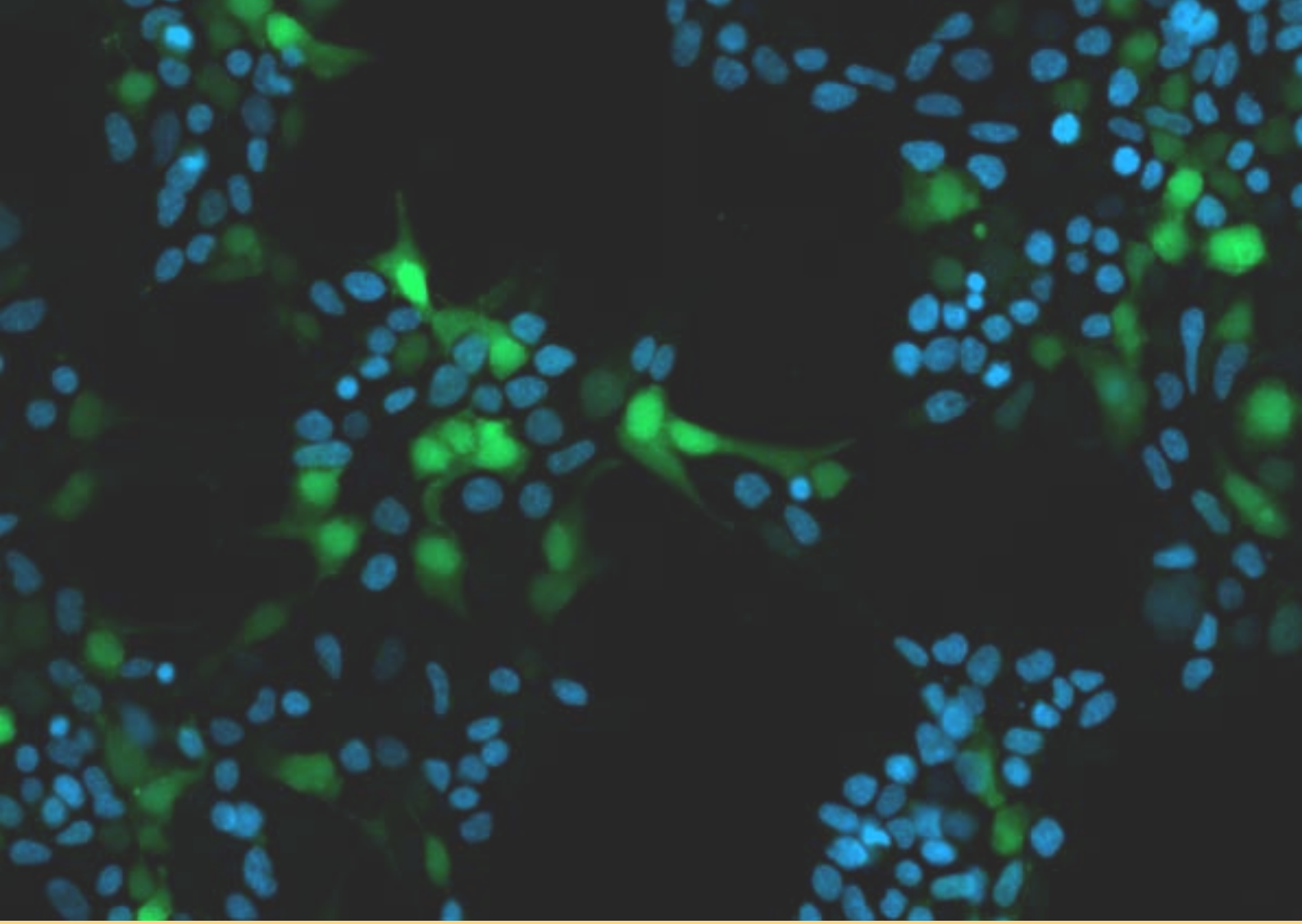

Nânci Lúcia dos Santos Ferreira

Optimization of a Viral Culture System to Evaluate Antiviral Activity

Tese de Mestrado em Análises Clínicas, orientada pela Professora Doutora Célia Nogueira e apresentado à Faculdade de Farmácia da Universidade de Coimbra

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GLOSSARY

3' UTR - 3'-noncoding regions

5' UTR - 5'-noncoding regions

AIDS - Acquired immunodeficiency syndrome

ATCC - American tissue culture centre (http://www.lgcstandards-atcc.org/)

BSA - Bovine serum albumin

Caco-2 - Human colorectal adenocarcinoma cell

cDNA - complementary Deoxyribonucleic Acid

CO - Cobrançosa olive extract

C_T - Cycle Threshold

CVA12 - Coxsackievirus A12

CVA6 - Coxsackievirus A6

DAPI - 4',6-diamidino-2-phenylindole

DMEM - Dulbecco's Modified Eagle's Medium

DMSO - Dimethyl sulphoxide

EDTA - Ethylenediamine Tetraacetic Acid

EGFP - Enhanced green fluorescent protein

Env - Envelope

FBS - Fetal bovine serum

FG - Female gametophyte

Gag - Group specific antigen

HEK-293T - Human embryonic kidney cell

HEPES - N-(2-Hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid

HFMD - Hand, foot and mouth disease

HIV - Human Immunodeficiency Virus

IRES - Internal ribosome entry site

IN - Integrase

ISO - International Organization for Standardization

LV - Lentivirus

MO - Maçanilha olive extract

OP - *Osmundea pinnatifida* algae

PBS - Phosphate buffered saline

PCR - Polymerase chain reaction

PDL - Poly-D-Lysine

Pol - Polymerase

PR - Protease

qPCR - quantitative PCR

RNA - Ribonucleic Acid

RT - Reverse transcriptase

T - Tetrasporophyte extract

Tm - Melting temperature

VPg - Virion protein genome linked

ABSTRACT

Optimization of a viral culture system to evaluate antiviral activity of natural compounds have great importance nowadays due to the lack of antiviral treatment for the majority viral infections.

This study involves the in vitro optimization of two models of viral infection, HEK-293T/Lentivirus model and Caco-2/Coxsackievirus A12 model; evaluation of antiviral potential at different treatment conditions and evaluation of extracts cytotoxicity on the studied cell lines, HEK-293T and Caco-2 cell line.

This models were applied on the screening of the antiviral potential of five natural extracts: Cobrançosa Olive (CO) and Maçanilha Olive (MO) extracts, Tetrasporophyte (T) and Female gametophyte (FG) of *Chondracanthus teedei var. lusitanicus* algae and Osmundea pinnatifida (OP) extract. Prior to antiviral assays it was evaluated the cytotoxicity of the listed extracts. Only OP extract reveal cytotoxicity on the cell lines studied.

On the HEK-293T/Lentivirus model, all extracts presented antiviral potential by protecting the cell (i) and virucidal effect (ii). The infection in this model was quantify by fluorescence microscopy.

On Caco-2/Coxsackievirus A12 model, all extracts reveal potential as virucidal agents (ii). Only CO and MO reveal potential to protect cells against infection (i) and only OP extract reveal antiviral effectiveness when added post-infection (iii). CVA12 infection was relatively quantify by RT real time PCR and normalized with 18S.

These results suggest that extracts CO, MO and OP have a inhibitory effect of LV infection at an early stage and can therefore being candidates for prophylactic intervention, as well as extracts CO and MO for Coxsackievirus A12 infection. OP extract it may be a candidate for therapeutic intervention on Coxsackievirus A12 infection because exhibits inhibitory effect in viral replication.

RESUMO

A otimização de um sistema de cultura viral para avaliar a atividade antiviral de compostos naturais tem grande importância hoje em dia devido à falta de tratamento para a maioria das infeções virais.

Este estudo envolve a otimização de dois modelos de infeção, modelo HEK-293T/Lentivirus e modelo Caco-2/Coxsackievirus A12; avaliação do potencial antivírico com diferentes tratamentos e avaliação da citotoxicidade dos extratos nas linhas celulares utilizadas nos modelos, linha celular HEK-293T e linha celular Caco-2.

Estes modelos de infeção foram utilizados no *screening* do potencial antiviral de cinco extratos naturais: extrato de azeitona Cobrançosa (CO) e azeitona Maçanilha (MO), extrato do tetrasporófito (T) e gametófito feminino (FG) da alga *Chondracanthus teedei var. lusitanicus* e extrato da alga *Osmundea pinnatifida* (OP). Antes da realização dos ensaios antivirais foi testada a citotoxicidade dos extratos. O extrato OP foi o único que se revelou tóxico nas linhas celulares estudadas.

No modelo HEK-293T/Lentivirus todos os extratos apresentaram potencial antiviral ao induzir um efeito protetor na célula (i) e um efeito virucida (ii). A infeção foi quantificada por microscopia de fluorescência.

No modelo Caco-2/Coxsackievirus A12 todos os extratos revelaram potencial como agentes virucidas (ii). Apenas os extratos CO e MO induziram nas células um efeito protetor contra a infeção (i) e apenas o extrato OP revelou efeito antiviral quando adicionado após a infeção (iii). Uma quantificação relativa da infeção por CVA12 foi por RT-PCR em tempo real e normalizada com o gene 18S.

Estes resultados sugerem que os extratos CO, MO e OP têm um efeito inibitório na fase inicial da infeção pelo LV sendo, por isso, candidatos a ser usados na profilaxia desta infeção, assim como os extratos CO e MO na infeção por Coxsackievirus A12. O extrato OP parece ser um bom candidato para a terapêutica da infeção pelo Coxsackievirus A12 porque demonstra atividade inibitória a nível da replicação viral

1. Introduction

Viruses are obligate intracellular parasites with metabolically inert nucleoprotein particles containing a genome of either RNA or DNA, with or without a lipid-containing envelope, and utilises the host cell machinery to propagate new viruses.

The ability to persist within the host, efficient replication, variety in mode of transmission allows viruses to adapt to all forms of life, resulting in widespread diseases in human, livestock and plants (Chattopadhyay *et al.*, 2009).

1.1. Enterovirus

Coxsackievirus A12 (CVA12) is a serotype of Coxsackievirus group A that was first isolated in Texas, 1948. Coxsackievirus received this name after being discover in Coxsackie village, Greene County, New York (Pallansch e Roos, 2001). Coxsackievirus belong to the family *Picornaviridae* and the genus *Enterovirus*.

The genus *Enterovirus* is characterized by twelve different species – *Enterovirus* A to J and *Rhinovirus* A to C, wherein Coxsackievirus A12 serotype belong to *Enterovirus* A species (ICTV, 2014). There are two groups of human Coxsackievirus: Coxsackievirus group A (included 23 serotypes) and Coxsackievirus group B (include 6 serotypes). They are distinguish based on their pathogenicity in suckling mice: Group A Coxsackievirus produces a single generalized myositis lesion with flaccid paralysis of skeletal muscle; Group B Coxsackievirus produces a generalized infection that affects several organs (brain, pancreas, heart and brown fat).

Enterovirus is a genus of small non-enveloped icosahedral viruses with a single-stranded RNA genome of positive polarity. The enterovirus genome (Figure 1) is unique because it is covalently linked at the 5' end to a protein called VPg (virion protein, genome linked) by an O4-(5'-uridylyl)-tyrosine linkage. 5'-noncoding regions (5' UTR) of picornaviruses are long and highly structured, and contains the internal ribosome entry site (IRES), an element that directs translation of the mRNA by internal ribosome binding. 3'-noncoding region (3' UTR) of picornavirus is short and is not required for infectivity. However, RNA lacking this sequence has low infectivity and viruses replicate poorly (Racaniello, 2001). The 5' UTR and 3' UTR of enterovirus influence tissue tropism, virulence and viral pathogenesis (Lin e Shih, 2014).

Viral RNA encodes a single large polyprotein that, after several self-processing events mediated by virus encoded proteases (2A and 3C), produces mature viral proteins (11 mature proteins and numerous partially processed products). Four of these proteins (VP1-VP4) encoded by P1 region of polyprotein constitute the virus capsid, and the others encoded by P2 and P3 region participate in viral replication (Lin *et al.*, 2009).

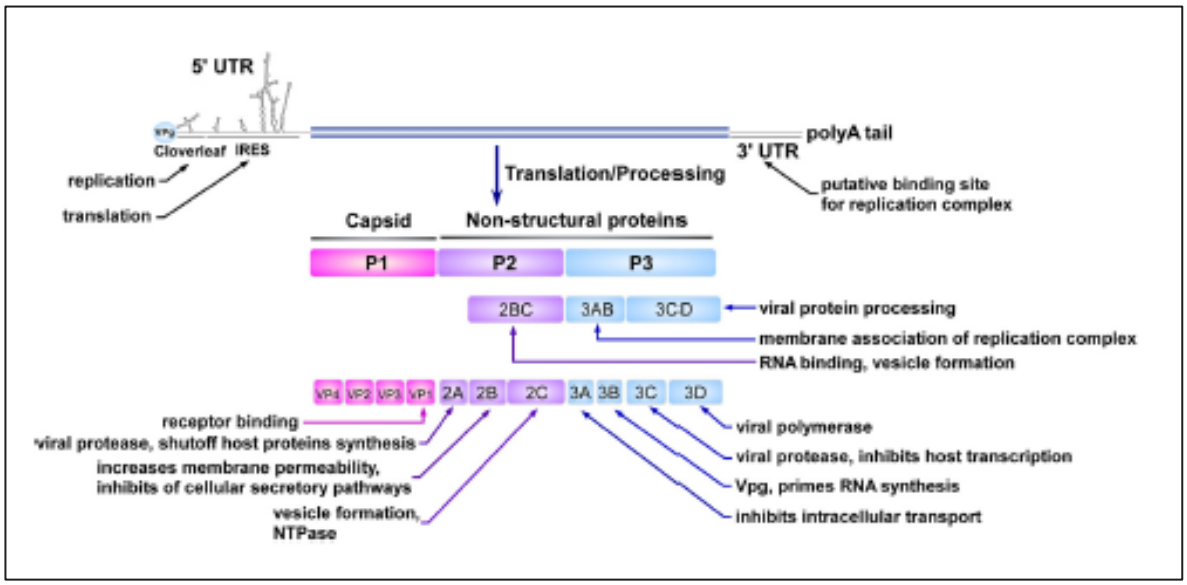

Figure 1 - Schematic of the enterovirus genome, the polyprotein products and their major function (Lin *et al.*, 2009).

Enteroviruses capsid proteins are encoded by the P1 region of the genome. Is constitute by 60 copies of VP1, VP2, VP3 and VP4. VP1, VP2 and VP3 reside on the outer surface of the virus and the shorter VP4 is located completely on the inner surface of the capsids (Figure 2).

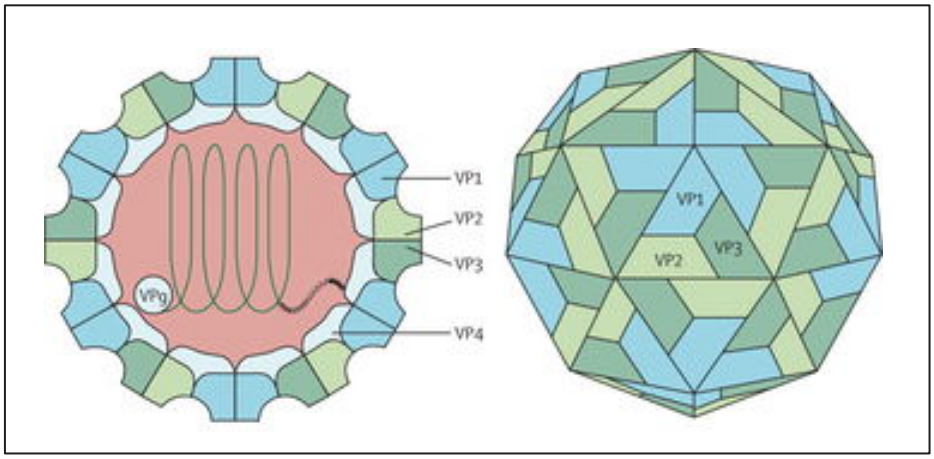

Figure 2 - Schematic of the enterovirus capsid, showing the packing arrangement of VP1, VP2 and VP3 (http://viralzone.expasy.org/all_by_species/97.html).

Replication cycle of enteroviruses occurs in the cytoplasm of the cell (Figure 3). After attachment to a cell receptor, the RNA genome is uncoated. Once the positive-stranded viral RNA enters the cytoplasm, it is translated to provide viral proteins for genome replication and production of new virus particles. Viral genome replication occurred through a virus–specific RNA-dependent RNA-polymerase ($3D^{pol}$), inside small membranous vesicles that are induced by virus proteins. A single replication cycle takes 5 to 10 hours to occur. New virus particles are released from the cell by lysis (Racaniello, 2001).

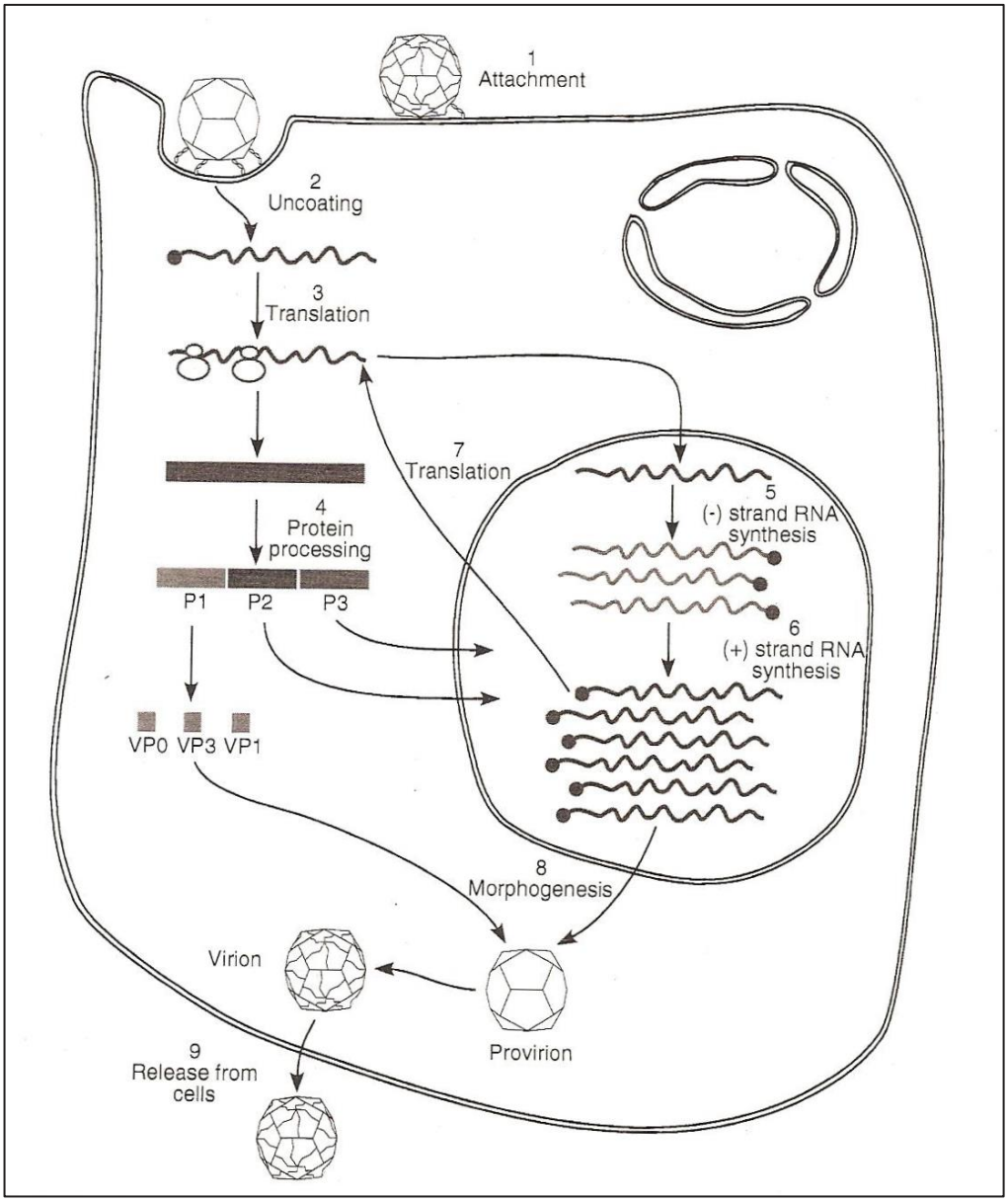

Figure 3 - Schematic representation of the picornavirus replication cycle (Racaniello, 2001).

These virus are spread by the faecal-oral route, person-to-person through respiratory secretions and by fomites. The primary site of infection is the respiratory tract

and small intestine. However, gastroenteritis is not a major feature of enterovirus infections. Infection proceeds to blood that lead to spreading of the virus to secondary target organs (Vuorinen *et al.*, 1999). Enterovirus is the most common cause of aseptic meningitis. Although the majority of enterovirus infections do not cause significant illness, infants and immunocompromised can develop serious disease (Pallansch e Roos, 2001). Coxsackievirus infections have a worldwide distribution and can cause several clinical syndromes: aseptic meningitis, paralysis, exanthema, Hand, foot and mouth disease (HFMD), herpangina, rashes, acute hemorrhagic conjunctivitis and undifferentiated febrile illness, among others (Figure 4).

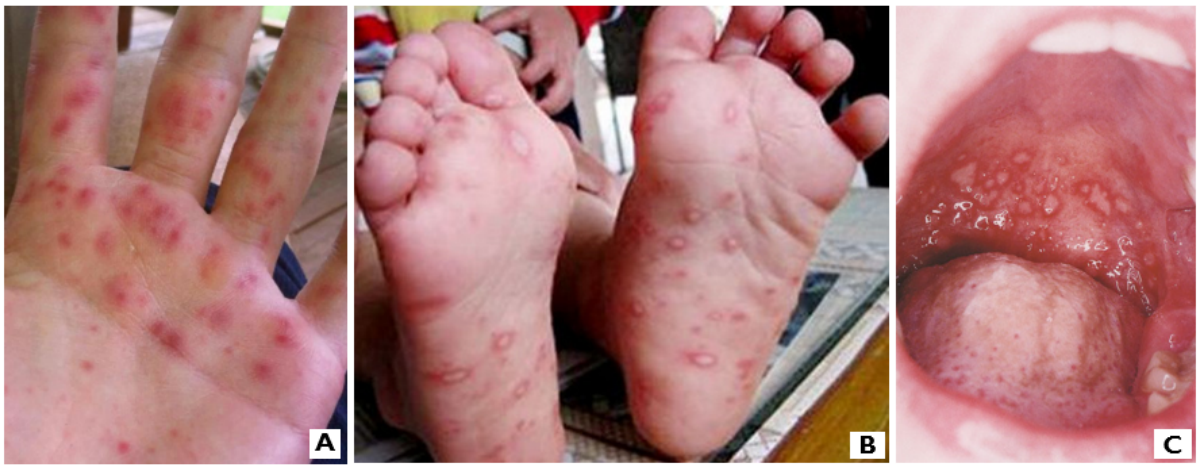

Figure 4 - Clinical manifestations of Coxsackievirus infection: Hand, foot and mouth disease (HFMD) (A and B) and Herpangina (C).

Since 1948 until 2011, CVA12 strains were only found in three countries: USA, Japan and China, indicating a limited geographical distribution of CVA12 infections. It is one of the virus responsible for the epidemics outbreaks of hand, foot and mouth disease in Southeast Asian countries. Even though is limited distribution in the world, CVA12 is consider an important emerging pathogen because of its potential to recombine with other human enterovirus (CVA16 and EV71) during a co-infection in HFMD outbreaks (Liu, X., Mao e Yu, 2014).

Treatment of enterovirus infections is only symptomatic. To date, no effective antiviral agent have been approved for treatment of diseases caused by viruses of *Picornaviridae* family (Song *et al.*, 2014; Wang *et al.*, 2012).

1.2. Lentivirus

Lentivirus is a genus of viruses of the *Retroviridae* family. This family comprises seven major genera: *Alpharetrovirus*, *Betaretrovirus*, *Deltaretrovirus*, *Gammaretrovirus*, *Epsilonretrovirus*, *Lentivirus* and *Spumavirus* (ICTV, 2014). The most important human lentivirus are the human immunodeficiency virus (HIV) 1 and 2, responsible for the acquired immunodeficiency syndrome (AIDS). These viruses infect and destroy certain cells of the immune system, especially T-helper cells, leading to high susceptibility to other infections.

HIV is an enveloped virus with elongated conical capsid and two copies of positive single-stranded RNA. Viral particle also includes virus replication enzymes (Figure 5). This virus is characterized by a long incubation period and, have de ability to infect non-dividing cells and integrate permanently viral DNA into cell genome (Freed e Martin, 2001).

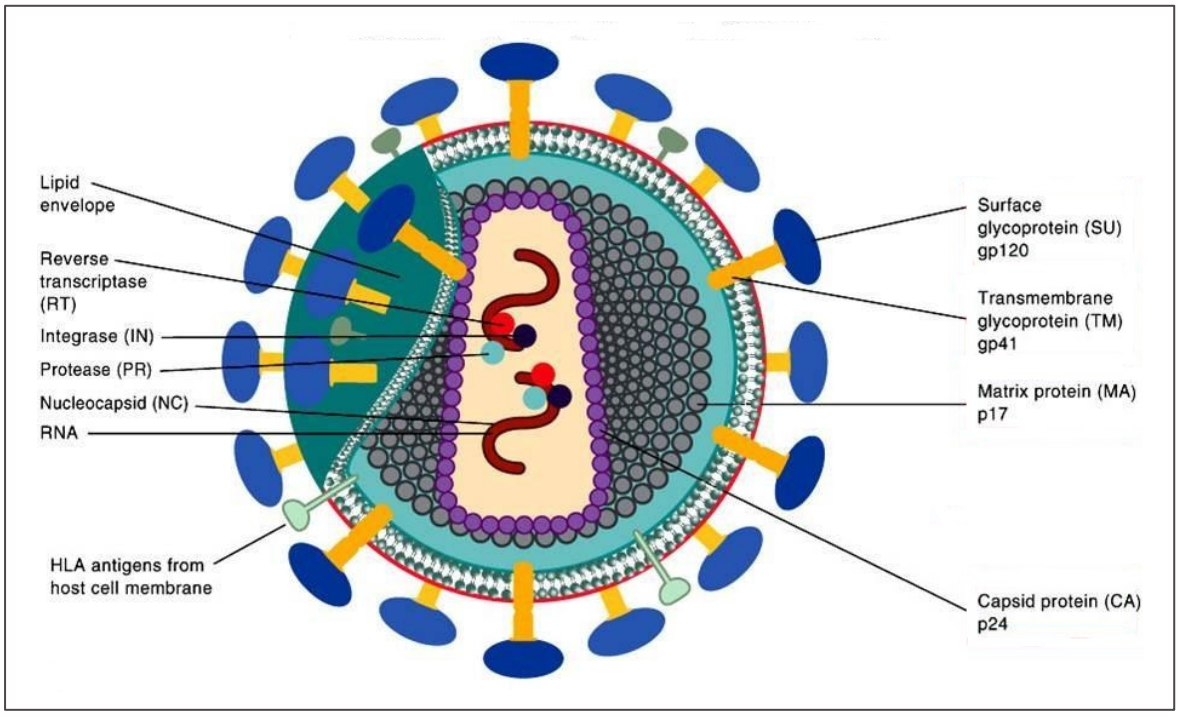

Figure 5 - Schematic representation of HIV-1 virion (https://www.studyblue.com/notes/note/n/mc2-02-hiv-and-aids-part-1/deck/13153178).

HIV genome (Figure 6) is flaked at both ends by a long terminal repeat promoter region (LTR) and encode three polyproteins (gag, pol and env), four accessory proteins (Vif, Vpr, Vpu and Nef) and two regulatory proteins (Rev, Tat). Accessory and regulatory proteins control replication functions, transcription, RNA processing, virion assembly and host gene expression.

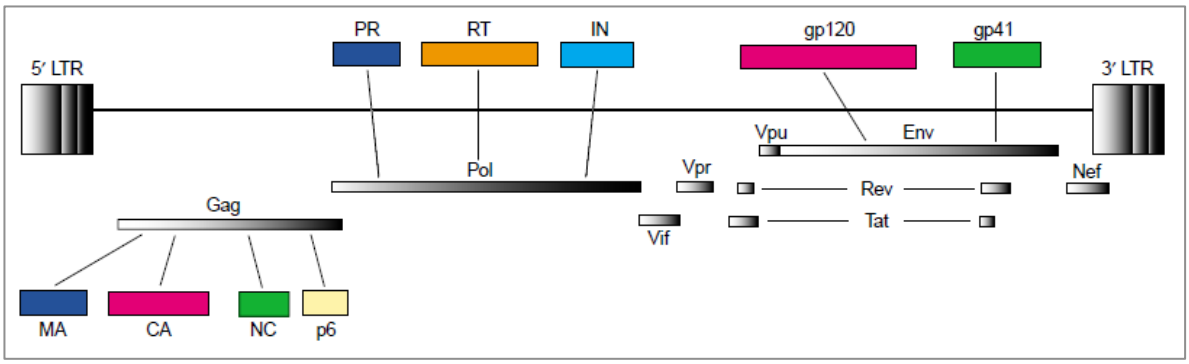

Figure 6 - Schematic diagram of HIV-1 genome (9.7 kb) (Freed, 2004).

Gag (group specifc antigen) gene encode a structural protein from capsid and nucleocapsid, Pol (polymerase) gene encode a protease (PR), a reverse transcriptase (RT) and an integrase (IN), and Env (envelope) gene encode proteins from the envelope (gp120 and gp40) (Freed, 2004).

The life cycle of HIV and replication of viral genome includes the formation of a DNA intermediate (Figure 7). Infection of host cell by HIV requires fusion of the viral membrane within the membrane of host cell. After binding to CD4 and a co-receptor (CCR5 or CXCR4), fusion with the cell membrane results in entry of viral core into the citoplasm. Uncoating and reverse transcription occur. Then the preintegration complex is imported into the nucleus and become integrated into a host chromosome. After this point, viral DNA is transcribed by cellular RNA polymerase into viral RNAs. New virus particles are assembled at the inner surface of cell membrane and immature particles are bud out of the membrane and released (Grandgenett *et al.*, 2015).

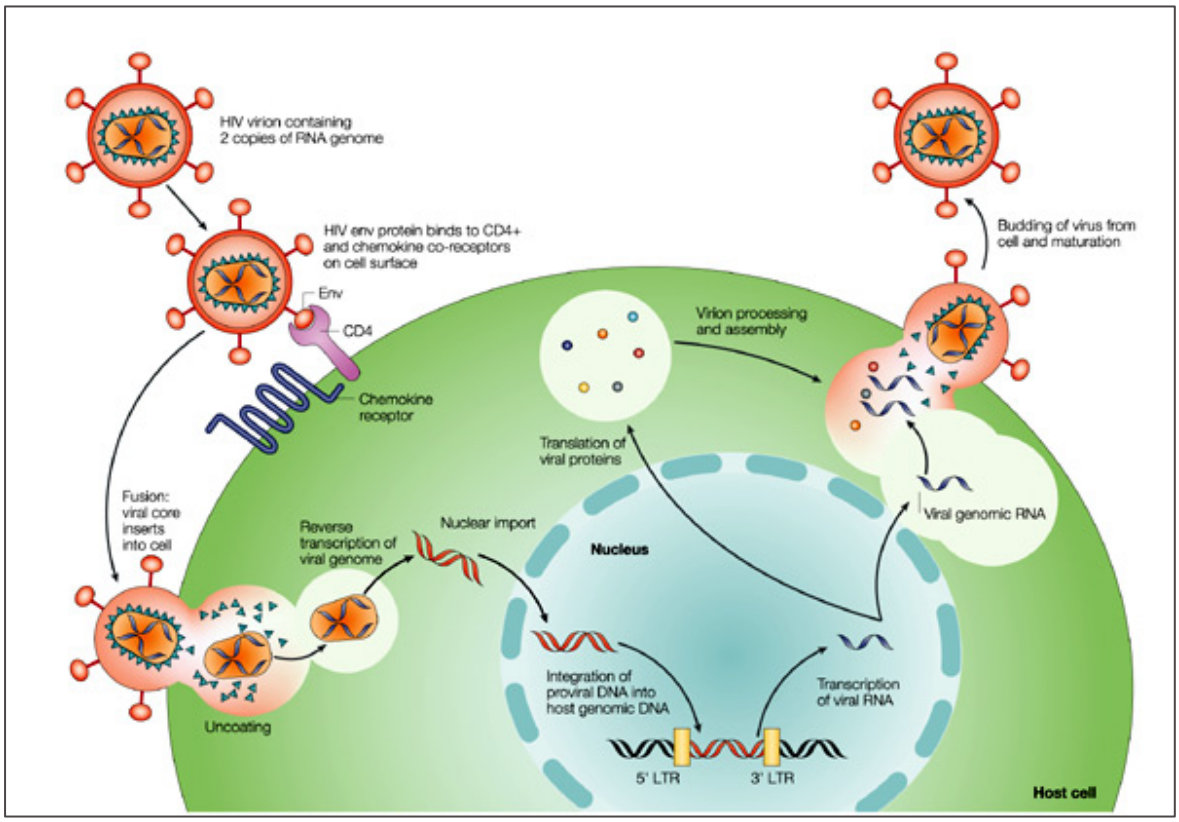

Figure 7 - Schematic representation of HIV replication cycle (http://www.nature.com/nrg/journal/v5/n1/fig_tab/nrg1246_F1.html).

The unique characteristics of lentivirus infection make them an important tool in biotechnology. The ability of integrate permanently viral DNA into chromosomal DNA, that is replicated during the cell cycle and subsequently passed to daughter cells, makes lentivirus extremely useful in gene therapy as lentiviral vectors (Buchschacher e Wong-staal, 2000). Lentivirus are also used to produce *in vivo* models of diseases, being widely used in neurodegenerative diseases, as Machado-Joseph disease (Nascimento-Ferreira *et al.*, 2011) and Huntington disease (Almeida, de *et al.*, 2002). Incorporation of fluorescent protein into HIV virions allows the identification of virions that have productively entered the target cell (Campbell *et al.*, 2007).

1.3. Natural compounds

The need of new efficient antiviral agents to combat viral infections is notorious. The current antiviral drugs in clinical use only target some viral infections: HIV, hepatitis B virus, herpes simplex virus, varicella-zoster virus, cytomegalovirus, influenza virus, respiratory syncytial virus and hepatitis C virus infections (Clercq, 2004). Several virus infections have never had treatment and the ones that have are starting to fail due to anti-viral resistance.

Natural products have been one of the most important sources of novel anti-viral agents. Many compounds derived from plants, specially higher plants, algae and liquens, have shown some antimicrobial properties (Abonyi *et al.*, 2009; Chattopadhyay *et al.*, 2009). An advantage of natural compounds is their presence in nature, their multiple targets, minor side effects, low potentials to cause resistance and low cost (Song *et al.*, 2014).

1.4. Aims

The discovery and development of new antiviral drugs are urgently needed, mainly for treatment of viral diseases responsible for high mortality and/or high incidence in immunocompromised patients.

The aim of this study was to optimize a viral culture system to evaluate the antiviral activity of natural compounds. The experiment was performed in a cell culture-based assay using two cell lines, HEK-293T and Caco-2 cell line, and two viruses, a modified Lentivirus and an Enterovirus (Coxsackievirus A12). Two models of viral infection were optimized: HEK-293T/Lentivirus model (293T/LV model) and Caco-2/Coxsackievirus A12 model (Caco/CVA12 model). The system was tested by evaluating the antiviral potential of five natural extracts from olive and algae at various stages of viral replication cycle.

2. Materials and methods

2.1. Preparation of extracts

Five extracts were tested to determine their potential as antivirals (Table 1). Two extracts correspond to the aqueous fraction of Portuguese olives: Cobrançosa Olive (CO) and Maçanilha Olive (MO). The aqueous extracts of olives were lyophilized and stored at -20°C.

The other tree extracts tested were from two algae: *Chondracanthus teedei* var. *lusitanicus* (Filo *Rhodophyta*, Order *Gigartinales*) and *Osmundea pinnatifida (Filo Rhodophyta, Order Ceramiales)* algae. The extracts of algae belong to master colleagues that are evaluating the antimicrobial activity of these algae.

Table 1- Natural extracts tested.

Extract	Fraction of extraction	Solvent
Cobrançosa Olive (CO)	Aqueous	Ultra-pure water
Maçanilha Olive (MO)	Aqueous	Ultra-pure water
Chondracanthus teedei var. lusitanicus – Tetrasporophyte **(T)**	Alkaline	Ultra-pure water
***Chondracanthus teedei var. lusitanicus* –** Female gametophyte **(FG)**	Alkaline	Ultra-pure water
***Osmundea pinnatifida* (OP)**	n-Hexane	DMSO

A stock solution of 10 mg/mL of each extract was prepared. The solvent of primary choice was dimethyl sulfoxide (DMSO) (*Sigma-Aldrich*) (Cos *et al.*, 2006). Extracts unable to dissolve in DMSO were first dissolved in ethanol and following in ultra-pure water. Due to extraction characteristics, only extract from *Osmundea pinnatifida* was dissolved in DMSO. The other extracts were dissolved in ultra-pure water. Dilutions of 10x (1000 μg/mL) and 100x (100 μg/mL) of extracts were made to use on the assays. The concentration of DMSO during the assays never exceeded 2%. All the solutions were stored at -20°C.

2.2. Cells and viruses

Two viral models were optimized to test the extracts: HEK-293T/Lentivirus model and Caco-2/Coxsackievirus A12 model. Two different cell lines were needed to cultivate viruses and perform the assays. All cells and viruses stocks were stored at -80°C.

2.2.1. HEK-293T and Caco-2 cells

HEK-293T [293T] (ATCC® CRL-3216™) is an adherent epithelial cell line from Human Embryonic Kidney cells derivative of the HEK293 cell line, that was generated by transforming normal human embryonic kidney cells with adenovirus 5 DNA. The HEK-293T cell line stably and constitutively expresses the SV40 large T antigen that facilitate optimal lentivirus production (PHE, 2013).

HEK-293T cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (*Sigma-Aldrich*) with 10% of heat inactivated Fetal Bovine Serum (FBS) (*Gibco*). Culture medium was supplemented with 1% of penicillin-streptomycin (*Gibco*) and sodium bicarbonate (*Sigma-Aldrich*) as pH buffer. HEK-293T are small round/oval cells with very fine short extensions that attach and grow in touch with each other (Figure 8). The population doubling time of this cell line is approximately 20h.

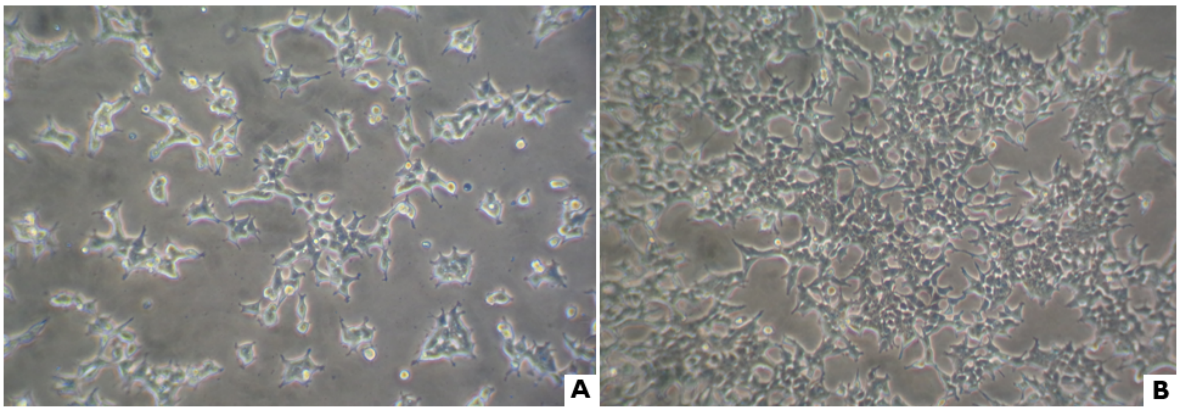

Figure 8 - HEK-293T cells morphology at different incubation times: (A) 24h and (B) 72h.

Caco-2 [Caco2] (ATCC® HTB-37™) are an adherent epithelial cell line from Colorectal Adenocarcinoma of 72 years Caucasian male. This cells have been widely used for screening of intestinal permeability of pharmaceutical drugs (Ungell, 2004).

Caco-2 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 20% of heat inactivated FBS. Culture medium was supplemented with 1% of penicillin-

streptomycin and HEPES (*Sigma-Aldrich*) and sodium bicarbonate as pH buffer. At first, it was used a culture medium with 10% of FBS but growth of Caco-2 cells is much improved using 20% of FBS.

Caco-2 cells have a very heterogeneous morphology. Most cells are cuboidal or epithelial-like with some apparent giant cells. When confluent, the cells appear to have a very definitive border (Figure 9). This cells attach to each other by tight junctions (Balimane e Chong, 2005). The population doubling time of this cell line is approximately 32h.

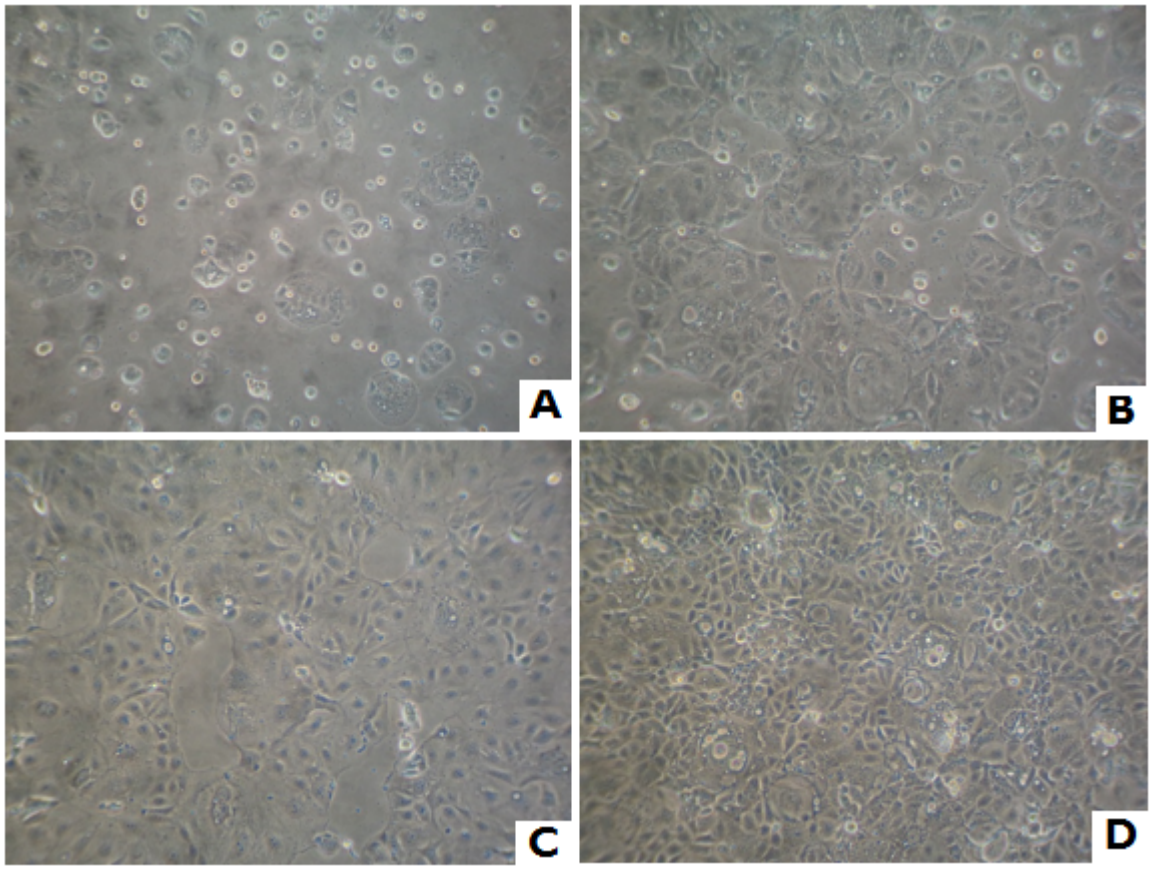

Figure 9 - Caco-2 cells morphology at different incubation times: (A) 24h, (B) 48, (C) 72h and (D) 96h.

2.2.2. Cell culture

Cell lines were manipulated in aseptic environment and with sterile material. All procedures were conducted in a class 2 safety cabinet and following the guidelines to a correct manipulation of cells.

2.2.2.1. Resuscitation of frozen cells

The vial with frozen cells was thaw by gentle agitation in a 37°C water bath. All of the operations from this point on were carried out under strict aseptic conditions. The content of the vial was transfer to a centrifuge tube containing 9.0 mL of culture medium and spin at approximately 9000 rpm for 5 minutes. The supernatant was discarded and the cell pellet was resuspend with 10 mL of the medium and dispense into a 25 cm^2 culture flask. The culture was incubated at 37°C in a suitable incubator with 5% CO_2 air atmosphere.

2.2.2.2. Subculture of adherent cells

Cells were subcultured when density reaches approximately 80% of confluence. Subculture procedure was based on the transference of cells from one culture vessel to another by dissociation of the monolayer of cells adhered to bottom flask. After every passage the number of cell generations increase one time.

HEK-293T cells

Old medium was removed and the cell layer was briefly rinsed with 5 mL of phosphate buffered saline (PBS). HEK-293T easily detach from the culture flask, therefore cells need to be handle gently during washing or replacing the culture medium. After remove all traces of serum, 2 mL of 0.05% Trypsin/0.025% EDTA (Ethylenediamine tetraacetic acid) solution (*Sigma-Aldrich*) was added to flask. Trypsin-EDTA solution was gently dispersed with a pipette against the wall of the flask to detach cells. Then, 4 mL of culture medium was added and the cell suspension was homogenized gently by pipetting. Appropriate aliquots of the cell suspension were transfer to new culture vessels and completed with culture medium to make 10 mL of final volume in 75 cm^2 flasks. The cultures were incubated at 37°C in a suitable incubator with 5% CO_2 air atmosphere. The subculture ration of HEK-293T cells are 1:3 to 1:8.

When a higher number of cells were needed, HEK-293T cells were subculture in 150 cm^2 flasks. On this vessels the volume of culture medium added was to complete a final volume of 20 mL.

Caco-2 cells

Subculture procedure on Caco-2 cells was similar to HEK-293T but Caco-2 cells have stronger ligations between cells and to the bottom of flask, so after addition of trypsin-

EDTA solution the flask was incubated at 37°C for 5 minutes to facilitate dispersal. The optimal activity temperature of trypsin is at 37°C. Caco-2 cells were subcultured in a ratio of 1:4 to 1:6.

Caco-2 cells attach to flasks in islands and cannot grow isolated. If a higher number of cells were required, Caco-2 cells were subculture in more than one vessel and never in 150 cm^2 flasks.

2.2.2.3. Cell maintenance

According to the growth rate of the cells, fresh culture medium was added to cells normally 1 to 2 times per week. An appropriate volume of pre-warmed culture medium was added to cells (10 mL to a 75 cm^2 flask or 20 mL to a 150 cm^2 flask). If after thawing a high degree of dead cells or debris in the culture was notice, cells were first rinse with PBS and then new culture medium was added. After changing the culture medium, cells return to the 37°C incubator with 5% CO_2.

2.2.2.4. Cell quantification

The procedure used to harvest cells from the vessel was the same described to subculture cells. After detaching cells with trypsin-EDTA solution, 7 mL of culture medium was added and the suspension of cells was transfer to a centrifuge tube and spin at approximately 1000 rpm for 5 minutes. The pellet of cells was resuspended in 4 mL of culture medium and cells were gently homogenized to separate the clumps of cells. A dilution of the suspension of cells was made and counted in a haemocytometer. The cell counting was performed in two chambers of the Neubauer counting chamber and cells were counted on the four big corner squares of each chamber. After determine the number of cells, a suspension with the required density of cells was made with fresh culture medium and distributed on the multiwell desired. The plate was placed at 37°C in an incubator with 5% CO_2 air atmosphere.

2.2.2.5. Cryopreservation of cell lines

The procedure used to harvest cells and centrifugation was the same described to cell quantification. After centrifugation, the supernatant was discard and the cell pellet was

resuspended in heat inactivated FBS with 10% DMSO. The suspension was distributed on cryogenic vials (one confluent 75 cm^2 flask gives 2 vials with 1 mL of cell suspension/vial and one confluent 150 cm^2 flask gives 4 vials with 1 mL of cell suspension/vial) and store at -80°C.

2.2.3. Lentivirus

Lentivirus used in 293T/LV model was gently provide by Doctor Luís Pereira de Almeida. It is a self-inactivating lentiviral transfer vector encoding for the enhanced green fluorescent protein (EGFP). This viral vector was produced in human embryonic kidney 293T cells and the concentration was determined by p24 antigen ELISA (Nascimento-Ferreira *et al.*, 2011).

2.2.4. Coxsackievirus A12

The enterovirus utilized on this experiment was Human Coxsackievirus A12 (CVA12), strain Texas 12, obtained from ATCC (Cat. #VR-1018). CVA12 was cultivated on Caco-2 cells. CVA12 was tried to replicate in HEK-293T cells but these cells weren't permissive to viral infection.

A stock of virus was produced by infecting $1.4x10^7$ Caco-2 cells with 2 mL of CVA12 from the original vial and incubating for 8 days. Virus was collected after 2 cycles of freeze/thaw and centrifugation. Supernatant was aliquot and storage at -80°C.

2.3. Cytotoxicity assay

The cytotoxicity of the extracts was evaluated on the HEK-293Tcell line and Caco-2 cell line. Cell viability was measured by the alamarBlue® assay following exposure to different concentrations of the extracts.

AlamarBlue® is a colorimetric/fluorescent assay, based on oxidation-reduction of resazurin sodium salt ($C_{12}H_6NNaO_4$) indicator. Resazurin (oxidized form) is blue and non-fluorescent dye that is reduced to resorufin (reduced form) pink/red and highly fluorescent (Figure 10) (O'Brien *et al.*, 2000). This occurs because the dye acts as an intermediate electron acceptor in the electron transport chain, there for reduction of the dye only happens in living and metabolic active cells. The quantity of reduced dye is proportional to the number of cells.

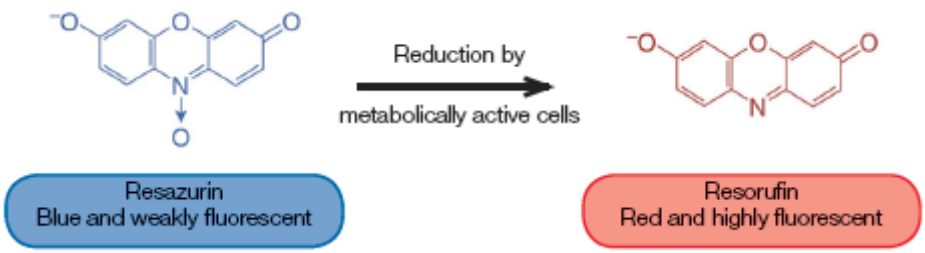

Figure 10 - alamarBlue® assay principle (alamarBlue® - Cell Proliferation and Viability Reagent, 2015).

Quantitative determination of cell viability can be performed by colorimetric or fluorometric methods. Colorimetric quantification is measure reading the absorbance at 570 and 600 nm (*alamarBlue® Technical Datasheet*, 2013). The viability can also be measure qualitatively: a visible change in colour indicates more or less viable cells.

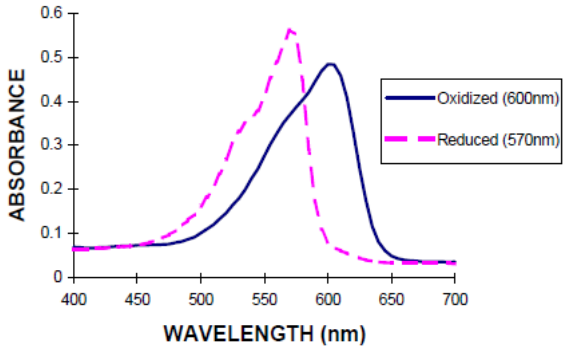

Figure 11 - alamarBlue® absorbance spectra (tools.thermofisher.comcontentsfsmanualsPI-DAL 1025-1100_TI%20alamarBlue%20Rev% 201.1.pdf).

Resazurin is water-soluble compound, stable in culture medium, non-toxic and permeable through cell membranes. Because of is non-toxicity cells can be reused for further investigations.

HEK-293T cells

To evaluate the toxicity of the extracts on HEK-293T cells an alamarBlue® assay was performed after 8h, 24h and 48h of incubation with extracts. Cells were seeded in 24-well plates at a density of 100.000 cells/well and incubated at 37°C/5% CO_2 for 24h to facilitate adherence and normal growth of HEK-293T. Different concentrations of the extracts (1, 10, 50, 100 and 200 ug/mL) were added to cells (Figure 6). A control, cells only, was included. After the incubation for 8h, 24h or 48h, medium was substituted for 500 µL of cultured medium with 10% of resazurin sodium salt (0,1 mg/mL) (Sigma-Aldrich). Plates were incubated for approximately 2h to permit the metabolization of the resazurin. Incubation was stopped when the control well (only cells) had a pink coloration.

The supernatant was read in duplicate in 96-well plates on a microplate reader spectrometer (SPECTRAmax PLUS 384, Molecular Devices) at the wavelength of 570 and 600 nm. If a high cytotoxicity was showed at 48h of exposure, the assay will be longstanding until 72h. After every alamarBlue® assay, cells were washed with PBS and added new medium with extract to continue the incubation.

Caco-2 cells

To evaluate the toxicity of the extracts on Caco-2 cells a similar procedure was applied but with adaptations to this cell line: after seeding cells were incubated at 37°C for 36h to facilitate adherence and normal growth of Caco-2 cells; the incubation with medium culture with 10% of resazurin occurred for approximately 1h30 to permit the metabolization of the resazurin.

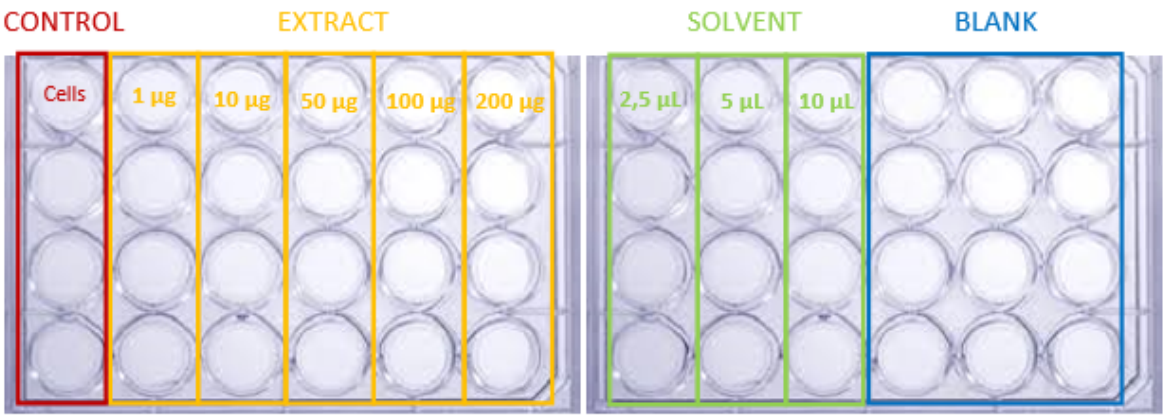

Figure 6 - Schematic representation of cytotoxicity assay on 24-well plates.

Each assay was performed in duplicate and with 4 replicates per condition. Cytotoxicity of solvent of extracts (ultra-pure water and DMSO) was also tested at the volumes they are added during the assays (2.5 µL, 5 µL and 10 µL). During the incubation with resazurin some wells without cells and with culture medium with 10% resazurin were include to perform the blank.

To perform alamarBlue® some conditions should be establish to obtain the most accurate cell viability measurement. The 96-well plates used on the assays were all the same type (flat-bottomed) and from the same manufacturer. The incubation and storage of resazurin was done in the dark because resazurin is photosensitive. Prolonged incubation times were avoided to prevent the formation of dihydroresofurin (colourless) that occurs by reduction of resorufin. All the assay was carried out under aseptic conditions to avoid contamination, which could increase the quantity of reduced resazurin.

2.4. Antiviral assays

The antiviral activity of the extracts was tested at the higher nontoxic concentration to the cells and under several conditions. Each treatment was performed in triplicate.

2.4.1. HEK-293T/Lentivirus model

The lentivirus used in this study was genetic modified to no replicate after enter the cell, so two conditions were tested in this model of infection: (i) Cell protection assay (pre-treatment) and (ii) Virucidal assay. The infection was evaluated by fluorescence microscopy.

2.4.1.1. Infection

HEK-293T/LV model was evaluated by infecting HEK-293T cells with LV. To quantify the infection by fluorescence microscopy cells were plated in a 12-well plate with 16 mm sterile cover-glass. As the cells weren't adhering to the cover-glass, cover-glass were coated with poly-D-lysine (PDL). PDL is a polymer of the amino acid D-lysine that facilitate the attachment of cells to solid surfaces.

Incubation of infected HEK-293T cells needs to be extended in order to cells divide, synthetize and express GFP. An equilibrium between number of cells, concentration of virus and incubation time needed to be found to obtain a monolayer of HEK-293T cells with a bright expression of GFP. If cells weren't in monolayer it was impossible to count them on a microscope (Figure 12).

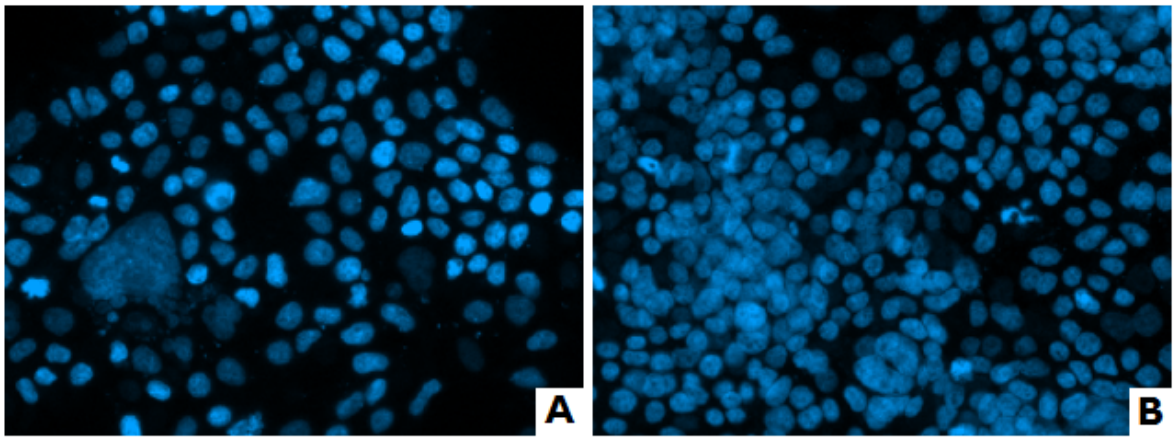

Figure 12 - HEK-293T monolayer (A) and HEK-293T over grown (B). Cells were stained with DAPI.

For optimization of viral infection different times of incubation and infection were tested in different densities of cells, namely - 50.000, 100.000 and 200.000 cells infected 24h and 48h after seeding, and incubated 72h and 96h post-infection. The best balance was achieved by infecting 100.000 HEK-293T 24h after seeding and incubate at 37°C with 5% CO_2 for 72h.

Several concentration of LV were tested. HEK-293T cells were infected with 20 ng, 40 ng 50 ng/µL of LV. These concentrations of virus weren't producing a good rate of infection. A concentration of 200 ng/µL was tested with and without polybrene (*Sigma-Aldrich*). Polybrene (hexadimethrine bromide) is a cationic polymer that neutralize the charge repulsion between virions and sialic acid on the cell surface. It is used to increase retrovirus infection efficiency (Davis, Morgan e Yarmush, 2002). 200 ng/µL of LV produced a much bigger rate of infection and with polybrene the infection was more efficient (when viewed under the microscope cells were brighter). Polybrene wasn't used on the assays to not introduce another external factor that could interfere or interact with extracts.

Optimized procedure

HEK-293T (100.000 cells/well 500 µL) were seeded into 12-well plates with cover-glass coated with Poly-L-Lysine (Figure 13), incubated at 37°C with 5% CO_2 for 24h and then the assays were performed.

(i) Cell protection assay: extracts were added at the maximum nontoxic concentration and incubated at 37°C with 5% CO_2 for 2 hours. Cells were washed twice with warm PBS, infected with 200 ng/µL of LV and incubated overnight.

(ii) Virucidal assay: LV was treated with the extracts in culture medium at 4°C for 2 hours and then added to cells and incubated overnight.

In both assays, after incubation with virus overnight, cells were washed twice with warm PBS to remove unabsorved virus and incubated at 37°C with 5% CO_2 for 72h. During the infection, the volume of the well was reduced to 400 µL to facilitate the entry of the virus. Cells with virus but without extracts were considered as a positive control. A control with only cells were included to normalize auto-fluorescence of HEK-293T. Cells were collected 72h post-infection and the rate of infection was quantified by fluorescence microscopy. Each condition was performed in triplicate.

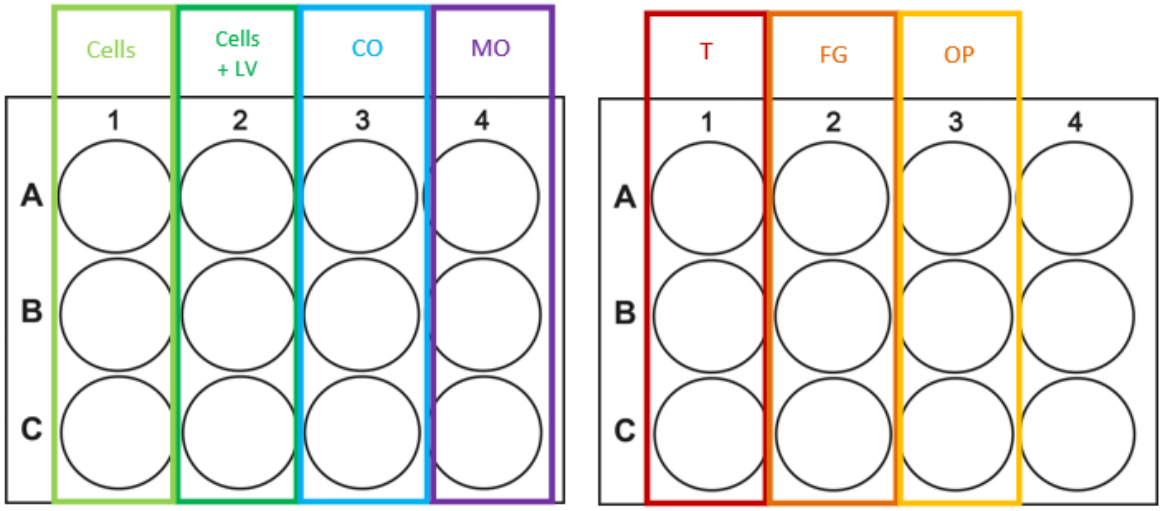

Figure 13 - Schematic representation of 293T/LV model of infection on 12-well plates with 16 mm cover-glass coated with PDL. LV – Lentivirus; CO – Cobrançosa olive extract; MO – Maçanilha olive extract; T – Tetrasporophyte extract of *Chondracanthus teedei* var. *lusitanicus*; FG – Female gametophyte extract of *Chondracanthus teedei* var. *lusitanicus*; OP – *Osmundea pinnatifida* extract.

2.4.1.2. Fluorescence microscopy

Infection of HEK-293T with LV was quantified by fluorescence microscopy. LV carries the gene of green fluorescent protein (GFP) so infected cells will express GFP. GFP is a fluorescent protein isolated from the jellyfish *Aequorea victoria*. This protein has a wide application in biotechnology because the fluorophore is part of the peptide chain and does not necessitate a cofactor to fluoresce (Pakhomov e Martynov, 2008). The excitation wavelength of EGFP is 488 nm and emission wavelength is 510 nm.

Cells were stain with DAPI (4',6-Diamidino-2-phenylindole). DAPI is a blue fluorescent nucleic acid stain that attaches to AT clusters in the DNA (Kapuscin'ski e Szer, 1979). The excitation wavelength of DAPI is 358 nm and emission wavelength is 463 nm.

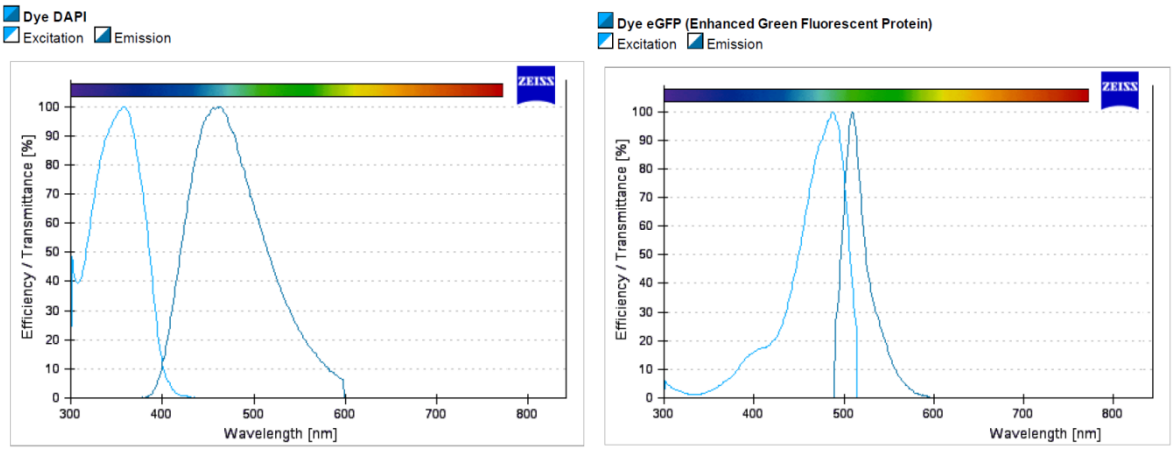

Figure 14 - Excitation and emission spectrum of DAPI and EGFP (https://www.micro-shop.zeiss.com/index.php?s=20727184472d856&l=en&p=de&f=f&a=d)

Optimized procedure

After 72h post-infection, HEK-293T were placed on ice and washed twice with warm PBS. Cells were incubated with formaldehyde 4% (Sigma-Aldrich®) at room temperature for 15 minutes, washed twice with cold PBS and then incubated 15 minutes at room temperature with DAPI (Sigma-Aldrich®).

Cells were washed again with PBS and the cover-glass was assembled on slide with DACO mounting medium (Palex Medical SA). After this, slides were sealed with polish and watch under a fluorescence microscope.

Axio Imager Z2 (Zeiss) was the microscope utilized to obtain the pictures to posterior counting. Ten pictures of each cover-glass were taken covering the cover-glass on cross with the objective Zeiss Plan-Apochromat 20x, in brightfield, DAPI and EGFP channel (Figure 16). Preparations were observed at a maximum of ten days after staining.

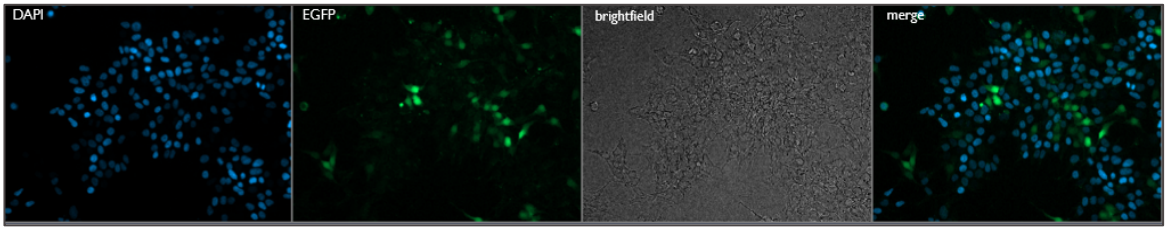

Figure 15 - Infection of HEK-293T with 200 ng/µL of LV.

2.4.2. Caco-2/Coxsackievirus A12 model

The infection of Caco-2 cells with CVA12 was evaluated on tree different conditions: (i) Cell protection assay (pre-treatment), (ii) Virucidal assay and (iii) Post-infection assay. The rate of infection was quantify by real time-PCR.

2.4.2.1. Infection

For optimization of viral infection we have tested: 2h and 5h for viral adsorption and 24h and 48h for viral replication. The best relation between time and rate of infection was obtained with 2h of adsorption and an incubation time of 48 hours.

Some enterovirus produces cytopathic effect. In order to evaluate the ability of CVA12 produce cytopathic effect on Caco-2, cells were infected with 30 µL of virus and observed under an inverted microscope every day during 120h of incubation at 37°C/5% CO_2. Due to the variability of Caco-2 appearance no specifically change was observed in cell morphology.

Optimized procedure

Caco-2 (200.000cells/well 400 µL) were seeded into 24-well plates (Figure 16), incubated at 37°C/5% CO_2 for 24h and then the assays were performed.

(i) Cell protection assay: extracts were added at the maximum nontoxic concentration and incubated at 37°C/5% CO_2 for 2 hours. Cells were washed twice with warm PBS and infected with 30 µL of CVA12. After 2h of incubation with virus, cells were again washed with warm PBS and incubated at 37°C/5% CO_2 for 46h.

(ii) Virucidal assay: CVA12 was treated with the extracts in culture medium at 4°C for 2 hours. The mixture was added to cells and after 2h of incubation at 37°C/5% CO_2, cells were washed twice with warm PBS and incubated at 37°C/5% CO_2 for 46h.

(iii) Post-infection assay: cells were firstly infected with 30 µL of CVA12 and incubated at 37°C/5% CO_2 for 2h. Then cells were washed twice with warm PBS, extracts were added and cells were incubated at 37°C/5% CO_2 for 46h.

Cells with virus but without extracts were considered as a positive control. A control with only cells were included to behold the normal grow of cells. Supernatant and cells were collected 48h post-infection after 2 cycles of freeze/thaw and storage at -80°C until viral quantification. Each condition were tested in triplicate.

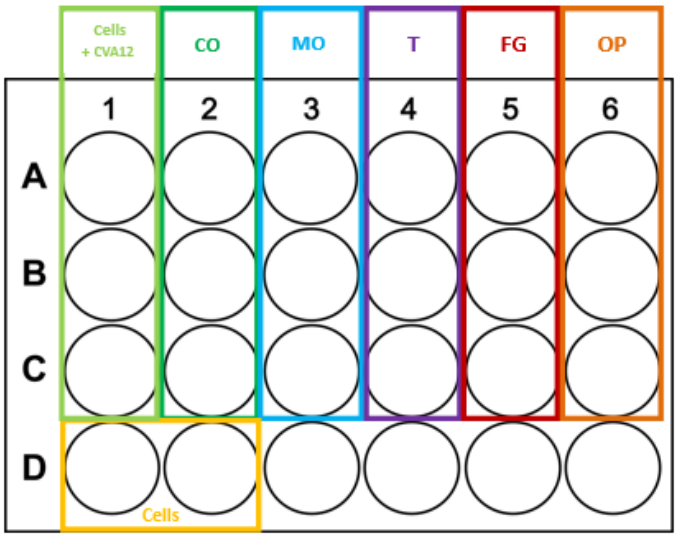

Figure 16 - Schematic representation of Caco-2/CVA12 model of infection on 24-well plates. CVA12 – Coxsackievirus A12; CO – Cobrançosa olive extract; MO – Maçanilha olive extract; T – Tetrasporophyte extract of *Chondracanthus teedei* var. *lusitanicus*; FG – Female gametophyte extract of *Chondracanthus teedei* var. *lusitanicus*; OP – *Osmundea pinnatifida* extract.

2.4.2.2. Real time RT-PCR

A Reverse Transcriptase (RT) reaction followed by real time Polymerase Chain Reaction (PCR) was used to quantify the viral charge of Coxsackie virus A12 on Caco-2 cells. The primary aim was to do an absolute quantification. A RNA control of CVA6 (AmpliRun®Coxsackievirus A6 RNA control, *Vircell*) was purchased but the concentration weren't high enough to do a standard curve. So a relative quantification was performed using the rRNA 18S gene as housekeeping gene.

2.4.2.2.1. Extraction of RNA

Isolation of total RNA was performed using MagNA Pure Compact Nucleic Acid Isolation Kit (*Roche*) on the automatic extractor MagNA Pure Compact System (*Roche*), using the "Total_NA_Plasma_100_400_v3_2" protocol and following the manufacturer's instructions. Total RNA was extracted from 300 µL of sample and eluted in 50 µL.

After extraction, RNA was quantify on NanoDrop 2000 Spectrophotometer (*ThermoFisher Scientific*). The yield and purity of RNA was determined by optical density measurements at 260 and 280 nm.

2.4.2.2.2. Reverse transcriptase reaction

The reverse transcription (RT) reaction, that converts RNA into cDNA, was performed immediately after extraction to avoid degradation of the RNA, using the Transcriptor First Strand cDNA Synthesis Kit (*Roche*) and following the manufacturer instructions.

The reaction was prepared to a final volume of 20 µL, containing the following components: 4 µL of Transcriptor RT Reaction Buffer, 1mM of each Deoxynucleotide, 20 U of Protector RNase Inhibitor, 10 U of Transcriptor Reverse Transcriptase, 60 µM of Random Hexamer Primer and 10 µg/mL of RNA. The volume was made up to 20 µL with ultrapure water.

The reactions occurred on Perkin Elmer-Gene Amp PCR System 2400 Termocycler at 25°C for 10 min followed by incubation at 50°C for 1h and then inactivation at 85°C for 5 min. After RT, cDNA was stored at -20°C until further analysis.

2.4.2.2.3. Real time PCR of Enterovirus and 18S

Real time PCR (qPCR) of Enterovirus cDNA was performed on the LightCycler® 2.0 (*Roche*) using the LC FastStart DNA Master Plus SYBR Green I Kit (*Roche*). This kit utilizes SYBR Green I dye to measure the fluorescence signal of PCR products. SYBR Green I intercalates into the DNA double helix and emits fluorescence at 530 nm. During PCR, increase in SYBR Green I fluorescence is proportional to the amount of double-stranded DNA generated.

The specificity of reaction is given by using primers of the 5'-non coding region (NCR) of enterovirus (Kares *et al.*, 2004). The nucleotide sequence of primers utilized to perform enterovirus qPCR was the one listed on table 2.

Table 2 - Sequence of primers used in real time PCR of Enterovirus and 18S.

Primer	Nucleotide sequence	Fragment size (bp)	References
Ent S (forward)	5'-CGG CCC CTG AAT GCG GCT AA-3'	120	(Kares *et al.*, 2004)
Ent R RT (reverse)	5'-GAA ACA CGG ACA CCC AAA GTA-3'		
18 S - F (forward)	5'-CGG CTA CCA CAT CCA AGG AA-3'	187	(Marques *et al.*, 2006)
18 S - R (reverse)	5'-GCT GGA ATT ACC GCG GCT-3'		

A reaction mixture was prepared to a final volume of 20 µL, containing the following components: 4 µL of LC FastStart DNA Master SYBRGreen kit, 0.5 µM of Ent S primer, 0.5 µM of Ent R primer, 9.8 µL of ultrapure water and 5 µL of cDNA. Real time PCR occurred following the conditions described on table 3.

Table 3 - Number of cycles and temperature settings for PCR reaction.

PCR	Stage no.	No. of cycles	Temperature (°C)	Time
ENT	1	1	95	10 min
	2	45	95	10 s
	3		55	15 s
	4		72	5 s
18S	1	1	95	10 min
	2	45	95	10 s
	3		55	5 s
	4		72	10 s

1 – Pre-incubation; 2 – Denaturation; 3 – Annealing; 4 – Extension.

To normalized number of cells, a real time PCR of an reference gene to human cells, 18S, was performed (Liu, L. *et al.*, 2015). 18S PCR was performed using the same instrument and SYBR Green kit as enterovirus PCR. Nucleotide sequence of primers are listed on table x. A reaction mixture was prepared to a final volume of 20 µL, containing following components: 4 µL of LC FastStart DNA Master SYBRGreen kit, 0.4 µM of primers, 13.2 µL of ultrapure water and 2 µL of cDNA. Real time PCR occurred following the conditions described on table 4.

Fluorescence was measured in the end of each extension step in the 530 nm channel. Melting analysis was achieved with continuous monitoring of fluorescence from 65°C to 95°C at a temperature transition rate of 0.1°C/sec. A specimen was considered positive for enterovirus if a single melting peak was measured between 86°C and 87°C, and for 18S if a single melting peak was measured between 85°C and 86°C. To validate the amplification process and exclude carryover contamination, positive and negative controls were included in each PCR run. PCR reactions were performed in duplicate for each sample.

2.4.2.2.5. Analysis of real time PCR results

After PCR reaction, a qualitative analysis and melting temperature (Tm) were executed. Specificity of the amplified PCR product was assessed by performing a Melting Curve analysis (Figure 17). Melting curves enable discrimination between primer-dimers and specific PCR product because Tm is a function of product length and base composition.

Qualitative analysis includes the analysis of Ct (cycle threshold) (Figure 18). Ct is a relative measure of the concentration of target in PCR reaction. Is define as the number of cycles required for the fluorescent signal cross the threshold. Ct levels are inversely proportional to the amount of target nucleic acid in the sample.

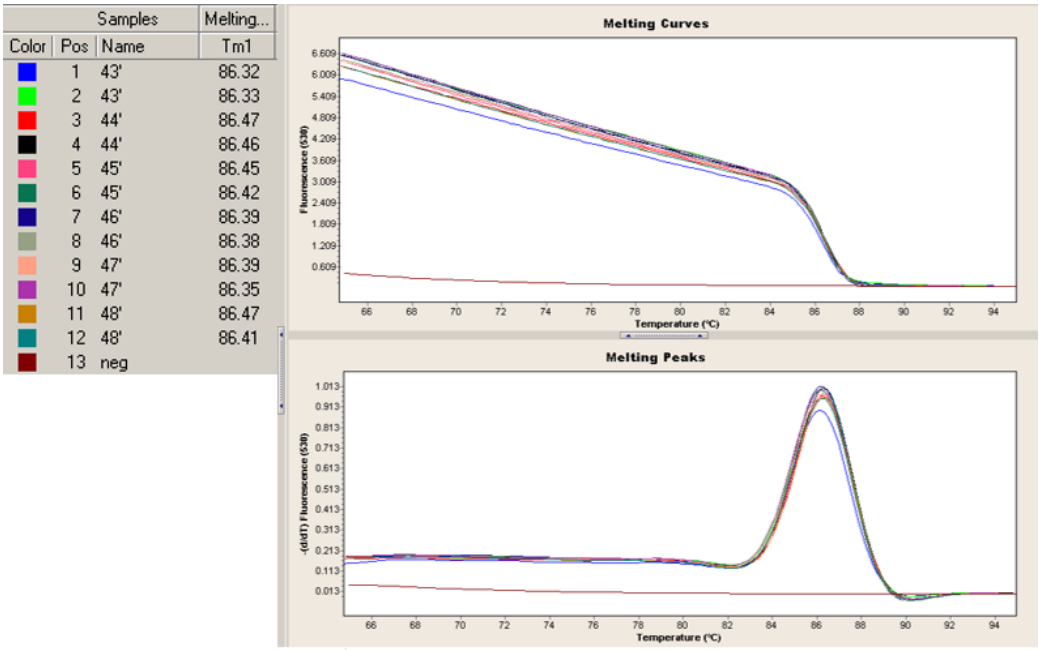

Figure 17 - Melting curve analysis on LightCycler 2.0 instrument (*Roche*).

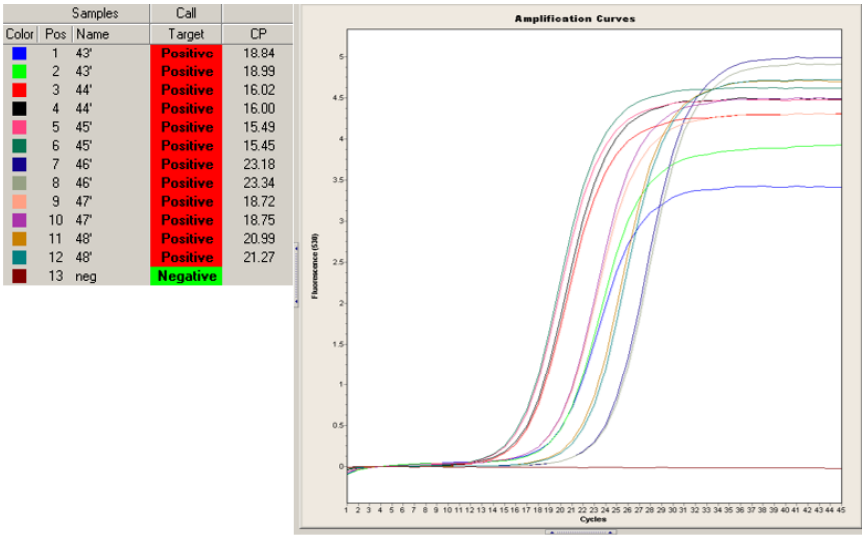

Figure 18 - Qualitative detection on LightCycler 2.0 instrument (*Roche*).

Efficiency of amplification for each gene was already been assessed and was placed between 1.8 and 2.2.

The relative quantification of Coxsackievirus A12 was obtained and analysed using the comparative Ct method. In relative quantification, the real time PCR data is presented relative to a reference gene (housekeeping gene). Values of relative expression from the gene of interest for all samples and respective reference gene was calculated applying the $2^{-\Delta\Delta Ct}$ formula (Schmittgen e Livak, 2008). The result is presented as fold change.

$$\text{Fold change} = 2^{-\Delta\Delta Ct} = 2^{-}\,[(Ct\ ENT\ treated - Ct\ 18S\ treated) - (Ct\ ENT\ untreated - Ct\ 18S\ untreated)]$$

If the value of $2^{-\Delta\Delta Ct}$ is inferior to one (< 1), a reduction in the expression due to treatment occur. Making the negative inverse of $2^{-\Delta\Delta Ct}$ provide the fold change reduction in expression.

3. Results

After the optimization of the two models – HEK-293T/Lentivirus model (293T/LV model) and Caco-2/Coxsackievirus model (Caco/CVA12 model) – it was tested the antiviral potential of five natural compounds. However, the presented results, discussed in the next chapters, are just about the Olive extracts (CO and MO) and *Osmundea pinnatifida* extract (OP). Results of Tetrasporophyte (T) and Female gametophyte (FG) of *Chondracanthus teedei var. lusitanicus* algae were presented in the master thesis of the person responsible for the extracts.

Results are presented as the mean value of two independent experiments (n=2). The standard deviation of the mean was used to evaluate the variability of data.

3.1. Optimization of the experiment

3.1.1. Preparations of the extracts

Cobrançosa olive (CO) and Maçanilha olive (MO) extracts correspond to the aqueous fraction of the olives and were dissolved in sterile ultrapure water for use in assays. The extract of *Osmundea pinnatifida* (OP) correspond to the n-Hexane fraction and was dissolved in DMSO. The concentrations of extract studied was 1, 10, 50, 100 and 200 ug/mL.

3.1.2. Cytotoxicity assay

The cytotoxicity of extracts was evaluated by the alamarBlue® assay. This method consist in add a solution of medium culture containing 10% of resazurin. The assay was interrupted for quantification after 2h of incubation for HEK-293T cells and 1h30 of incubation for Caco-2 cells.

3.1.3. Antiviral assays

3.1.3.1. HEK-293T/Lentivirus model

In the HEK/LV model the density of cells used for infection was 100.000 cells per well of 500 µL in a 12-well plate (Figure 19). During infection volume of the well is reduce to 400 µL of culture medium.

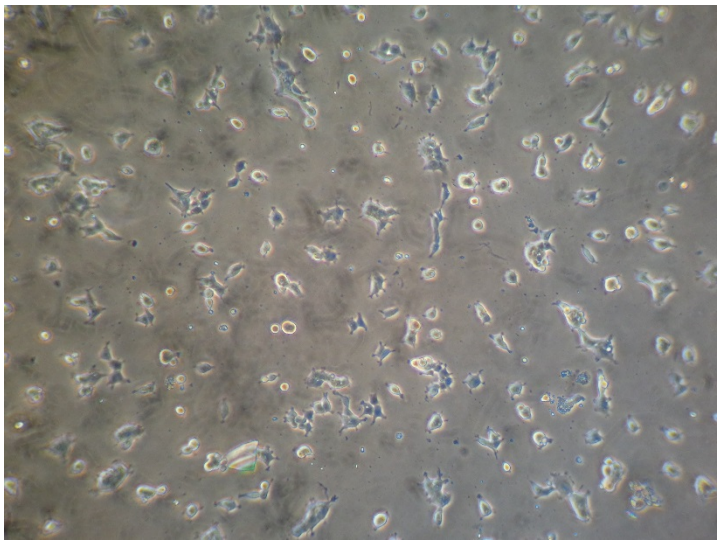

Figure 19 - HEK-293T prior to infection, 24h after seeding (density of 100.000 cells).

Only two conditions were tested with this model: (i) Cell protection assay and (ii) Virucidal assay. It would be ineffective test the post-treatment because the modified lentivirus are unable to replicate so extracts would have no effect. The infection was evaluated by fluorescence microscopy.

Quantification of LV infection in cells was performed by fluorescence microscopy determining the ratio of infected cells/total cells. Total cell number was achieve by nuclear staining with DAPI (Nascimento-Ferreira *et al.*, 2011). However, a phenomenon of DAPI quenching was found between cells that express GFP (Figure 20). Assessment of total cell number was made by evaluating brightfield, DAPI and EGFP channels.

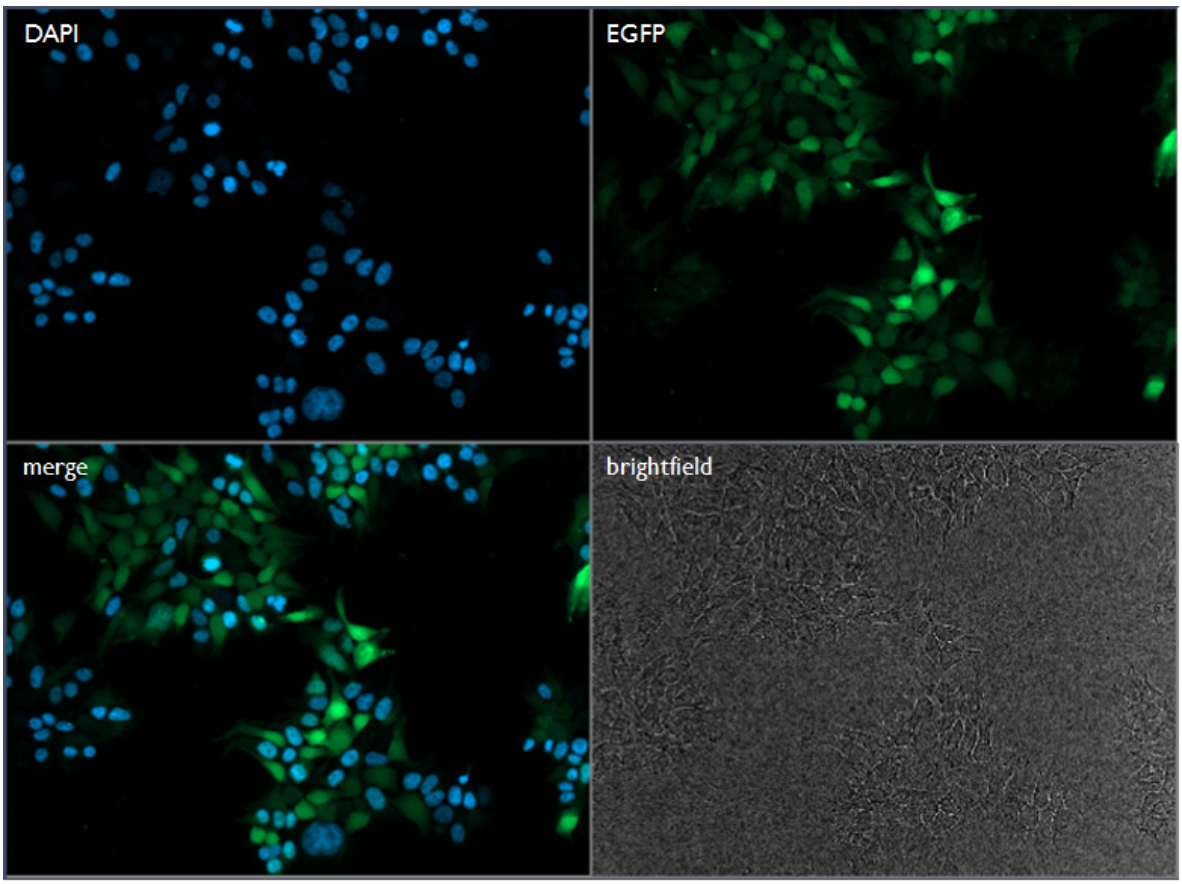

Figure 20 - Fluorescence microscopy of HEK-293T cells infected with lentivirus expressing EGFP. The analysis was performed at 72h post-infection by nuclear staining (DAPI, blue) and infected cells (EGFP, green). Pictures were taken on Axio Imager Z2 (Zeiss) with Zeiss Plan-Apochromat 20x objective. DAPI = 4',6-diamidino-2-phenylindole; EGFP = Enhanced green fluorescent protein.

3.1.3.2. Caco-2/Coxsackievirus A12 model

In the Caco/CVA12 model the density of cells used for infection was 200.000 cells per well of 400 µL in a 24-well plate.

Quantification of CVA12 infection was performed by real time PCR. The absolute quantification of viral concentration includes a calibration curve made from a standard with known concentration. Quantification of a CVA6 RNA control and stock of CVA12 was performed (Table 4). The concentration of CVA6 RNA was very low comparing to virus stock, so utilize this standard it would be inappropriate.

Quantification of CVA12 infection was performed by a relative quantification using the comparative Ct method (Schmittgen e Livak, 2008). The PCR results obtained were analysed with and without normalization of the housekeeping gene 18S. The importance of normalized the number of cells is exemplified on table 5. Without correction of the cell number, MO extract induces an 11.6-fold increase in viral infection. In contrast, if the cell

number was normalized, MO extract induces a 4.8-fold reduction in viral concentration, resulting in a contrary action of MO extract in viral infection. This proves the effect of the cell number in viral concentration, being fundamental eliminate the interference of cell number to quantify only the action of extracts in viral infection. It was chosen a relative quantification with 18S normalization to determine the viral charge.

Table 4 - Relative quantification of CVA6 RNA standard and CVA12 stock.

Sample	Concentration (copies/µL)	RNA concentration (ng/µL)	Ct
CVA6	19.9×10^3	2,9	29.96
CVA12 stock	unknown	34,0	11.63

Table 5 - Fold change in viral charge with and without 18S normalization.

(ii)	PCR ENT (Ct)		PCR 18S (Ct)		$2^{-\Delta\Delta Ct}$	$2^{-\Delta Ct}$
Control	20.31	20.51	13.07	13.02		
	20.7		12.96			
MO	16.95	16.98	9.81	9.79	- 4.8	11.6
	17		9.76			

(ii) - Virucidal assay; MO - Maçanilha olive extract; ENT - Enterovirus.

3.2. Antiviral activity of natural extracts

3.2.1. Cytotoxicity assay

Cytotoxicity of Cobrançosa olive (CO), Maçanilha olive (MO) and *Osmundea pinnatifida* (OP) extracts was evaluated at different concentrations (1, 10, 50, 100 and 200 ug/mL) in HEK-293T and Caco-2 cell lines after incubation for 8h, 24h and 48h using alamarBlue® assay. Results are presented as a percentage of cell viability relative to the negative control (100% viability).

According to ISO 10993-5:2009(E) (ISO, 2009), a compound is cytotoxic when reduces cell viability by more than 30%. For this reason, cell viabilities less than 70% were used as cut-off values.

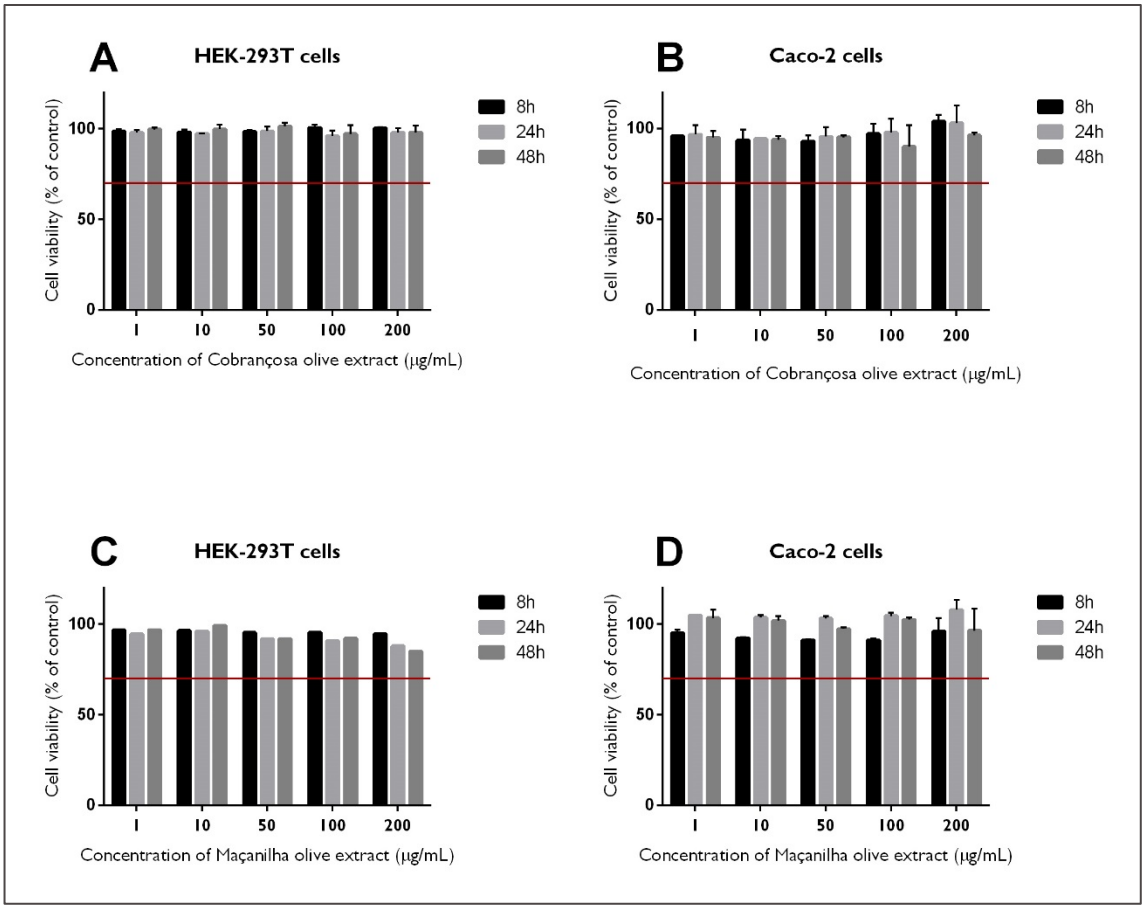

Figure 21 - Effects of Cobrançosa olive and Maçanilha olive extract on HEK-293T and Caco-2 cell viability until 48h of incubation. The red line separates the cell viability values above and below 70%. Data were obtained by alamarBlue® assay and are presented as mean ± SD from two independent experiments (n=2) each carried out in quadruplicate.

The results of the present study indicate that there was no significant cytotoxicity associated with Cobrançosa olive (CO) extract and Maçanilha olive (MO) extract at the tested concentrations and incubation times for HEK-293T and Caco-2 cells because in all tested concentrations there was no decrease in cell viability for more than 30% (Figure 21). CO and MO extracts were further evaluated for antiviral activity at the maximum concentration tested, 200 μg/mL.

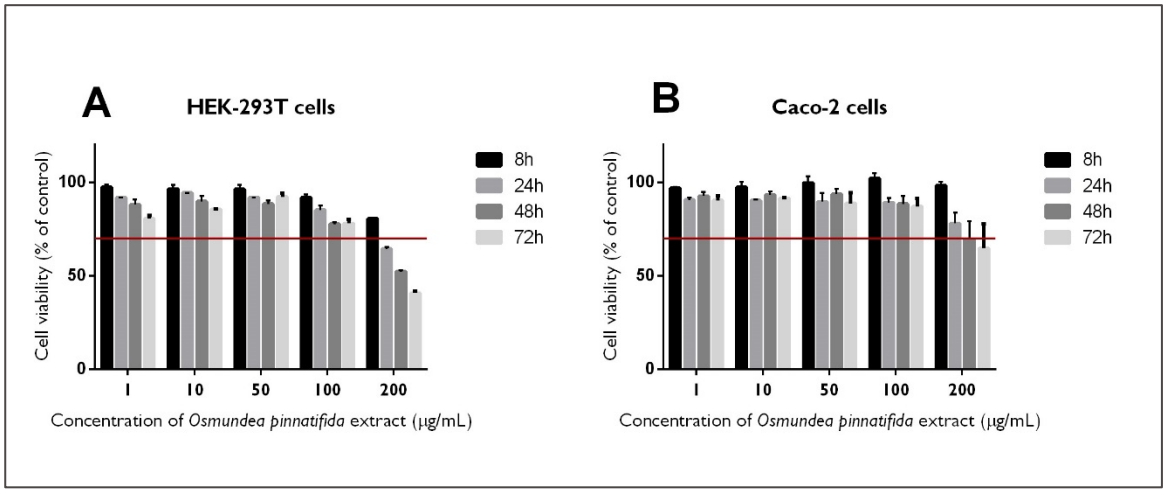

Figure 22 - Effects of Osmundea pinnatifida (OP) extract on HEK-293T and Caco-2 cell viability until 72h of incubation. The red line separates the cell viability values above and below 70%. Data were obtained by alamarBlue® assay and are presented as mean ± SD from two independent experiments (n=2) each carried out in quadruplicate.

Osmundea pinnatifida (OP) extract reveal cytotoxicity in both cell lines when used at the higher concentration tested, 200 μg/mL (Figure 22). alamarBlue® assay for this extracts was longstanding until 72h because at 48h a qualitative alteration on cell viability was observed (Figure 23).

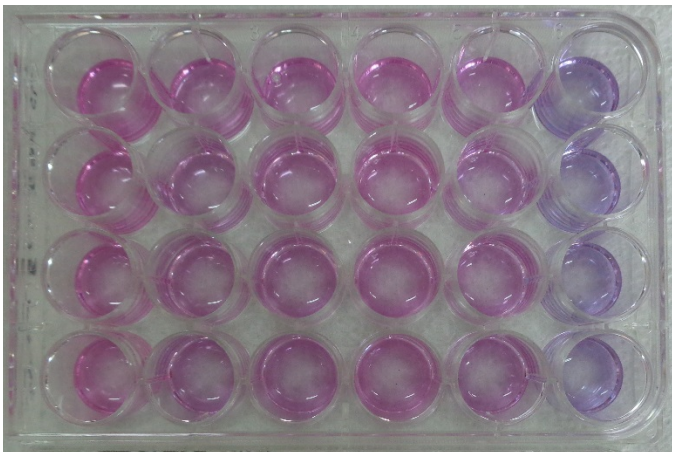

Figure 23 - alamarBlue® assay at 48h of incubation with OP extract. First column of wells correspond to negative control and the last column of wells correspond to 200 μg/mL of OP extract.

At 72h of incubation, HEK-293T cells have a decrease of 55% in cell viability (Figure 22A) and Caco-2 have a reduction of 35% in cell viability (Figure 22B) when incubated with 200 µg/mL of OP extract. Alterations in HEK-293T cell morphology was also detected when cells were incubated with OP in this conditions (Figure 24). At the concentration of 200 µg/mL, OP extract inhibit HEK-293T cytoplasmic extensions (Figure 24 D).

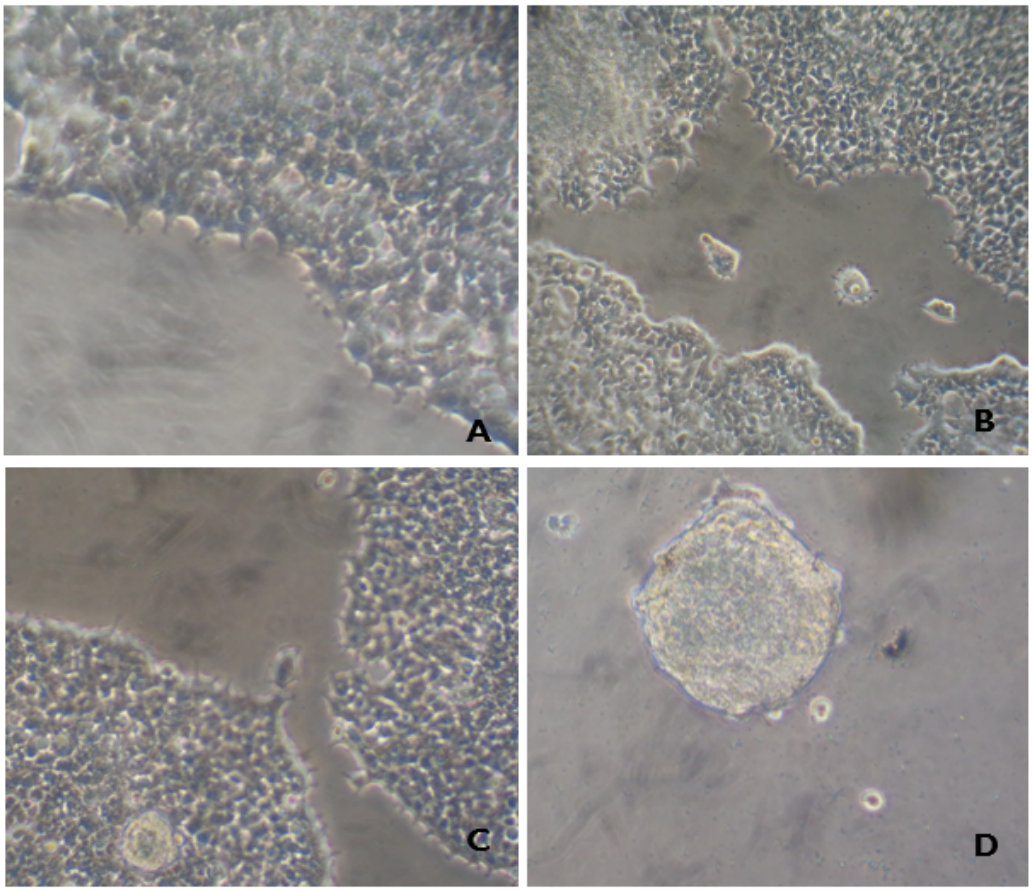

Figure 24 - HEK-293T cells morphology after 72h of incubation with *Osmundea pinnatifida* extract at different concentrations: A - 10 µg/mL; B - 50 µg/mL; C - 100 µg/mL; D - 200 µg/mL.

OP extracts was further evaluated for antiviral activity at the maximum nontoxic concentration of 100 µg/mL, according to ISO 10993-5:2009(E) indications.

In addition to CO, MO and OP extracts, evaluation of cytotoxicity of the extracts solvents (ultrapure water and DMSO) was also performed.

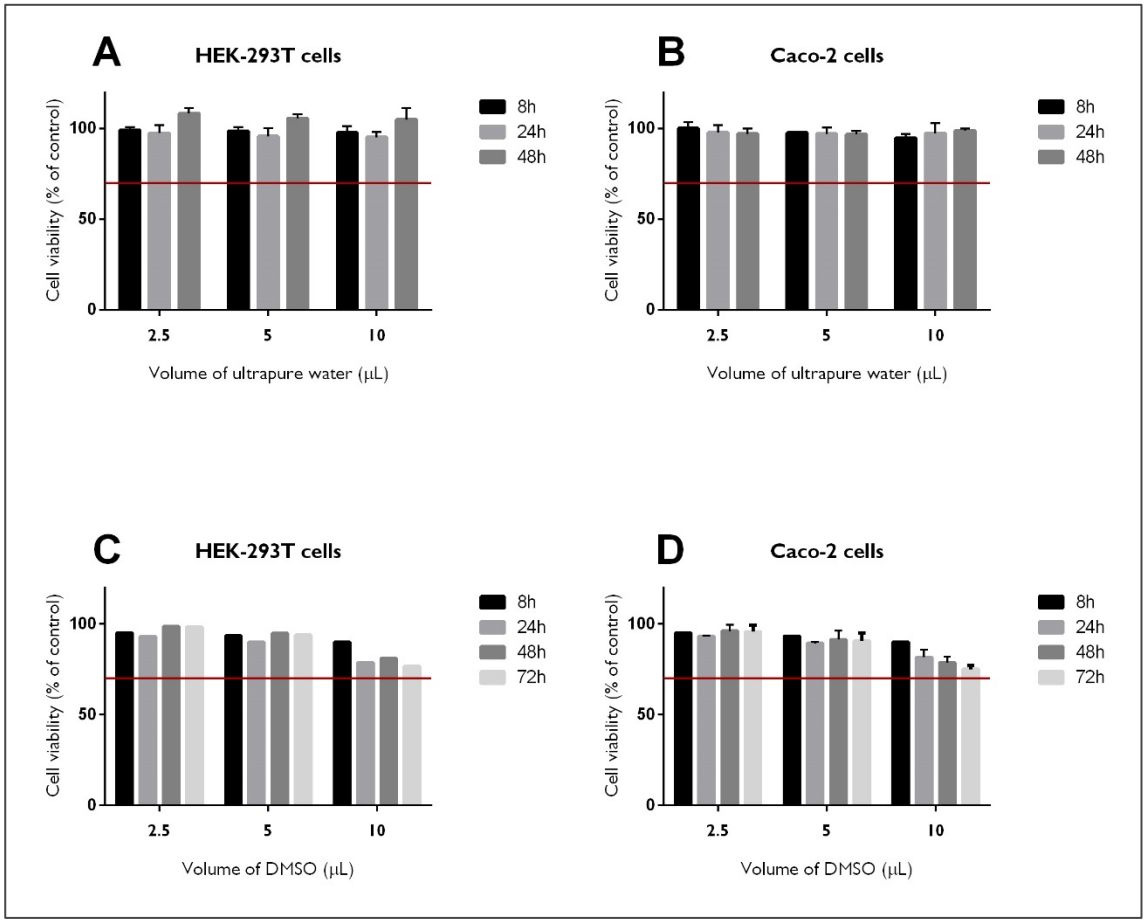

Figure 25 - Effects of extract solvents on HEK-293T and Caco-2 cell viability. The red line separates the cell viability values above and below 70%. Data were obtained by alamarBlue® assay and are presented as mean ± SD from two independent experiments (n=2) each carried out in quadruplicate.

Data showed that ultrapure water had no effects in cell viability (Figure 25 A and B). In contrast, DMSO reveal cytotoxicity when used at the volume of 10 µL, decreasing Caco-2 viability by 25% (Figure 25D) and HEK-293T viability by 24% (Figure 25C) after 72h of incubation. This means that cytotoxicity of OP extract was also due to the solvent. DMSO do not induce alterations in HEK-293T morphology (data not shown), so HEK-293T change in morphology was only due to extract.

3.2.2. Antiviral assays

3.2.2.1. HEK-293T/Lentivirus model

Quantification of LV infection in cells was performed by fluorescence microscopy determining the ratio of infected cells/total cells.

Antiviral activity of CO, MO and OP extracts against the genetic modified lentivirus was evaluated in two different conditions. (i) Cell protection assay: cells were pre-incubated for 2h with 200 μg/mL of CO and MO extracts and 100 μg/mL of OP extract; (ii) Virucidal assay: CVA12 was pre-incubated at 4°C for 2h with the respective concentrations of extracts. Infected cells without extracts were considered as a negative control (100% of infection). A control with only cells was included to evaluate cell auto-fluorescence in each assay. Results are presented as rate of infection comparing to control (Figure 26).

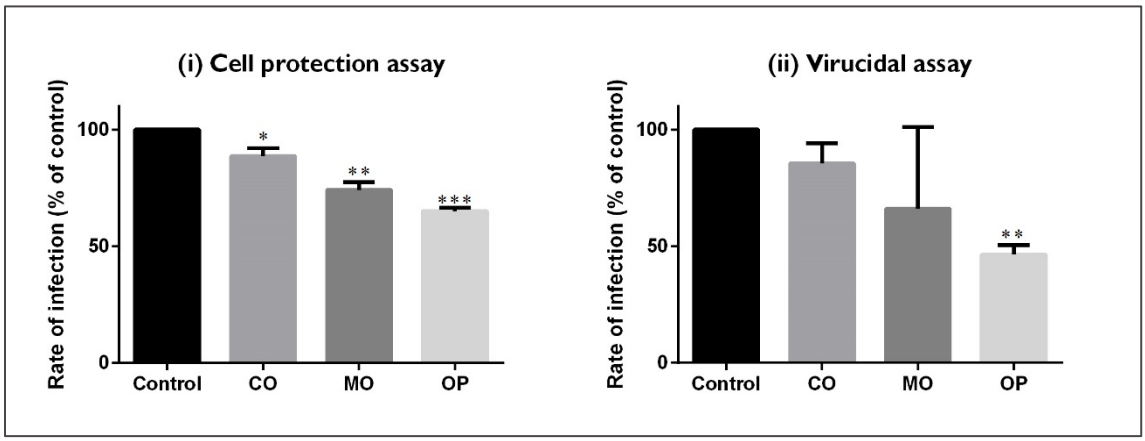

Figure 26 - Antiviral activity of CO, MO and OP extracts against Lentivirus infection in HEK-293T cells after different treatment conditions: (i) Cell protection assay and (ii) Virucidal assay. Extracts CO and MO were evaluated at 200 μg/mL. Extract OP was evaluated at 100 μg/mL. Results are expressed as the percentage of infection relative to a negative control (100% infection). Data are presented as mean ± SD from two independent experiments (n=2) each carried out in triplicate. For comparison of antiviral activity between treatment and control an unpaired Student's t-test was performed (GraphPad Prism 6). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

HEK-293T pre-incubated with CO extract showed a reduction of 12% in LV infection ($P = 0.0428$), while cells pre-incubated with MO extract showed a reduction of 26% in infection ($P = 0.0087$), both results are statistical significant (Figure 26 (i)).

When CO and MO extracts were pre-incubated with lentivirus prior to infection, a 15% and 34% reduction in viral infection was also, respectively, detected (Figure 22 (ii)).

These results do not have statistical significance. However, a tendency to reduce infection is clearly noticed.

Osmundea pinnatifida extract had a great statistical significant effectiveness against LV infection, reducing viral infection by 35% (P = 0.0009) when pre-incubated with cells and by 54% (P = 0.003) when pre-incubated with virus.

3.2.2.2. Caco-2/Coxsackievirus A12 model

Antiviral activity of extracts against Coxsackievirus A12 was evaluated in three different conditions. (i) Cell protection assay: cells were pre-incubated for 2h with 200 μg/mL of CO and MO extract and 100 μg/mL of OP extract; (ii) Virucidal assay: CVA12 was pre-incubated at 4°C for 2h with the respective concentration of extracts; (iii) Post-infection assay: cells were treated with extracts 2h after infection. Infected cells in culture medium without extracts were considered as a negative control. A positive control was not included because does not exist available drugs against CVA12.

Relative quantification of infection was determined by real time PCR. A housekeeping gene (rRNA 18S) was utilized to normalize the number of cells from each well. Reduction or increase in viral concentration due to treatment are presented as fold change and was calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen e Livak, 2008). The results are presented in figure 27.

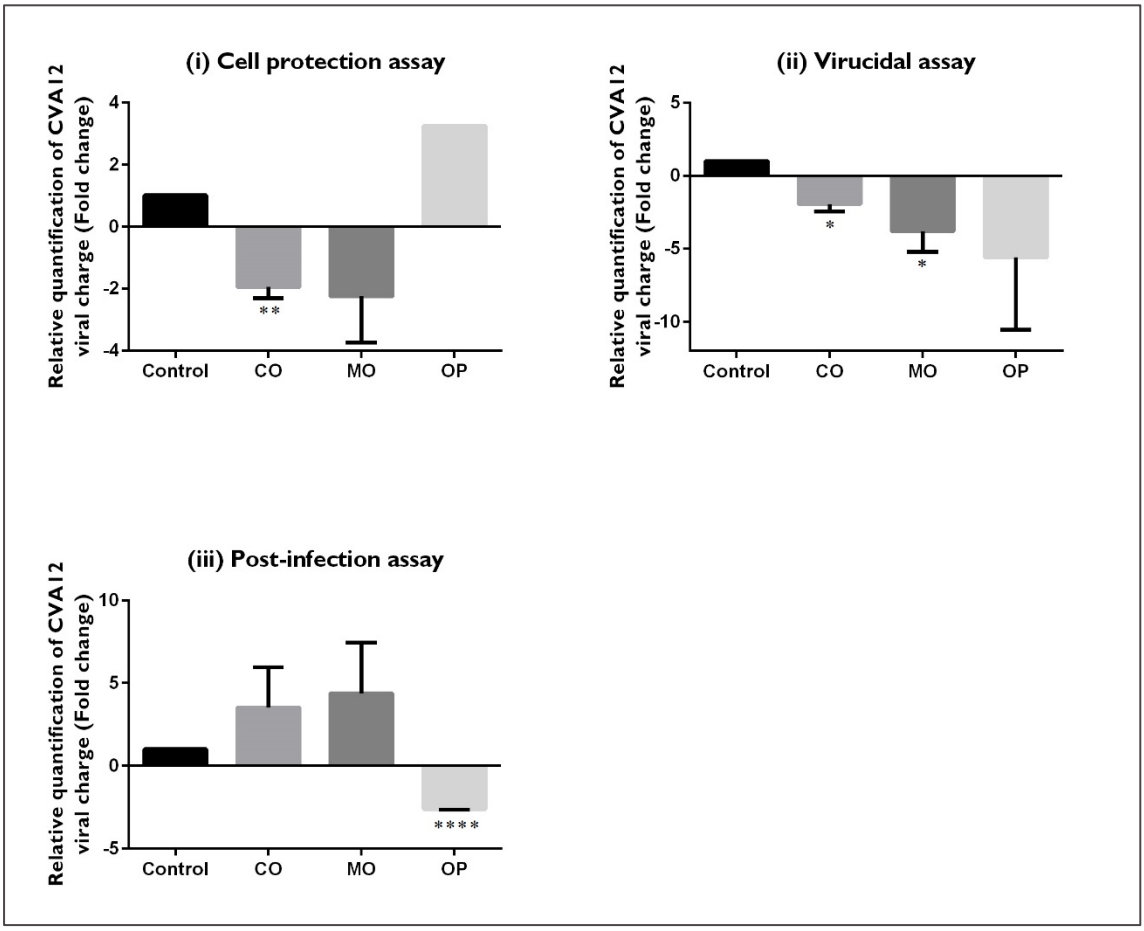

Figure 27 - Antiviral activity of CO, MO, and OP extracts against Coxsackievirus A12 infection in Caco-2 cells after different treatment conditions: (i) Cell protection assay; (ii) Virucidal assay and (iii) Post-infection assay. Extracts CO and MO were evaluated at 200 μg/mL. Extract OP was evaluated at 100 μg/mL. Results are expressed as fold change in viral charge due to treatment. Data are presented as mean ± SD from two independent experiments (n=2) each carried out in duplicate. For comparison of antiviral activity between treatment and control an unpaired Student's t-test was performed (GraphPad Prism 6). * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$.

Cobrançosa olive (CO) extract showed statistical significant antiviral activity against CVA12 when added prior to infection (i) and pre-incubated with virus (ii), inducing a 2-fold reduction in viral concentration due to treatment in both assays ($P = 0.0071$ and $P = 0.0138$, respectively) comparing to untreated control. When CO extract is added after infection (iii) a 3.5-fold increase in viral titration is observed.

Maçanilha olive (MO) extract induce a 2.3-fold reduction in CVA12 quantification when added prior to infection (i) and a statistic significant 3.8-fold reduction ($P = 0.0408$) when pre-incubated with virus (ii). When MO extract is added after infection (iii) a 4.4-fold increase in viral expression is observed.

OP extract increases viral charge by a 3.3-fold change when pre-incubated with cells. In contrast, OP extract reveal great inhibitory activity when pre-incubated with virus

and added to cells after infection by inducing a 5.6-fold and 2.6-fold reduction ($P < 0.0001$, respectively, in viral charge.

Osmundea pinnatifida extract reveal great activity against LV infection, as virucidal agent and with the ability of induce an antiviral state in the cell by reducing in 54% ($P = 0.003$) and in 35% ($P = 0.0009$) the LV infection, respectively. In CVA12 infection, OP extract was the only extract that reveal antiviral activity when added after virus replication and induce a 2.6-fold reduction on CVA12 infection ($P < 0.0001$). Also reduces CVA12 charge when pre-incubated with virus by a 5.6-fold decrease in viral charge, revelling virucidal potential. However, OP extract does not demonstrate ability to protect cell prior to infection.

4. Discussion

Viral infections are a problem of public health due to the variability of virus, multiple ways of transmission and lack of effective treatment. Treatment of viral infections is mostly symptomatic due to the absence of antiviral drugs.

Mechanisms of action of antiviral agents are based on their antioxidant activities, scavenging capacities, inhibition of DNA or RNA synthesis, inhibition of the viral entry or inhibition of the viral reproduction (Chattopadhyay *et al.*, 2009). The majority of antiviral drugs are specific to viruses which prevents the development of broad-spectrum antiviral agents (Zhu *et al.*, 2015). Some viruses also persist in a latent infection reducing the effectiveness of antiviral drugs (Abonyi *et al.*, 2009).

HIV infection does not have a cure. Viral load is controlled through antiretroviral therapy (ART) that consists in combined therapy with antiretrovirals that act in different phases of HIV life cycle. Owing to the incapacity of lentivirus reverse transcriptase to correct errors during retrotranscription and co-infection with different strains, HIV suffers several mutations per cycle of replication, leading to antiretroviral resistance (Cohen e Fauci, 2001).

Enterovirus infections are a worldwide distributed disease, mainly because of its easy transmission, and currently there are no specific antiviral agents available for enterovirus infection (Pallansch e Roos, 2001). Much effort is being applied to discover efficient treatment for the principal human enterovirus: Enterovirus 71 (Deng *et al.*, 2014; Kok, 2015; Zhao *et al.*, 2014), Coxsackievirus and Rhinovirus (Song *et al.*, 2014).

The huge inconvenient of some already discovered efficient antivirals is their toxicity to the host. Viruses during their replicative cycle become physically and functionally incorporated into the host cells. Therefore, the antiviral agent needs to be able to distinguish biochemical features of the cell and virus, and attack only the virus (Abonyi *et al.*, 2009). Pleconaril, a synthetic drug, has demonstrated antiviral activity against enterovirus infections but due to its toxicity has not been approved for therapy (Pevear et al., 1999).

It is extremely important to discover new molecules capable of inhibit virus infection and replication. Natural products are one of the most important sources of novel antiviral agents (Chattopadhyay *et al.*, 2009). Antiviral activity of several natural extracts has been described in literature, amongst them are medicinal plants (Schnitzler, Astani e

Reichling, 2011), algaes (Kang, Seo e Park, 2015) and higher plants, as olive trees and their products (Pereira *et al.*, 2007).

There are several methods available to evaluate antiviral activities of compounds that could be used and modified taking into consideration the characteristics of the virus in study (Chattopadhyay *et al.*, 2009). Optimizing a system capable of screening the antiviral potential of natural compounds is of great importance.

4.1. Optimization of the experiment

DMSO was the polar solvent of choice to dissolve the tested extracts. Even though his toxicity for cells, DMSO is the standard solvent because of his stability, elimination of microbial contamination and have good compatibility with test automation and integrated screening platforms (Cos *et al.*, 2006). CO and MO extracts did not dissolve in DMSO because they are aqueous extracts. T and FG, like CO and MO, did not dissolve in DMSO. Instead these four extracts were dissolve in sterile ultrapure water. OP extract was the only that dissolve in DMSO.

The concentrations of extract (1, 10, 50, 100 and 200 ug/mL) studied was higher than the usual concentrations tested for this kind of study (Tan *et al.*, 2012; Zhao *et al.*, 2014) because, at first, natural compounds are less toxic than synthetic compounds.

Caco-2 cells have a doubling time of approximately 32h, taking about 7 days to obtain a confluent flask of cells. FBS is a complex mix of albumins and growth factors from bovine origin that increase cell grow (HPA, 2010). Enriching the culture medium with 20% of heat inactivated FBS takes only 5 days to obtain a confluent flask of cells, instead of 7 days with 10% of FBS.

When performing alamarBlue® assay, Caco-2 cells are more fast metabolizing resazurin than HEK-293T. Caco-2 cells are epithelial cells from the colon, therefore have much higher capacity to metabolize compounds (Marino *et al.*, 2005). This cells undergo spontaneous enterocyte differentiation in culture after stablish a confluent monolayer. Cells are linking by tight junctions (Balimane e Chong, 2005). Caco-2 cells should be detach from the flask by trypsinization when confluency reach 80% and never after stablish a confluence monolayer because after this point ligations between cells and to the bottom of the flasks are stronger, making detach of cells more difficult and with lots of clumps.

Determination of extracts cytotoxicity using alamarBlue® has many advantages. alamarBlue® assay is a simple, fast, sensitive and economical method to evaluate cell viability

and because of its non-toxicity to cells, allows an overtime assessment of cell viability (Rampersad, 2012).

During LV infection of HEK-293T cells the volume of the well was reduce to 400 µL of culture medium to facilitate virus entrance since polybrene was not use. Polybrene is a cationic polymer commonly used in lentivirus infections (Nasri, Karimi e Allahbakhshian Farsani, 2014) but was not used in this study to not introduce another variable that could interact with the extracts.

HEK-293T cells were infected with LV when reaching about 20% of confluence, allowing a longer incubation always in monolayer and giving time for infected cells to synthetize GFP. This method enable quantification of LV infection by fluorescence microscopy determining the ratio of infected cells/total cells. Since total cell number was assessed by nuclear staining with DAPI and infected cell number with GFP, an automatized quantification of fluorescence was to be performed on the microscope software by reading total fluorescence in each channel (DAPI and EGFP). This was not possible because infected cells, that express GFP, were doing quenching of DAPI, decreasing the fluorescence of DAPI and by consequence decreasing the total number of cells (Figure 20). In order to make a correct count, total cell number was determined in brightfield, DAPI and EGFP channels.

CVA12 infections was performed in a density of 200.000 Caco-2 cells. This density correspond to a monolayer of cells which is the desired for infection because CVA12 is a cytolytic virus.

To perform an absolute quantification of CVA12 a calibration curve using a standard of known concentration needs to be created. Serial dilutions of the standard are made and quantify to make the curve points. CVA6 RNA pattern ($19.9x10^3$ copies/µL) had a Ct of 29.96 then the dilutions only gives 2 or 3 more points of the curve because the PCR protocol utilized have 45 cycles.

CVA6 pattern had a low concentration comparing to CVA12 stock because the concentration of $19.9x10^3$copies/µL correspond a Ct of 29.96 which means that CVA12 stock concentration is much higher (Table 4), so the extrapolation of CVA12 stock concentration from standard curve will have a great bias. Therefore we choose not performed an absolute quantification.

Instead, a relative quantification of CVA12 infection on Caco-2 cells was implemented using the comparative Ct method. However, as Caco-2 cells detach is more difficult and form many clumps, changing the number of cells, seed the same number of cells

in each well is extremely difficult. Viruses as intracellular parasites need to enter the cells in order to replicate (Condit, 2001). If more cells are available to infect, more virus will enter and higher will be the final viral charge. For all this, normalization of the number of cells is necessary and in our study we use the 18S rRNA (18S ribosomal RNA) gene, present in all cells as an housekeeping gene (Wisnieski *et al.*, 2013). An example of the influence of cell number in the infection is shown in table 5. Without normalization with 18S, when CVA12 is pre-incubated with MO extract an 11.6-fold increase comparing to control is noted but when number of cells are normalized with 18S, a 4.8-fold decrease in viral charge is noted. An opposite change on the action of the extract occurs when the number of cells is not evaluated.

4.2. Antiviral activity of natural extracts

Evaluation of antiviral activity in vitro includes antiviral efficacy and cell toxicity evaluation (Cos et al., 2006). Before test the antiviral activity of the extracts, their cytotoxicity on HEK-293T and Caco-2 cells was assessed. alamarBlue® assay revealed that incubation with CO and MO extracts for 48h at a maximum concentration of 200 μg/mL does not cause HEK-293T and Caco-2 cell death. On the other hand, OP extract showed a large increase of cytotoxicity on the study cell lines along the time when incubated at the maximum concentration studied (200 μg/mL).

Antimicrobial activity of olive oil and olive leafs extracts has already been demonstrated an inhibitory effect against several pathogens, including bacteria, fungi, mycoplasma and virus (Lee-Huang et al., 2003; Sudjana et al., 2009). Marine algae potential has also been studied (Bouhlal *et al.*, 2011).

The presented study demonstrates that pre-treatment of cells with CO and MO extracts inhibited infection of CVA12 and LV (Figure 27 (i) and figure 26 (i)). As in the pre-treatment extracts were removed before adding the virus, these results suggest that the extracts induce an antiviral state on cells making them more resistant to virus attack, perhaps by inhibiting the viral attachment and entry.

The results presented here also show that both CO and MO extracts have inhibitory effects when the viruses were incubated with the extracts before infecting the cells (Figure 27 (ii) and 26 (ii)), suggesting a direct inactivation effect on CVA12 and lentivirus, turning them in ineffective viral particles.

Therefore, Olive extracts demonstrated potential as antiviral agent and virucidal agent. Antiviral agents interfere with one or more dynamic processes during virus biosynthesis and are consequently candidates for clinical use, whereas virucidal substances inactivate virus infectivity extracellularly and are candidates as biocides (Cos et al., 2006).

Otherwise, CO and MO extracts could not reduce CVA12 infection when added after virus replication, and instead the viral infection is much more increased (Figure 27 (iii)).

Osmundea pinnatifida extract reveal great activity against LV infection, as virucidal agent and with the ability of induce an antiviral state in the cell. In CVA12 infection, OP extract was the only extract that reveal antiviral activity when added after virus replication and also reduces CVA12 charge when pre-incubated with virus, suggesting that it may act at an intracellular stage of infection. However, OP extract does not demonstrate ability to protect cell prior to infection.

This extract demonstrated great cytotoxicity in HEK-293T and Caco-2 cells. Part of this toxicity is due to the solvent where OP extract was dissolved, DMSO.

5. Conclusion

Despite expensive, real time RT-PCR are a fast, sensible and highly specific method to evaluate changes in viral charge. Normalization of cell number with 18S is fundamental to correct the amount of virus adsorbed in the beginning of infection. Fluorescence microscopy is a cheaper method to assess viral infection but is more susceptible to variation and is a time consuming technic, requiring a trained and experiment technician. Is important in both methodologies increase the number of independent experiences (n) in order to reduce the error associated with the variability of results.

Due to the lack of effective drugs for treatment of enterovirus infections, amongst them CVA12 infections, natural products are being a relevant search source of antiviral agents.

The results demonstrates that CO, MO and OP extracts were effective antiviral agents against human CVA12 and Lentivirus in this study. CO and MO extracts have inhibitory effects against the studied viruses when added prior to infection, so they are an excellent candidate for prophylactic intervention, and as virucidal agents. In addition, OP extract also reveal great activity in virus replication, suggesting that it can act at an intracellular stage of infection, being as well, a great candidate for therapeutic intervention.

In conclusion, data from the present study collectively suggest that Cobrançosa Olive and Maçanilha Olive extracts and *Osmundea pinnatifida* extract should be further evaluated as antiviral agents.

6. Future perspectives

One perspective for the future is test both models of infection – HEK-293T/Lentivirus model and Caco-2/Coxsackievirus model - with other treatment condition: treatment of cells simultaneously with virus infection. In order to understand the mechanism of action by which extracts inhibit viral infection, targeted studies should be performed taking into account the stage where the extracts seems to be active.

Another future perspective is optimized and adapt a similar procedure to a DNA virus, like Herpes Simplex Virus (HSV).

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