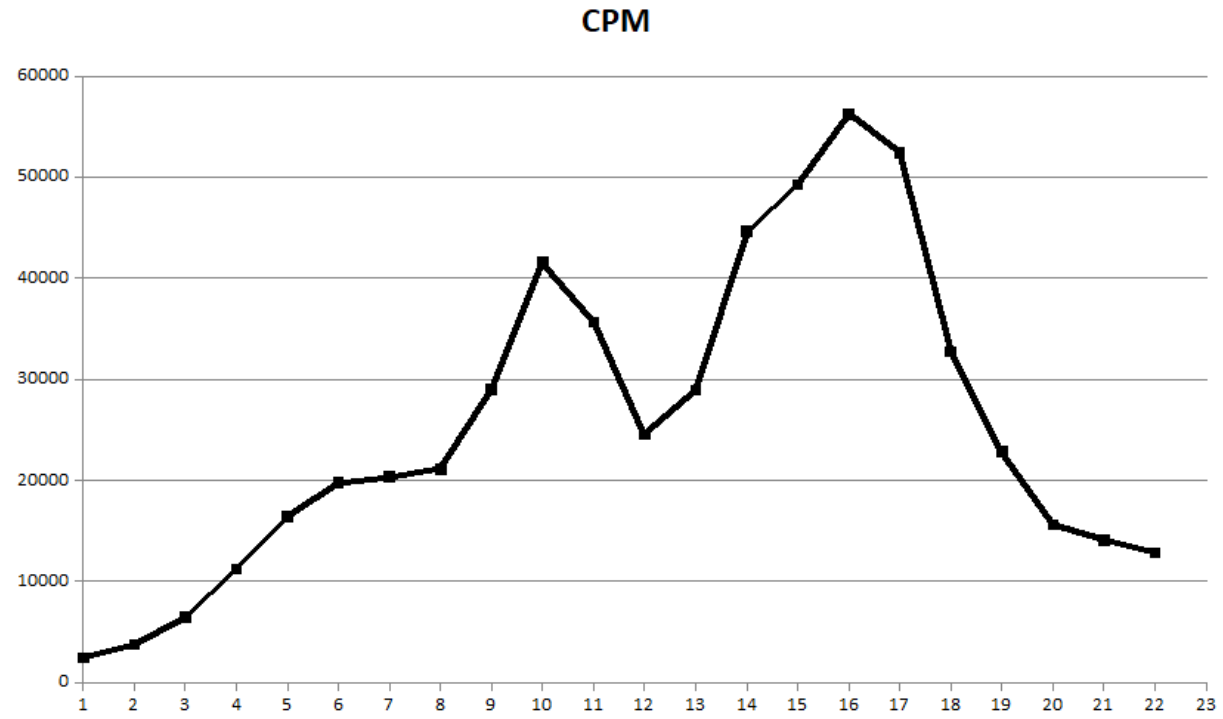

Figure 3. CVB3 radioactive labeling. The graph shows the measured CPM of each of the fractions collected from the sucrose gradient. The first major peak represents the empty virus particles (fractions 9-11). Typically, they reside in fractions 8-10. Around fraction 14 is the second peak that indicates the UIM particles and the final peak at 15-17 is the intact normal virus particles.

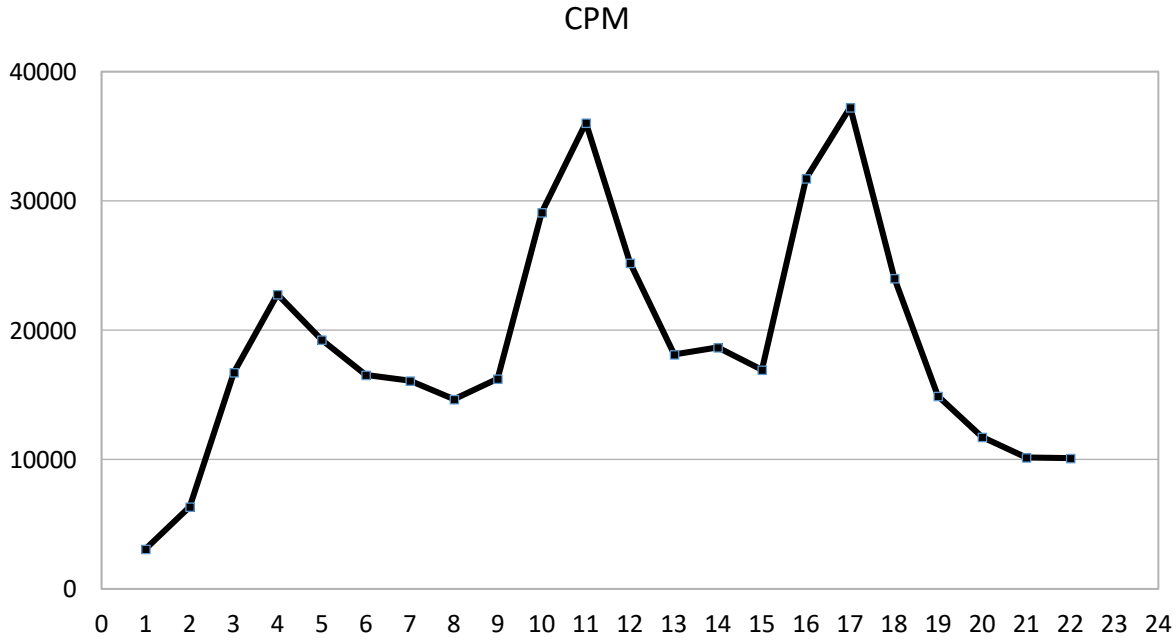

Figure 4. CVA9 radioactive labeling. The graph shows the CPM of the fractions

collected from the sucrose gradient. The peak at fraction 11 represents the empty virus particles. The peak at 16-18 includes the UIM particles as well as the intact particles. Therefore, fractions 16-18 were collected. The minor peak at fraction 4 is of unknown origin.

4.2 Binding Assay

The binding assay was performed to determine the effect of the extract on the ability of the virus to bind to the cell surface. The radioactivity measured correlates with the amount of viruses remaining after the washes. The data from the binding assay experiments was averaged within the 3 replicates of each sample and normalized within the experiment with the control having a value of 1 (Figure 5). In the CVB3 binding assays, extracts 4, 7, 21, 22 and 27 caused increased binding in the first and second experiment. In both of them 13 showed decreased binding affinity, while 31 had no major effect. The third repeat suffered from greater variation and it is hard make any conclusions with the exception that 13 still seems to reduce binding. CVA9 experiments show increased binding with extracts 6, 9, 22 and 23 in each experiment while the effect of extracts 10, 15 and 28 are inconclusive. No statistical significance was found in the results.

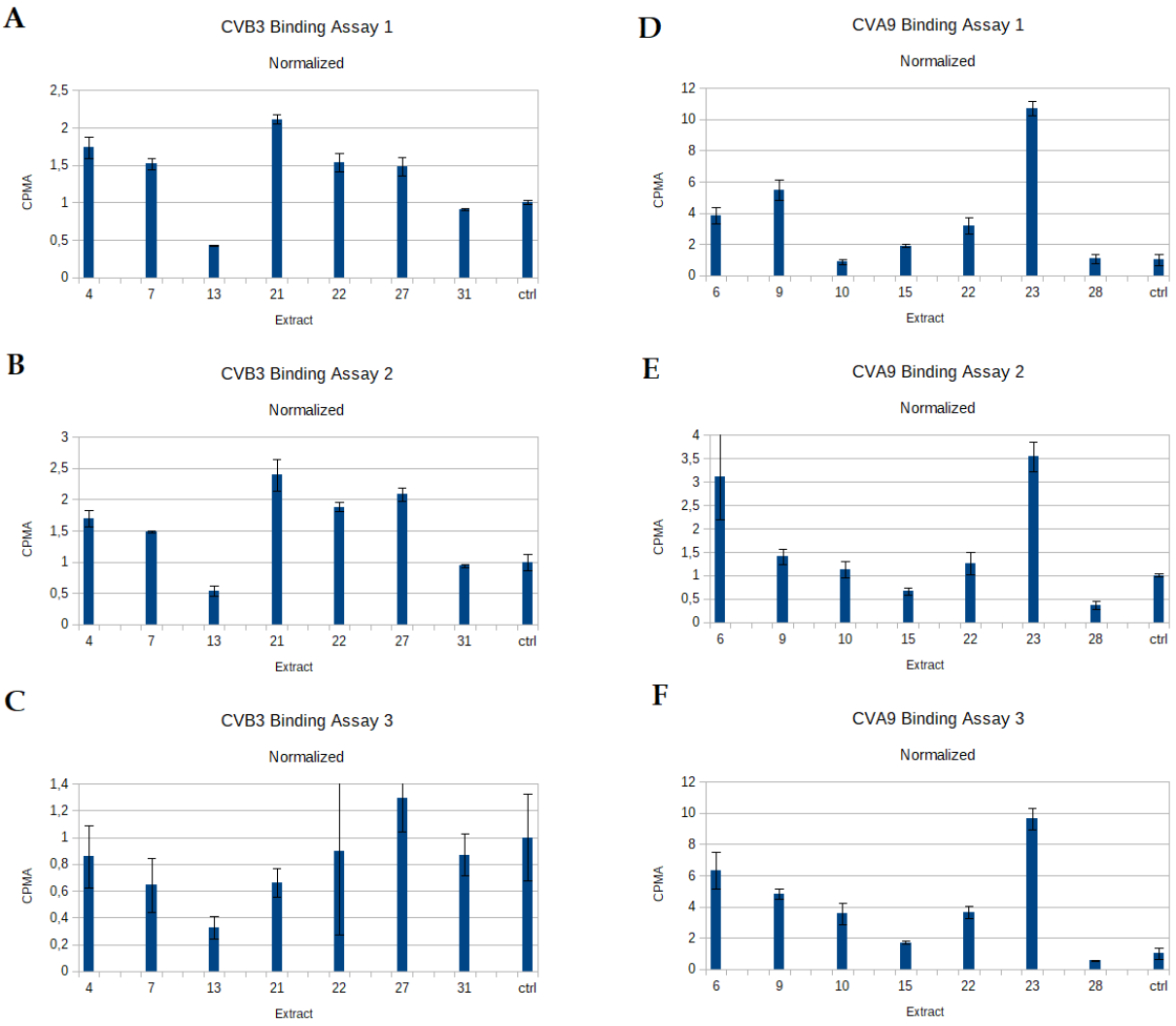

Figure 5. Binding Assays with CVB3 (**A-C**) and CVA9 (**D-F**). Control samples were infected cells without extract. Counts per minute average (CPMA) represents the radioactivity and thus the amount of viruses bound to the cells. The different samples are labeled according to the extract used. Results are the mean of three replicates with standard error of the mean (SEM). They were normalized within each experiment with control as 1. (**A**) The first experiment shows a slight increase in extracts 4, 7, 21, 22 and 27 with 21 being the highest, while 13 seems to have a diminishing effect on binding. Extract 31 seems to have no effect either way. (**B**) The second experiment follows the trend of the first one with 13 being the most effective at inhibiting binding. (**C**) The third experiment had a lot more deviation in the results and thus is more unclear. However extract 13 is the lowest and 27 the highest in binding. (**D**) The first CVA9 experiment shows higher binding with extracts 6, 9, 15, 22 and 23 with the last being the highest. The other extracts seem to have little effect. (**E**) Extracts 6 and 23 have higher binding but extracts 15 and 28 seem to have a diminishing effect. (**F**) Higher binding with extracts 6, 9, 15, 22 and 23. Lower with extract 28. No statistical significance was found

in any of the results.

4.3 Cytotoxicity Assay

The cytotoxicity assay was performed to discover the effect of the extracts on the normal function of cells. The results show luminescence measured that represents the ATP activity in the cells in each sample. ATP activity is a good indicator of a cell's viability (Riss *et al.* 2004). The data is presented in figure 6 and 7. While extracts 7, 9, 15, and 25 seem to have caused some reduced ATP activity in one or both experiments, extracts 6, 10, 21 and 28 seem to have had either no visible effect or even increased ATP activity. Others like, extract 31 seems to fluctuate between experiments. No statistical significance was found in the results and that indicates the extracts to be non-cytotoxic.

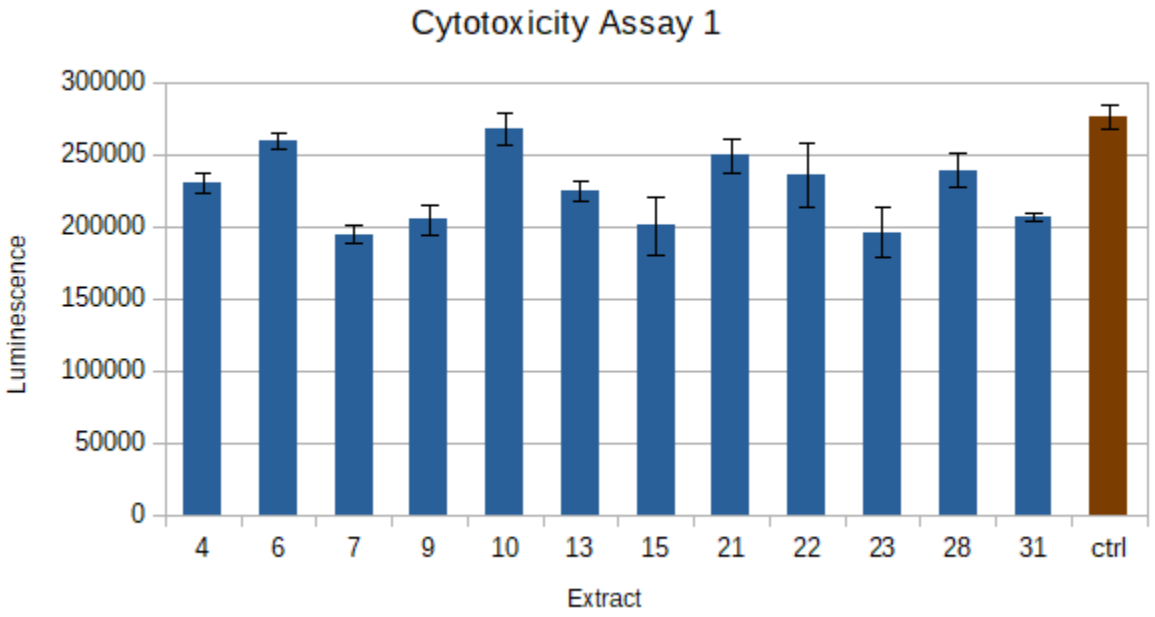

Figure 6. Cytotoxicity Assay 1. The samples were cells treated with the numbered extract. Control cells were without extract. Luminescence measured indicates the ATP activity and thus viability of the cells. The result is the mean of three replicates with SEM. As it looks, all the extracts (possibly excluding 10) had a slight negative effect to ATP activity. No statistical significance was found, however.

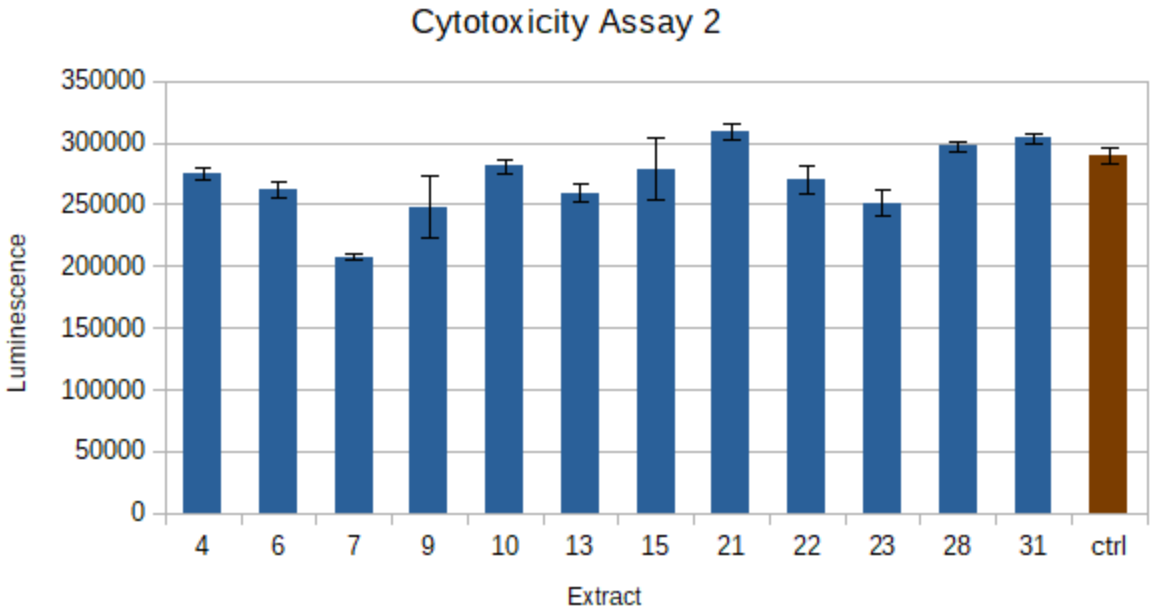

Figure 7. Cytotoxicity Assay 2. The samples were cells treated with the numbered extract. Control cells were without extract. Luminescence measured indicates the ATP activity and thus viability of the cells. The result is the mean of three replicates with SEM. Extract 7 had the most negative effect on ATP activity. Some of the extracts seem to possibly increase the ATP activity in the cells. No statistical significance was found, however.

4.4 Pretreatment Assay

The purpose of the pretreatment assay was to assess the role of cellular factors in the extract's antiviral activity. The data presented measures the number of viable cells through absorbance caused by the CPE dye absorbed by the living cells (figure 8). The dye containing cells were lysed to create a homogenous mixture with an even and comparable absorbance between samples. When compared to the virus control, no statistically significant difference is detected. All the samples and virus controls had a significant (P>0,05) reduction in viable cells compared to the cell controls in experiments with both viruses. It can be assumed that the experiment was successful and that no antiviral effect was caused by the pretreatment process.

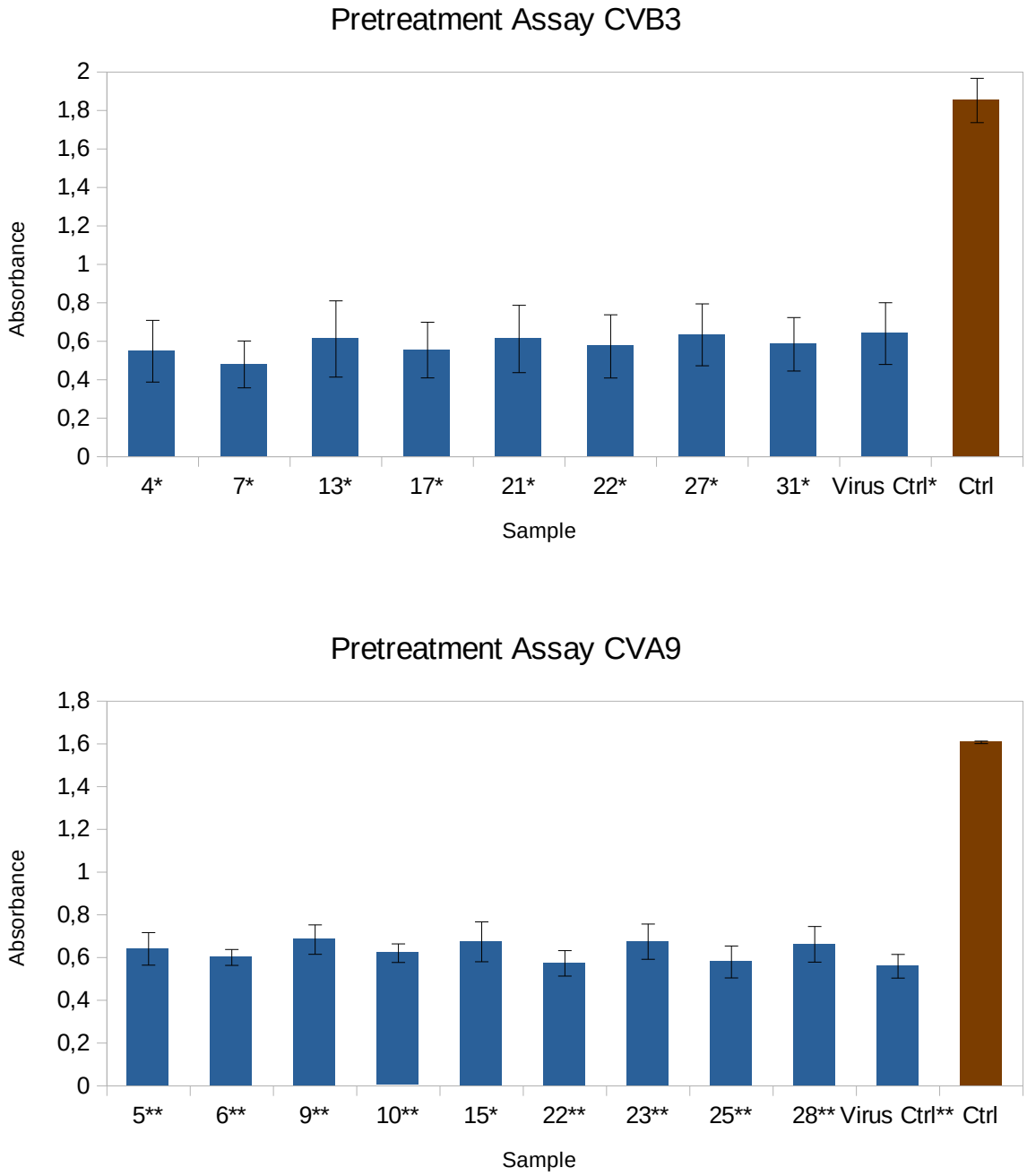

Figure 8. The pretreatment assays. The absorbance represents the amount of living cells left. The virus control was infected normally but did not undergo pretreatment with an extract. The control was made up of wells containing uninfected cells. The result is the mean of three replicates with SEM. The experiments with CVB3 or CVA9 show no significant difference in the pretreated cells when compared to the virus control. The samples and virus controls however have significantly less viable cells remaining when compared to the cell controls.

5. DISCUSSION

We are only beginning to grasp the variety and abundance of severe, chronic disease caused by enteroviruses worldwide. Diseases such as type-1 diabetes and atherosclerosis are severely affecting quality of life and thus countermeasures against these infections must be developed. Vaccines and antiviral drugs are the tools needed to combat enterovirus infections. The only available vaccine against enteroviruses in this point of time is against PV. However, due to the wide range of EV-B serotypes, developing a vaccine against all of them would be highly impractical. A promising source of antiviral compounds are endophytic fungi extracts. Previous experiments have already found them to contain antiviral agents (Reshamwala 2017; Linnakoski *et al.* 2018).

In this study endophyte extracts were used that were previously shown to have direct antiviral effects on the virions. Here, the aim was to study further if the extracts would exert their effect via affecting on the binding on cells, or via acting through the host cell. Radioactively labeled (^{35}S) CVB3 and CVA9 viruses were created and purified using a 5-20% sucrose gradient. These viruses where used to study the effects of the extracts effects on virus binding to the cell surface using a binding assay. In general, it was clear that most of the extracts did not have inhibitory effects on the binding. Only the number 13 showed a moderate inhibition on binding, while many caused an increase compared to the control sample. The results with CVA9 varied more from one experiment to the next. While there was no extract in particular that caused a reduction in binding, extracts 6, 9 and 23 caused mostly increases in binding. However, no statistical significance was found in either case confirming either. Why the extracts would cause an increase in the binding of viruses to the cell surface is a very interesting question. As this thesis did not study further the nature of the binding we can only guess, but there is a possibility that some of the extracts contain salts or other agents that cause the aggregation of viral particles on the cell surface (Floyd and Sharp 1978; Gerba and

Betancourt 2017). Further experiments studying this effect are warranted. Radioactive measurements are very sensitive and easily show even small pipetting errors and results between experiments can easily show variation. However, altogether, the results from both CVA9 and CVB3 show that the extract had more enhancing effect on the binding rather than inhibitory effects. Enhancing effects would possibly support entry of un-infective particles to enter the cells by endocytosis, which may be beneficial for vaccination purposes.

Cytotoxicity of the extracts was measured using an ATP assay. All of the extracts were found not to be cytotoxic. This is a promising sign if any extracts were to be used in drug development in the future. Other experiments involving biological extracts against viruses have found cytotoxic effects at quite low concentrations (Daoud and Soliman 2015). Most substances become cytotoxic at high enough concentration, but they can still have effective antiviral properties in safe concentrations (Nolkemper *et al.* 2006). Further research involving the antiviral effect of these extracts is worthwhile.

Finally, a pretreatment experiment was performed with the cells being exposed to the extracts for 2 hours before the infection, only to have the extracts removed as the infection was initiated. This was expected to reveal if any host mechanisms where influenced by the extracts to create an antiviral effect. No such evidence was found however, as the absorbance of the treated cells showed no statistical difference when compared to untreated infected cells (virus control). It could be concluded then, that whatever the antiviral target of the extracts is, it is not via a host mechanism.-Perhaps the effects that the extracts have is very short-lived and by removing the extract from the solution when infecting the cells, any effect they may have had was nullified after a 24-hour infection. Other experiments have done pretreatment by incubating the virus with biological extract before infection and found that to be effective (Micol et al. 2005; Daoud and Soliman 2015). In fact, the original antiviral hits found from the extracts used in this experiment were found this way. As the pretreatment had no antiviral

effect, it would be reasonable to assume the extracts interact directly with the virus particles. As the virus has been incubated with the extracts before infection and the cells have been incubated with the extract before infection, the final combination would be to introduce the extracts to the cells after infection to confirm these suspicions (McMahon *et al.* 1993).

Currently the composition of the extracts used in this study is unknown. If they were ascertained, the bioactive compounds could be screened for antiviral molecules or compounds. When narrowed down to this level, the target could be easier to determine. For example, computational methods could be used to find binding sites on the virus capsid for the antiviral agents. It is also possible that no single substance is responsible for the antiviral effect, but instead the combination of several present in the extracts is required. This factor makes it hard to compare the results with other experiments since the concentrations used are effectively unknown. Further research will be made into fractionating the extracts into their bioactive compounds and determining their structure, which will be then further tested for their antiviral mechanism.

The need for antivirals against EV-Bs is dire. Endophyte extracts, such as the ones studied in this thesis, could be an as of yet largely untapped source of novel antiviral compounds. While previously found to have antiviral effects, the extracts specific antiviral target is unknown. This study has shown that the extracts do not prevent binding of the virions on the cells, nor that the effects are acting through the host cells. The most likely target is the viral capsid itself. Further research is necessary to determine the exact effects. The composition of the extracts could provide insight into the identity of the effective agent.

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CITATIONS

Abzug M.J. 2014. The enteroviruses: Problems in need of treatments. *Journal of Infection* 68: S108–S114.

Bacon C.W. & White J. 2000. *Microbial Endophytes*.

Baker D., Mocek U. & Garr C. 2000. Natural products vs. combinatorials: a case studyle. *Biodiversity: New Leads for the Pharmaceutical and Agrochemical Industries*.

Bergelson J.M. & Coyne C.B. 2013. Picornavirus Entry. In: Springer, New York, NY, pp. 24–41.

Bergelson J.M., Cunningham J.A., Droguett G., Kurt-Jones E.A., Krithivas A., Hong J.S., Horwitz M.S., Crowell R.L. & Finberg R.W. 1997. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science (New York, N.Y.)* 275: 1320–1323.

Brewer S. 2000. The relationship between natural products and synthetic chemistry in the discovery process. *Biodiversity: New Leads for the Pharmaceutical and Agrochemical Industries*.

Chandra S. 2012. Endophytic fungi: novel sources of anticancer lead molecules. *Applied Microbiology and Biotechnology* 95: 47–59.

Clercq E. De & Herdewijn P. 2010. Strategies in the Design of Antiviral Drugs. In: *Pharmaceutical Sciences Encyclopedia*, John Wiley & Sons, Inc., Hoboken, NJ, USA, pp. 1–56.

Cohen C.J., Shieh J.T., Pickles R.J., Okegawa T., Hsieh J.T. & Bergelson J.M. 2001. The coxsackievirus and adenovirus receptor is a transmembrane component of the tight junction. *Proceedings of the National Academy of Sciences of the United States of America* 98: 15191–15196.

Coyne C.B. & Bergelson J.M. 2006. Virus-Induced Abl and Fyn Kinase Signals Permit Coxsackievirus Entry through Epithelial Tight Junctions. *Cell* 124: 119–131.

Coyne C.B., Shen L., Turner J.R. & Bergelson J.M. 2007. Coxsackievirus Entry across Epithelial Tight Junctions Requires Occludin and the Small GTPases Rab34 and Rab5. *Cell Host & Microbe* 2: 181–192.

Daoud H.M. & Soliman E.M. 2015. Evaluation of Spirulina platensis extract as natural antivirus against foot and mouth disease virus strains (A, O, SAT2). *Veterinary world* 8: 1260–1265.

Floyd R. & Sharp D.G. 1978. Viral aggregation: effects of salts on the aggregation of poliovirus and reovirus at low pH. *Applied and environmental microbiology* 35: 1084–1094.

Gerba C.P. & Betancourt W.Q. 2017. Viral Aggregation: Impact on Virus Behavior in the Environment. *Environmental Science & Technology* 51: 7318–7325.

Grabley S. (Susanne) & Thiericke R. (Ralf). 1999. *Drug discovery from nature*. Springer.

Hayden F.G., Herrington D.T., Coats T.L., Kim K., Cooper E.C., Villano S.A., Liu S., Hudson S., Pevear D.C., Collett M. & McKinlay M. 2003. Efficacy and Safety of Oral Pleconaril for Treatment of Colds Due to Picornaviruses in Adults: Results of 2 Double-Blind, Randomized, Placebo-Controlled Trials. *Clinical Infectious Diseases* 36: 1523–1532.

Heikkila O., Susi P., Tevaluoto T., Harma H., Marjomaki V., Hyypia T. & Kiljunen S. 2010. Internalization of Coxsackievirus A9 Is Mediated by β2-Microglobulin, Dynamin, and Arf6 but Not by Caveolin-1 or Clathrin. *Journal of Virology* 84: 3666–3681.

Hilszczańska D. 2017. Endophytes – characteristics and possibilities of application in forest management. *Forest Research Papers*.

Huttunen M., Waris M., Kajander R., Hyypia T. & Marjomaki V. 2014. Coxsackievirus A9 Infects Cells via Nonacidic Multivesicular Bodies. *Journal of Virology* 88: 5138–5151.

Isaka M., Berkaew P., Intereya K., Komwijit S. & Sathitkunanon T. 2007. Antiplasmodial and antiviral cyclohexadepsipeptides from the endophytic fungus Pullularia sp. BCC 8613. *Tetrahedron* 63: 6855–6860.

Kashimura T., Kodama M., Hotta Y., Hosoya J., Yoshida K., Ozawa T., Watanabe R., Okura Y., Kato K., Hanawa H., Kuwano R. & Aizawa Y. 2004. Spatiotemporal changes of coxsackievirus and adenovirus receptor in rat hearts during postnatal development and in cultured cardiomyocytes of neonatal rat. *Virchows Archiv* 444: 283–292.

Laajala M. & Marjomäki V. 2019. A Common Receptor Found for Echoviruses. *Trends in*

Microbiology 27: 475–477.

Laitinen O.H., Honkanen H., Pakkanen O., Oikarinen S., Hankaniemi M.M., Huhtala H., Ruokoranta T., Lecouturier V., André P., Harju R., Virtanen S.M., Lehtonen J., Almond J.W., Simell T., Simell O., Ilonen J., Veijola R., Knip M. & Hyöty H. 2014. Coxsackievirus b1 is associated with induction of β-cells autoimmunity that portends type 1 diabetes. *Diabetes*.

Linden L. van der, Wolthers K.C. & Kuppeveld F.J.M. van. 2015. Replication and inhibitors of enteroviruses and parechoviruses. *Viruses* 7: 4529–4562.

Linnakoski R., Reshamwala D., Veteli P., Cortina-Escribano M., Vanhanen H. & Marjomäki V. 2018. Antiviral agents from fungi: Diversity, mechanisms and potential applications. *Frontiers in Microbiology* 9.

McMahon J.B., Gulakowski R.J., Weislow O.S., Schultz R.J., Narayanan V.L., Clanton D.J., Pedemonte R., Wassmundt F.W., Buckheit R.W. & Decker W.D. 1993. Diarylsulfones, a new chemical class of nonnucleoside antiviral inhibitors of human immunodeficiency virus type 1 reverse transcriptase. *Antimicrobial agents and chemotherapy* 37: 754–760.

Marjomäki V., Turkki P. & Huttunen M. 2015. Infectious entry pathway of enterovirus B species. *Viruses* 7: 6387–6399.

Meijer D.K.F., Jansen R.W. & Molema G. 1992. Drug targeting systems for antiviral agents: options and limitations. *Antiviral Research* 18: 215–258.

Micol V., Caturla N., Pérez-Fons L., Más V., Pérez L. & Estepa A. 2005. The olive leaf extract exhibits antiviral activity against viral haemorrhagic septicaemia rhabdovirus (VHSV). *Antiviral Research* 66: 129–136.

Myllynen M., Kazmertsuk A. & Marjomäki V. 2016. A Novel Open and Infectious Form of Echovirus 1. *Journal of Virology*.

Nolkemper S., Reichling J., Stintzing F., Carle R. & Schnitzler P. 2006. Antiviral Effect of Aqueous Extracts from Species of the Lamiaceae Family against *Herpes simplex* Virus Type 1 and Type 2 *in vitro*. *Planta Medica* 72: 1378–1382.

Norn S., Permin H., Kruse P.R. & Kruse E. 2009. [From willow bark to acetylsalicylic acid]. *Dansk medicinhistorisk arbog* 37: 79–98.

Plevka P., Perera R., Cardosa J., Kuhn R.J. & Rossmann M.G. 2012. Crystal structure of human enterovirus 71. *Science (New York, N.Y.)* 336: 1274.

Reshamwala D. 2017. Master's Thesis: Fungal endophytes in search for novel anti-virals against enteroviruses Dhanik Reshamwala.

Riss T.L., Moravec R.A., Niles A.L., Duellman S., Benink H.A., Worzella T.J. & Minor L. 2004. *Cell Viability Assays*. Eli Lilly & Company and the National Center for Advancing Translational Sciences.

Roivainen M., Alfthan G., Jousilahti P., Kimpimäki M., Hovi T. & Tuomilehto J. 1998. Enterovirus infections as a possible risk factor for myocardial infarction. *Circulation* 98: 2534–2537.

Roivainen M. & Klingel K. 2009. Role of enteroviruses in the pathogenesis of type 1 diabetes. *Diabetologia* 52: 995–996.

Schaar H.M. van der, Linden L. van der, Lanke K.H.W., Strating J.R.P.M., Pürstinger G., Vries E. de, Haan C.A.M. de, Neyts J. & Kuppeveld F.J.M. van. 2012. Coxsackievirus mutants that can bypass host factor PI4KIIIβ and the need for high levels of PI4P lipids for replication. *Cell Research* 22: 1576–1592.

Schmidtke M., Schnittler U., Jahn B., Dahse H.-M. & Stelzner A. 2001. A rapid assay for evaluation of antiviral activity against coxsackie virus B3, influenza virus A, and herpes simplex virus type 1. *Journal of Virological Methods* 95: 133–143.

Selim K. 2012. Biology of Endophytic Fungi. *Current Research in Environmental & Applied Mycology*.

Strobel G. & Daisy B. 2003. Bioprospecting for microbial endophytes and their natural products. *Microbiology and molecular biology reviews : MMBR* 67: 491–502.

Triantafilou K. & Triantafilou M. 2003. Lipid raft microdomains: Key sites for Coxsackievirus A9 infectious cycle. *Virology* 317: 128–135.

Virucides - an overview | ScienceDirect Topics.

Wani M.C., Taylor H.L., Wall M.E., Coggon P. & Mcphail A.T. 1971. Plant Antitumor Agents.VI.The Isolation and Structure of Taxol, a Novel Antileukemic and Antitumor Agent from Taxus brevifolia2. *Journal of the American Chemical Society*.

Williams C.H., Kajander T., Hyypia T., Jackson T., Sheppard D. & Stanway G. 2004. Integrin v 6 Is an RGD-Dependent Receptor for Coxsackievirus A9. *Journal of Virology*.

Willis K.J. (ed. . 2017. State of the World's Plants 2017. *Royal Botanic Gardens, Kew*: 44–46,

49, 52, 59.

Zhang G., Sun S., Zhu T., Lin Z., Gu J., Li D. & Gu Q. 2011. Antiviral isoindolone derivatives from an endophytic fungus Emericella sp. associated with Aegiceras corniculatum. *Phytochemistry* 72: 1436–1442.

Zhao X., Zhang G., Liu S., Chen X., Peng R., Dai L., Qu X., Li S., Song H., Gao Z., Yuan P., Liu Z., Li C., Shang Z., Li Y., Zhang M., Qi J., Wang H., Du N., Wu Y., Bi Y., Gao S., Shi Y., Yan J., Zhang Y., Xie Z., Wei W. & Gao G.F. 2019. Human Neonatal Fc Receptor Is the Cellular Uncoating Receptor for Enterovirus B. *Cell*: 1–13.