

UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS DA UNIVERSIDADE DE LISBOA

DEPARTAMENTO DE BIOLOGIA VEGETAL



ACTIVITIES OF AN AQUEOUS PLANT EXTRACT AGAINST HERPES SIMPLEX VIRUS
TYPE 2 (HSV 2): ROLE IN VIRUS REPLICATION AND PROGENY YIELD

Ana Rita Mota Mendes

Trabalho orientado pela Professora Doutora Maria Filomena Caeiro (CESAM-
FCUL) e pela Doutora Sílvia Lopo Esteves (INSA)

Mestrado em Biologia Molecular e Genética

Dissertação

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ULisboa** Faculdade
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This thesis was mostly performed at Centro de Estudos do Ambiente e do Mar da Universidade de Aveiro – Pólo FCUL (CESAM FCUL) under the direct supervision of Professor Maria Filomena Caeiro, and partially performed at Instituto Nacional de Saúde Dr. Ricardo Jorge (INSA) under the supervision of Doctor Sílvia Lopo Esteves.

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Acknowledgements

À minha orientadora, Professora Doutora Filomena Caeiro, pelos melhores e mais úteis conhecimentos transmitidos, por toda a disponibilidade e ajuda.

À minha co-orientadora Professora Doutora Sílvia Lopo, pela ajuda prestada e pela disponibilidade.

À Professora Doutora Lia Ascensão e ao Professor Doutor António Pedro de Matos, pela disponibilidade prestada.

À Professora Doutora Luísa Serralheiro, pela generosidade e disponibilidade.

À Teresa Granja, Rute Miguel e Manuela Lucas, por todos os esclarecimentos, ajuda e disponibilidade.

À Mónica Barra e Nadieny Barbosa, pelos concelhos e esclarecimentos prestados.

À Egídia Azevedo e Marta Figueiredo, pelo apoio e boa disposição.

À Marta Costa, por toda a ajuda e disponibilidade constante.

Aos meus pais, pelo apoio e incentivo que me deram ao longo desta etapa académica.

Abstract

Herpes simplex virus type 2 (HSV-2) is widely distributed through the human population, infecting more than 500 million people globally. Although causing generally mild infections this virus may cause severe symptoms occasionally, mainly in immunocompromised patients. Presently, there are a number of systemic antiviral agents against herpesvirus, the most commonly used being acyclovir (ACV) and its prodrugs. However, long term treatments with these drugs may result in the development of resistance, especially in immunocompromised patients, which leads to a continuous search for new and better therapeutic alternatives. According to the World Health Organization (WHO) plants would be the best sources for obtaining a wide variety of drugs. In fact, in the last decades many pharmacological and chemical studies have focused on medicinal plants and the discovery of new natural therapeutic compounds. In this study the anti-herpetic action of an aqueous extract was evaluated. The product was obtained by decoction of stem and leaves from *Solidago virgaurea*, a perennial herb member of the Asteraceae family. The study of the aqueous extract activity included a preliminary evaluation of its cytotoxicity in Vero cells – by the MTT assay - in the same conditions that are applied for viral production. Extract direct effect on viral particles – virucidal effect – was also assessed, having proved null. Anti-herpetic activity was investigated through two kinds of experiments: treatment of infected cells during virus production that revealed a mean yield reduction of 94 % in treated relatively to non-treated cells and an IC_{50} of 35.1 $\mu\text{g}/\text{mL}$; and treatment of infected cells during virus titration, which revealed slighter inhibition but significant size differences between virus plaques formed in treated and control conditions (smaller in treatment conditions). To further evaluate the mechanisms that mediate the aqueous extract inhibitory effect, infected cells – treated and non-treated - and virus particles – produced in treated and non-treated cells - were visualized through Transmission Electron Microscopy (TEM), revealing less damage due to infection in treated cells and a reduced amount of viral particles in HSV-2 suspensions produced in treated cells, relatively to controls. A kinetic of the first hours of the infection was performed with and without treatment, to assess possible differences in DNA production. Extracted samples were subjected to qPCR and results showed that the amount of viral DNA raises significantly slower in treated versus non-treated infected cells, throughout the infection. This is consistent with the effective reduction of the extract when added at later infection times - 4-6 h p.i. - when DNA replication is already in an advanced stage. Our results suggest that the aqueous extract inhibits HSV-2 replication, when present at the beginning of the infection, possibly by interfering with the viral DNA synthesis.

Keywords: HSV-2; Anti-herpetic activity; Asteraceae; Vero cells; Cytotoxicity

Resumo

O vírus Herpes simples tipo 2 (HSV-2) é um vírus de simetria icosaédrica e grandes dimensões, pertencente à família *Herpesviridae*, subfamília *Alphaherpesvirinae* da ordem *Herpesvirales*. Infecta mais de 500 milhões de pessoas em todo o mundo, surgindo, em cada ano, mais de 20 milhões de casos. A sua prevalência é geralmente mais elevada entre mulheres do que homens e varia substancialmente entre diferentes regiões do globo, alcançando os mínimos na Europa Ocidental - 18 % para mulheres e 13 % para homens – e percentagens máximas na África Subsaariana – 70 % para mulheres e 55 % para homens. O HSV infecta as células da mucosa oral ou genital e tem a capacidade de infetar as dendrites e os gânglios sensoriais que enervam estes tecidos onde estabelece latência. Estas infeções causam normalmente patologias ligeiras – gengivite, *herpes labialis* ou *herpes genitalis* – podendo até ser assintomáticas. Porém, infeções com HSV-2 podem causar complicações sérias a nível ocular - sendo a primeira causa de cegueira devida a infeção no mundo Ocidental -, neurológico - causando meningites e encefalites, especialmente comuns em neonatos – e dermatológico. Estas patologias mais severas, associadas à infeção com HSV, ocorrem com maior frequência em pacientes imuno-comprometidas, nomeadamente em indivíduos HIV (Vírus da Imunodeficiência Humana) positivos. Aliás, a infeção com HSV-2 aumenta 3 a 4 vezes a probabilidade de adquirir HIV e a sua disseminação, sendo esta relação recíproca, uma vez que pacientes HIV positivos têm maior probabilidade de adquirir HSV-2 e sofrem mais reativações da infeção. Atualmente, existem vários agentes com atividade antiviral contra HSV, como o Aciclovir (ACV) e as suas pró-drogas, no entanto, tratamentos prolongados com estes compostos podem resultar no desenvolvimento de resistência. De acordo com a Organização Mundial de Saúde (OMS) as plantas são a melhor fonte para a obtenção de novas drogas e, nas últimas décadas, as plantas medicinais têm sido alvo de vários estudos químicos e farmacológicos. Neste estudo foi avaliado o efeito anti-herpético do extrato aquoso de *Solidago virgaurea*, uma planta perianal, membro da família Asteraceae.

O extrato utilizado foi obtido por decocções de caules e folhas de *S. virgaurea* e a solução *stock* foi preparada a 100 mg/mL em DMSO. Antes de ser sujeito a testes de actividade anti-herpética foi ensaiado o efeito do extracto aquoso na viabilidade de células hospedeiras não infectadas, através do método do MTT – um ensaio colorimétrico que se baseia na capacidade de reduzir metabolicamente o sal de tetrazolio das células viáveis. Para isso cultivaram-se células Vero em placas de 96 poços – em meio de cultura com FBS (Soro Fetal Bovino) a 10 % – até à sub-confluência. O meio de cultura foi depois substituído por meio com FBS a 2 % e diferentes concentrações de extracto (25 - 250 µg/mL), ou meio com FBS a 2 % sem adição de extrato no caso dos testemunhos, e as placas foram incubadas 24 ou 48 horas a 37 °C. No final do período de tratamento fez-se o teste do MTT para determinação da viabilidade das culturas tratadas, por comparação com as culturas não tratadas. Os conteúdos de cada

poço da P96 foi analisado espectofotometricamente num leitor de placas Elisa. Para caracterizar a actividade citotóxica do extracto foram comparadas as absorvâncias obtidas em poços com células tratadas e relação a células não tratadas e determinaram-se os parâmetros CMNC (Concentração Máxima Não Citotóxica) - 150 µg/mL - e CC₅₀ (Concentração que inviabiliza 50 % das células) – 177,3 µg/mL.

O efeito directo do extracto nas partículas de HSV-2 foi testado através da comparação dos títulos de suspensões virais previamente tratadas com 100 µg/mL de extrato em meio com FBS a 2 % com os títulos de suspensões não tratadas. O extrato não demonstrou efeito virucida sobre partículas virais em suspensão, uma vez que os títulos das suspensões tratadas foram muito semelhantes aos títulos dos testemunhos.

Para testar o efeito do extrato na formação de placas virais infetaram-se células, em placas de 24 poços, com diluições seriadas de HSV-2. Depois da entrada do vírus – 30 minutos pós infecção – os inóculos foram removidos e foi adicionado meio de cultura com FBS a 2 % com ou sem 100 µg/mL de extrato, às culturas tratadas e testemunho, respetivamente. No final do período de tratamento – 3 a 4 horas pós infecção (h p.i.) – foi adicionado meio com FBS e Sephadex a 2 %. A redução de título em culturas tratadas foi 71,6 ± 26,8 %, em relação às não tratadas. Foi ainda evidente uma redução no diâmetro das placas produzidas nas culturas tratadas.

Foram também levadas a cabo várias experiências envolvendo a produção de vírus em condições de tratamento, em que culturas celulares sub-confluentes foram infetadas com cerca de 3 pfu/célula e tratadas com extrato em diferentes concentrações e durante diferentes períodos da infecção. Os títulos das suspensões produzidas em condições de tratamento foram comparados com os títulos dos testemunhos para o cálculo das percentagens de inibição. A redução média de título – com concentrações de extrato não citotóxicas – foi de 93,65 ± 8 %, relativamente a vírus produzido na ausência do extrato. Para determinar os parâmetros IC₅₀ e IC₉₀ (concentrações que reduzem o rendimento viral em 50 e 90 %, respetivamente) foram produzidas suspensões de HSV-2 em meio com FBS a 2 % e diferentes concentrações de extrato (10-175 µg/mL) que foram posteriormente tituladas. Os IC₅₀ e IC₉₀ foram calculados através de regressão linear da curva dose-resposta e foram 35,1 e 128,1 µg/mL, respetivamente. Para investigar a etapa da infecção afetada pelo extrato produziram-se suspensões de HSV-2 sob tratamento - com 100 µg/mL de extrato – iniciado em diferentes horas pós infecção. Foi observável uma redução da percentagem de inibição do extrato para cerca de 50 % em tratamentos começados entre as 3 e as 6 h p.i. e para 0 % a partir das 15 h p.i. Células infetadas tratadas e não tratadas, e partículas virais produzidas em ambas as condições, foram observadas por Microscopia Eletrónica de Transmissão (TEM), revelando a existência de menos danos, devidos à

infecção, em células infetadas tratadas do que em não tratadas e uma quantidade significativamente menor de partículas de HSV-2 em suspensões virais produzidas em células tratadas, apesar dos viriões produzidos sob tratamento não apresentarem defeitos morfológicos. Por fim foi executada uma cinética da infecção em condições de tratamento (e uma cinética testemunho, em condições normais de produção viral), para tal, células infetadas – tratadas e não tratadas - foram raspadas para o meio de cultura a diferentes horas pós infecção – 2 a 7. O DNA de cada cultura infetada recolhida foi extraído, quantificado e submetido a PCR para a deteção de DNA viral. O resultado não permitiu visualizar diferenças significativas na quantidade de DNA de HSV-2 em células tratadas e não tratadas, mas mostrou um aumento considerável na quantidade de DNA a partir das 5 horas pós infecção. As mesmas amostras foram depois utilizadas para PCR em tempo real (qPCR) para avaliar possíveis diferenças na quantidade de DNA viral. O resultado mostrou que, embora nas horas iniciais – 3-4 h p.i. – as diferenças na quantidade de DNA sejam mínimas, entre amostras de DNA extraído de células tratadas e não tratadas, é visível um aumento muito mais lento no número de cópias de DNA viral ao longo da infecção em culturas tratadas do que em não tratadas, indicando que a replicação de DNA é afetada durante o tratamento com extrato aquoso de *S. virgaurea*.

Palavras-chave: HSV-2; Atividade anti-herpética; Asteraceae; Células Vero; Citotoxicidade

Abbreviations

ACV - Acyclovir

ATCC – American Type Culture Collection

bp – Base pair

CC₁₀ - 10 % Cytotoxic Concentration

CC₅₀ – 50 % Cytotoxic Concentration

CC₉₀ - 90 % Cytotoxic Concentration

CNS – Central Nervous System

D-MEM – Dulbecco’s Modified Eagle Medium

Da - Dalton

DMSO - Dimethyl sulfoxide

DNA - Deoxyribonucleic Acid

dsDNA – Double stranded DNA

EtBr – Ethidium Bromide

FBS - Fetal Bovine Serum

FCV - Famciclovir

FDA – Food and Drug Administration

FOS - Foscarnet

g – Gravitational acceleration

HIV – Human immunodeficiency virus

HK – Herpes Keratitis

h p.i. – Hours post infection

HSCT – Hematopoietic Stem Cell Transplant

HSV – Herpes simplex virus

HSV-1 – Herpes simplex virus type 1

HSV-2 – Herpes simplex virus type 2

HVEM – Herpesvirus Entry Mediator

IC₁₀ – 10 % Inhibiting Concentration

IC₅₀ – 50 % Inhibiting Concentration

IC₉₀ – 90 % Inhibiting Concentration

ICTV - International Committee on Taxonomy of Viruses

IDU - Idoxuridine

kDa - Kilodalton

kb – Kilobase

mg – Milligram

mL – Milliliter

mM – Millimolar

MNCC - Maximum Non-cytotoxic Concentration

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

ng - Nanogram

nM – Nanomolar

nm - Nanometer

PBS - Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

pfu – Plaque forming units

pH – Measure of the acidity or alkalinity of a solution

RNA – Ribonucleic Acid

mRNA – Messenger Ribonucleic Acid

rpm – Revolutions per minute

RT-PCR - Reverse Transcriptase Polymerase Chain Reaction

SI – Selective Index

TFT – Trifluorothymidine

TNF – Tumor Necrosis Factor

T₂₅, T₇₅ – Culture flasks with 25 or 75 cm² area

U_L – Unique long sequence

U_S – Unique short sequence

UV – Ultraviolet

VACV – Valaciclovir

vhs – Virus host shut off proteins

VP – Virion Polypeptide

VZV – Varicella zoster virus

WHO - World Health Organization

α-TIF – α-transinducing factor

μg – Microgram

μL – Microliter

1. Introduction

Herpes simplex viruses (HSV) have been interacting with humans for thousands of years ^[1] and occur worldwide, without seasonal variation ^[2]. HSV-2 infects more than 500 million people globally, and, each year, more than 20 million new cases arise. HSV-2 prevalence increases with age, peaking at 35-39 years of age, and is generally higher among females than males. It varies substantially by region, ranging from about 18 % among women and 13 % among men in Western Europe to 70 % and 55 % in Sub-Saharan Africa ^[3].

Herpetic lesions at the oro-labial area are typically caused by HSV-1 and genital lesions are commonly associated to HSV-2, but, up to 10 % of primary oro-facial herpes infections could be caused by HSV-2 ^[4] and, on the other hand, HSV-1 seems to be emerging as the most common cause of primary genital herpes ^[5]. Even though, recurrences of infection by HSV-1 represent less than 5 % of the total cases of genital herpes ^[6].

Although HSV typically causes mild diseases it may provoke severe infections in immunocompromised patients like HIV seropositives and recipients of solid organ or bone marrow transplants ^[7]. In fact, an important finding in the last decades is that infection with HSV-2 is associated with high susceptibility to HIV, and increases its shedding ^[8, 9]. This relationship is reciprocal, as infection with HIV also significantly increases the probability of acquiring HSV-2 and its reactivation has shown itself more frequent in HIV-1 positive patients ^[10].

Currently, there are several systemic antiviral agents, against herpesvirus, like acyclovir (ACV) and its prodrugs – valaciclovir (VACV), famciclovir (FCV), ganciclovir, valganciclovir, cidofovir and foscarnet (FOS) – approved by the US Food and Drug Administration (FDA) ^[11], but, long-term prophylaxis and treatment with ACV, VACV or FCV can result in the development of resistance, especially in the immunocompromised patients group^[7,12] where ACV-resistant HSV prevalence varies between 3.5 % and 10 % ^[7].

Plants are still an important source of widely used pharmaceuticals ^[13-19] and Asteraceae family has been one of the most studied lately, and specifically its inhibitory activity against HSV was even the subject for a few recent works ^[17, 19-21]. Therefore, and considering the serious negative clinical implications that HSV-2 infections cause globally, it's highly significant to further evaluate anti-viral activities of plants of the Asteraceae family, inclusively the anti-herpetic proprieties of *Solidago virgaurea*, the object of this study.

1.1. Herpes Simplex Virus

1.1.1. Classification

Herpesvirus classification was addressed for the first time in 1971 by the International Committee on Taxonomy of Viruses (ICTV) where the genus *Herpesvirus* was established [23]; this genus was later elevated to the family *Herpesviridae* [23]. A first attempt of grouping herpesvirus into subfamilies has been done based in biological criteria [24]; this division was successful but contained a few misclassifications, therefore, further distribution of the subfamilies into genera was based in molecular data – genome characteristics, such as size and structure - to a greater extent than before [25].

Nowadays, the former family *Herpesviridae* has been elevated to a new order, the *Herpesvirales*, which accommodates three subfamilies - *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae* - 17 genera and 90 species [26].

1.1.2. Virion Properties

HSV are large viruses, and their viral particles have complex and characteristic structures. The spherical virion includes four major elements: core, capsid, tegument and envelope [27, 28].

The core consists of the viral genome packed as a linear double-stranded DNA molecule - 150 kpb - into the icosahedral capsid. Herpes simplex virus genome has an unusual structure that consists of two covalently linked components, long (L) and short (S), and each of these components contains a stretch of unique sequences, U_L and U_S , flanked by inverted repeats (Fig. 1 a). The two components can invert relative to one another, creating four different types of DNA molecule that differ only in the orientation of DNA sequences (Fig. 1 b). The sequence repeat at the left end of U_L is designated ab , and the internal inverted repeat is designated $b'a'$. The inverted repeats flanking U_S are designated $a'c'$ and ca . Most viral genes are present in only one copy per genome; the U_L region contains 65 protein coding sequences, and the U_S region contains 14 [28].

Therefore, HSV contain at least 84 different genes. Presently, most herpes simplex genes are numbered sequentially from one end of the unique sequence in which they are found. Thus, genes U_L1 to U_L56 are located in the L unique segment and U_S1 to U_S12 are located in the S unique segment, and some genes lying within the b and c segments have names related to their time of expression or other properties. HSV genes can be classified in at least three groups – *immediate early* (α), *early* (β) and *late* (γ) - based on the time of their expression during the replication cycle [2, 28].

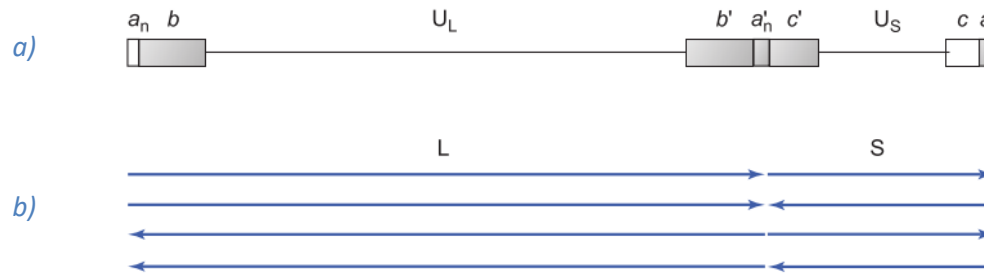


Fig. 1 - Genome of herpes simplex virus. a) Organization of unique and repeat sequence elements. Repeat sequence elements are shown in small letters (a,b,c) and elements shown with a prime are inverted. Unique sequence elements are shown in capital letters (U_L, U_S). b) Four isomeric forms of viral DNA with different orientations of the long (L) and (S) components due to frequent recombination at the repeated sequence elements. (Source: Acheson 2007)

The bilayered capsid (100-110 nm in diameter) is constructed with six proteins, of which the major one, VP5 (150 kDa), makes up the 162 capsomers, that are arranged as 12 pentamers at the vertices and 150 hexamers on the faces and edges of the icosahedron. Another capsid protein, VP26, is located at the tips of the capsomers. The capsid is enclosed in an envelope that contains at least ten different glycoproteins (gB to gM) and several other non-glycosylated membrane proteins [28, 29].

The virus envelope has a typical appearance and is thought to be derived from patches of host cell nuclear membrane modified by the insertion of virus glycoprotein spikes [29].

Unlike in most enveloped viruses, there is a large amorphous mass between the capsid and the envelope, called the tegument, which contains about 14 viral proteins like virus host shutoff (vhs), VP16 (α-TIF, ICP25), VP11-12, VP13-14 and VP1-2. These proteins are introduced into the cell upon infection by herpesviruses, and some of them carry out crucial functions in the virus growth cycle like activation of transcription and shutoff of host macromolecular synthesis [28, 29].

1.1.3. Replication

This virus is characterized by a short – 18 to 24 hours – and cytolytic replicative cycle. Initial attachment and penetration of the host cell is mediated via envelope glycoprotein gB that binds to heparan sulfate proteoglycans at the cell surface. Afterwards, envelope glycoprotein gD binds to at least two alternative receptors: nectin 1 – member of the nectin family of intercellular adhesion molecules – and herpesvirus entry mediator (HVEM) – member of the tumor necrosis factor (TNF) receptor family. Receptor binding triggers a pH-independent fusion of the viral envelope with the cellular membrane, which requires three conserved glycoproteins – gB, gH and gL [28, 29].

Fusion results in the introduction of tegument proteins and viral nucleocapsid into the cell cytoplasm and the rapid shut down of host macromolecular metabolism. Host DNA synthesis ceases, protein synthesis declines rapidly, ribosomal RNA synthesis is reduced and host protein glycosylation ceases. Most tegument proteins are believed to act directly or indirectly to produce early shutoff of the host macromolecular synthesis and to contribute to the early events of replication, like nucleocapsid attachment to nuclear pores and DNA release [29].

Nucleocapsids, along with some of the tegument proteins, are transported to the nuclear pores by dynein motors along the microtubular network of the cell and, at the nuclear pore, DNA is released and enters the nucleus. Empty capsids remain at the cytoplasmic side of nuclear pores for several hours until they disintegrate. Viral DNA in the nucleus is circularized, either by direct ligation of the ends or by recombination between the α sequences at the ends [28].

HSV gene expression is regulated and sequentially ordered as a cascade ($\alpha \rightarrow \beta \rightarrow \gamma$). Transcription of HSV *immediate early* (α) genes is carried out in the host nucleus, during the first several hours after infection, by cellular RNA polymerase II. Five of the six α gene products are regulatory proteins, involved in the regulation of viral and cellular gene expression, and their expression is required for the production of all subsequent polypeptide groups. To initiate transcription, tegument protein α -TIF or VP16, known as α -transinducing factor, forms a complex with at least two key cellular proteins - Oct-1 and HCF-1 - which binds to specific response elements in viral DNA that have a central consensus sequence - TAATGARATT. These elements, along with binding sequences for other cellular transcription factors, are found upstream of the promoters of all α genes, and act as transactivators to enhance the transcription of these genes. Transcripts are then transported to the cytoplasm where they are translated into five proteins - ICP0, ICP4, ICP22, ICP27 and ICP47 - that are transported back to the nucleus where they exert regulatory and activating functions relatively to β genes expression [28, 29].

Expression of *early* - or β - genes results in the production of proteins that work together to carry out essential steps in DNA replication. Maximum synthesis rates of these proteins are observed at 5-7 h p.i., and when sufficient levels have accumulated within the infected cell, viral DNA replication starts. Like other DNA viruses, herpesviruses make their own DNA polymerase that is encoded by a single major RNA and has its synthesis during β phase. This enzyme can begin DNA synthesis at any one of three viral origins of DNA replication on the herpes simplex virus genome. *oriL* is located in the middle of *U_L* region, and the other two - *oriS* - are in the *c* inverted repeats flanking the *U_S* region. Replicating DNA in infected cells is found as high molecular weight, branched head-to-tail multimers called concatemers, suggesting that DNA is made by a rolling circle mechanism, which can produce multimers

from a circular template. However, herpesvirus initially sets up bidirectional replication on the circular viral DNA, and only later shifts to rolling circle replication [28, 29].

Although γ_1 genes can be transcribed in the absence of viral DNA replication their optimal expression requires viral DNA synthesis, presumably because of the increase in the number of template DNAs available for transcription; in contrast, the transcription of γ_2 genes is totally dependent on viral DNA synthesis. Late mRNAs enter the cytoplasm for translation into late proteins which include the capsid structural proteins that travel back to the nucleus, envelope proteins that are produced in the endoplasmic reticulum (ER) and become incorporated in the nuclear membrane and other structural and packaging related proteins [28].

Immature capsids lacking DNA are assembled in the nucleus by accumulation of the major capsid protein VP5 and several other proteins around a scaffold formed by three late proteins – VP21, pre-VP22a and VP24. Viral DNA - in the form of head-to-tail concatemers - is cleaved and stuffed into the preformed capsids by the interaction of several viral late proteins with high conserved packaging sites – pac1 and pac2 – located in the α sequence near the termini of the DNA. Entry of viral DNA into the capsids is carried out by a multimers of U_L6 protein located at one vertex of the capsid and involves several other nonstructural proteins. DNA packaging is complete when a sufficient length of the DNA is introduced and when the packaging machinery encounters the next α sequence along the DNA in the same orientation. The packaged DNA is cleaved from the concatemers at a specific site within a 20-nt direct repeat flanking the terminal α sequence. The DNA filled capsids proceed to bud through the modified inner nuclear membrane, into the lumen between the two nuclear membranes, acquiring an envelope and a layer of tegument proteins. Virions are then transported from the lumen to the outside of the cell [28, 29].

Three distinct theories account for the mechanism by which virus particles exit the cell. According to one theory, virions retain their envelopes and are transported to the Golgi membranes within vesicles that bud from the outer nuclear membrane. According to another theory, virions lose their envelope by fusion with the outer nuclear membrane, releasing nucleocapsids into the cytoplasm; these nucleocapsids subsequently reacquire an envelope by budding into Golgi membranes. In either case, the envelope proteins would be glycosylated within the Golgi, and further transport of mature virions to the extracellular space is via secretory vesicles. A third theory is based on the observation that nuclear pores become enlarged and the Golgi membranes are fragmented during viral replication; according to this hypothesis, nucleocapsids exit through the enlarged nuclear pores without an envelope and become enveloped at multivesicular bodies derived from fragmented Golgi membranes [28, 29].

1.1.4. Clinical Manifestations

HSV infections cause usually mild pathologies – gingivostomatitis, *herpes labialis* and *herpes genitalis* – and may be, many times, asymptomatic [28, 30]. Transmission occurs by intimate contact with an infected individual either with evident infection or asymptomatic excretion of viral particles, and, after primary HSV replication, at the oral or genital mucosa, the virus is able to infect neuronal dendrites of sensory ganglia that innervate these tissues and establishes long-term latency, from which it can reactivate episodically [31, 32].

Besides common herpetic lesions HSV-1 and HSV-2 infections can also result in other skin diseases such as herpetic whitlow, which manifests as lesions at the finger tips [33], and eczema *herpeticum* in patients underlying atopic dermatitis – the most common chronic inflammatory skin disease - which may be life-threatening for children [34].

These viruses are also linked with a variety of ocular complications, mostly epithelial and stromal keratitis. HSV can cause stromal opacification and is estimated to be the first cause of blindness due to infection in the Western world [35].

In addition to establish latent infection in the human nervous system these neurotropic viruses can also infect the central nervous system (CNS) causing diseases as meningitis and encephalitis. These kinds of infections more often occur in neonates as a result of congenital infection from the mother to the new born at birth - even when the mother shows no clinical signs of infection - and can lead to severe neurological damage as well as death [36].

An important finding in the last decades is the fact that infection with HSV-2 increases 3-4 times the likelihood of acquiring HIV [8] and considerably increases the dissemination of HIV in the genitalia of co-infected patients [9]. Interestingly this is a reciprocal relationship, since infection with HIV also significantly increases both HSV-2 reactivations and the probability of acquiring it [10].

1.1.5. Therapeutic

The antiviral therapies for herpes were first approached at Yale, USA in 1959 where a team synthesized idoxuridine (IDU) - the first Food and Drug Administration approved antiviral drug - followed by trifluorothymidine (TFT), both used to treat Herpes Keratitis (HK) [11].

Vidarabine was the first antiviral drug selective enough to be used for systemic treatment of herpesvirus (HSV and Varicella zoster virus, VZV) [11, 37]. This drug was later abandoned for reasons

including relative insolubility in aqueous medium and rapid deamination, but, most of all, by being surpassed by much safer and potent drugs ^[11].

In December 1977 ^[38] and April 1978 ^[39] it was announced the discovery of acyclovir (9-(2-hydroxyethoxymethyl guanine)), a guanosine nucleoside analog that once internalized into infected cells is processed into a monophosphorylated form – by HSV thymidine kinase - that is subsequently phosphorylated into acyclovir triphosphate by host kinases. In this form ACV triphosphate is incorporated by the viral DNA polymerase disrupting the viral genome replication by a chain termination mechanism ^[40-42].

In vitro, ACV is most potent against HSV-1, approximately half as potent against HSV-2, 1/10th as potent against VZV and Epstein-Barr virus (EBV) and least potent against human cytomegalovirus (HCMV)^[7]. Its main limitation resides in its rather poor oral bioavailability, which led to the development of valacyclovir (VACV), an L-valyl-ester prodrug of acyclovir ^[43, 44].

Drug-resistant strains of HSV frequently develop following therapeutic treatment, particularly in immunocompromised patients group ^[7, 12] where its prevalence was reported between 3.5 % and 7 % in HIV patients, 2.5 % and 10 % in solid organ transplant patients and 4.1 % and 10.9 % in hematopoietic stem cell transplant (HSCT) patients ^[7].

1.2. Medicinal Plants

Evidence for the use of medicinal plants dates back to 60,000 years ago ^[45] and, in the last decades, many pharmacological and chemical studies concerning medicinal plants have been made, with the discovery of new therapeutic compounds as a goal ^[13]. In fact, according to the World Health Organization (WHO), these would be the best sources for obtaining a wide variety of drugs and could benefit a large population ^[14]. It has been estimated that more than 50 % of available drugs have, in some way, originated from plants ^[15, 16], examples being morphine, emetine, vincristine and colchicine ^[13].

1.2.1. *Solidago virgaurea*

Solidago virgaurea is a perennial herb member of the Asteraceae family, one of the largest plant families with more than 1,600 genus and 24,000 to 30,000 species ^[46, 47]. This family has a wide distribution, existing in every continent, except Antarctic ^[17, 18]. *Solidago L.* genus includes about 125 species that are widely present in the north hemisphere. *Solidago virgaurea*, commonly designated

Goldenrod, being the single member of this genus native in Europe ^[47, 48]. This species is recognizable by its elongated and branched golden yellow flower heads that bloom in summer and has been traditionally used to treat urinary tract, nephrolithiasis and prostate diseases ^[49, 50].

1.3. Objectives

Considering the high prevalence of this virus and the serious clinical implications that HSV-2 infections may cause to seropositive patients, along with the cooperative relation it can establish with HIV and the increasing occurrence of ACV-resistant strains, especially in immunodeficient individuals, the search for new and effective treatments is an extremely important matter. As two previous studies ^[17, 19] had concluded that *S. virgaurea* aqueous extract exhibited anti-herpetic activity, this work aims to further characterize its inhibitory effect and investigate the associated mechanisms.

2. Materials and Methods

Working with virus and, therefore, with cell cultures demands really strict sterility conditions. Thus, most procedures described were prepared in a class II laminar flow cabinet (Biohazard), irradiated with U.V. light for 30 minutes before and after each utilization. Most materials used were sterile, either disposable or glass, the last ones were sterilized in an autoclave (121 °C, 15 minutes). All culture media were supplemented with antibiotic and kept at 4 °C.

2.1. Cell Culture

Cells used were Vero – a continuous lineage isolated from the renal epithelium of the green African monkey (*Cercopithecus aethiops*) – and were obtained from the American Type Culture Collection (ATCC) with the reference CCL81. These cells form a monolayer adherent to the flask surface and are widely used in virology studies for being permissive for a large variety of virus.

Cultures were grown in T25 and T75 flasks (Nunc) at 37 °C in CO₂ Independent Medium (Gibco) or Dulbecco's Modified Eagle Medium (D-MEM, Gibco) supplemented with 10 % Fetal Bovine Serum (FBS, Gibco), 0.1 % gentamicin sulfate (50 µg/mL) and glutamax (5 µg/mL) with or without a 5 % CO₂ atmosphere, respectively for each medium. The cultures were observed through an inverted optic microscope (Zeiss IM).

Cells were transferred to a new flask when cultures reached 90 % confluence. The culture medium was removed and the cultures washed, twice, with 2.5 mL of PBS (Phosphate Buffered Saline, Gibco), then the cultures were trypsinized with 1 mL of trypsin (Gibco) at 37 °C for 5 minutes. After that, 5 mL of 10 % FBS culture medium – D-MEM or CO₂ Independent Medium - were added to the flask, to disable trypsin and homogenize the suspension. Then the cell suspension was distributed accordingly to the flask/plate size.

2.2. Aqueous extract stock preparation

The aqueous extract used in this study was obtained from stems and leaves of the Portuguese Asteraceae family member *Solidago virgaurea* L. The lyophilized extract was supplied by Prof. Dr. Luísa Serralheiro from CQB (Centro Química e Bioquímica), FCUL (Faculdade de Ciências da Universidade de Lisboa) and kept at -20 °C. Stock solution was prepared at 100 mg/mL concentration in DMSO

(Dimethyl sulfoxide) and kept at -20 °C, the work solutions were prepared at 10 mg/mL in PBS and kept at 4 °C.

2.3. Virus

The viruses used in this study were acquired from ATCC, and are wild strains of HSV-2 (Herpes simplex type 2 HD) and HSV-1 (Herpes simplex type 1 SC 16). Viruses were kept in suspension with 2 % FBS culture medium (D-MEM or CO₂ Independent Medium) at 4 °C.

2.3.1. Virus Production

The virus suspensions were produced in T75 flasks with sub confluent cell cultures (about 10⁷ cells). Culture medium was removed and cells were infected with, approximately, 10⁶ pfu, in a 1 mL volume. After the adsorption period – 30 to 60 minutes at 37 °C – 12 mL of 2 % FBS culture medium (D-MEM or CO₂ Independent Medium) were added. Flasks were kept at 37 °C until total cytopathic effect – characterized by severe morphological changes in the culture and loss of adherence by most or all the cells - could be observed, what takes, usually, 48 to 72 hours of infection.

Virus suspensions were collected in 15 mL *Falcon* tubes and centrifuged at 3,000 g for 5 minutes, the supernatants were transferred to new, similar, tubes and the *pellets* were discarded. Produced viruses were kept at 4 °C.

2.3.2. Virus Titration

To determine the virus titer – the number of plaque forming units (PFU) per mL – each virus suspension was serially diluted (1:10) in 2 % FBS culture medium (D-MEM or CO₂ Independent Medium) to 10⁻⁶. Then, in 24 or 48 well plates with sub confluent Vero cell cultures, the 10% FBS culture medium was removed and 0.1 mL of each viral dilution was inoculate in duplicates.

The plates were then incubated for 30 minutes at 37 °C, to allow viral entry, before the addition of 500 µL of titration medium – 2 % FBS culture medium with 2 % Sephadex G-75 (Pharmacia Fine Chemicals). Plates were kept, absolutely still, at 37 °C for 2 days – HSV-2 – or 3 days – HSV-1.

To end the titration, the infected cultures were fixated with 0.5 mL of 10 % (v/v) formaldehyde for 30 minutes, at room temperature, with stirring. After that, formaldehyde was removed and the plates

washed with tap water. The cells were then dyed with 0.2 % crystal violet for 15 minutes, with stirring, then the stain was removed and the plates were washed with tap water and dried in an oven at 37 °C.

All countable viral plaques were counted and virus titer was calculated by the formula:

$$\text{Virus titer (pfu/mL)} = \# \text{plaques} \times \left(\frac{1}{\text{Dilution}} \right) \times \left(\frac{1}{\text{Inocule (mL)}} \right)$$

2.4. Cytotoxicity Assays

The aqueous extract cytotoxicity was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method, which is a colorimetric assay that relies on viable cells ability to metabolically reduce tetrazolium salt - through mitochondrial enzyme succinic dehydrogenase – into purple formazan crystals that accumulate inside the cells. ^[51]

Cells were grown in 96 well plates, at 37 °C, with 10 % FBS culture medium until sub confluence was reached. Afterwards, the 10 % FBS culture medium was replaced by 200 µL of 2 % FBS medium with different extract concentrations (25 – 250 µg/mL). Wells with 2 % FBS culture medium were kept as control. The cell cultures were then incubated at 37 °C, and regularly observed, for 24 or 48 hours. When the incubation periods were concluded all inoculums were removed and the wells were washed with 100 µL of PBS.

Then 100 µL of 0.5 mg/mL MTT solution (Sigma) in 2 % FBS culture medium was added to each well and the plate was incubated at 37 °C for 2 hours, to allow MTT reduction. After that, the solution was carefully removed and the formazan crystals were dissolved with the addition of 100 µL of DMSO. Following 30 minute incubation at room temperature the wells content was spectrophotometrically analyzed in a microplate reader (Tecan Sunrise) with a 570 nm wavelength and a 630 nm reference filter.

To characterize the extract cytotoxicity parameters as MNCC (maximum non-cytotoxic concentration), CC₁₀, CC₅₀ and CC₉₀ (cytotoxic concentration that destroy 10, 50 and 90 % of the cells, respectively) were defined, by means of comparison of treated and control wells average absorbance values.

2.5. Virucidal Effect

The virucidal effect of a compound is its ability to directly inactivate a viral particle, outside or inside the host cell. To determine whether or not the aqueous extract exhibit this effect on HSV-2 particles,

viral suspensions of 100 – 200 μL ($\sim 10^6$ pfu) were treated, in 1.5 mL micro tubes, with different extract concentrations for 1-3 hours at room temperature with stirring. A control suspension, with the addition of the same volume of PBS, was prepared in parallel to each treated suspension.

Following the treatment each viral suspension was titrated as well as the respective control. After titers determination, the percentage of virus inactivation by each extract concentration was calculated by the formula:

$$\text{Virus inactivation} = \left(1 - \left(\frac{\text{Treated virus titer}}{\text{Non - treated virus titer}} \right) \right) \times 100$$

2.6. Effect on HSV-2 replication

To uncover the extract influence in HSV-2 replication cycle, two kinds of approaches were employed: HSV-2 titration under treatment and HSV-2 production under treatment.

2.6.1. Extract effect on viral plaques formation

HSV-2 was serially diluted (1:10) to 10^{-6} , in 2 % FBS culture medium (D-MEM or CO₂ Independent Medium) and, in 24 (or 6) well plates with sub confluent Vero cell cultures, the 10 % FBS culture medium was removed and 0.1 mL (or 0.4 mL) of each viral dilution was inoculated in duplicates. After a 30 minute incubation - to allow viral entry – 0.15 (or 0.7 mL) of culture medium with or without 100 $\mu\text{g}/\text{mL}$ extract were added, respectively, to each treated and control infected cells.

Plates were then incubated for 10 minutes to 4 hours at 37 $^{\circ}\text{C}$, before the addition of 0.5 (or 1.5 mL) of titration medium – 2 % FBS culture medium with 2 % Sephadex G-75 – with or without 100 $\mu\text{g}/\text{mL}$ extract, respectively to treated and control infected cells.

2.6.2. Extract effect on virus yield

Sub confluent cell cultures, grown in 6 well plates, were inoculated with 3 to 30 pfu/cell of HSV-2 and incubated at 4 $^{\circ}\text{C}$ for 1 to 1.5 hours at a plate rocker to allow viral adsorption to cell membrane. After the adsorption period, plates were incubated at 37 $^{\circ}\text{C}$ for 30 minutes, for viral entry, and inoculums were removed. Infected cells were then treated with 500 to 900 μL of 2 % FBS culture medium with or without extract (100 $\mu\text{g}/\text{mL}$), and incubated at 37 $^{\circ}\text{C}$, in the presence of the extract, for 2 to 24 hours. The content of all wells was replaced by 1.4 mL of 2 % FBS culture medium, following the treatment

period. Produced HSV-2 were subsequently titrated in 24 well plates as described before, and treated suspensions titers were compared to control titers for evaluation of the yield reduction percentage.

2.6.3. IC₅₀ and IC₉₀ determination

Sub confluent cell cultures, grown in a 12 well plate, were inoculated with 3 pfu/cell of HSV-2 and incubated at 4 °C for 1 hour at a plate rocker. After that, plates were incubated at 37 °C for 30 minutes and then inoculums were removed. Infected cells were then treated with 550 µL of 2 % FBS culture medium with or without extract – at variable concentrations -, and incubated at 37 °C, in the presence of the extract, for 4.5 hours. The content of all wells was replaced by 1.4 mL of 2 % FBS culture medium following the treatment period. Produced HSV-2 were subsequently titrated in 24 well plates, as described before, and treated suspensions' titers were compared to control titers for evaluation of the yield reduction percentage.

Concentration that reduces viral yield in 50 % (IC₅₀) and concentration that reduces viral yield in 90 % (IC₉₀) were calculated through linear regression of the dose-response curve.

2.6.4. Determination of the affected stage during the virus replication

Sub confluent cell cultures, grown in 6 well plates, were inoculated with 3 pfu/cell of HSV-2 and incubated at 4 °C for 1 hour at a plate rocker. After the adsorption period plates were incubated at 37 °C for 30 minutes and inoculums were removed. 800 µL of 2 % FBS culture medium without extract were added to each well and, at each treatment starting point – 0.5 to 20 h p.i. -, 800 µL of 2 % FBS culture medium with extract (200 µg/mL) were added to treated cultures. Four controls were kept - two for treatment starting at 0.5 h p.i., one for 6 h p.i. and one for 20 h p.i. – where 800 µL of 2 % FBS culture medium without extract were added at the indicated times. Plates were incubated at 37 °C, in the presence of the extract, for 24 hours. Produced HSV-2 were subsequently titrated in 24 well plates, as described before, and treated suspensions titers were compared to control suspensions titers for evaluation of the yield reduction percentage.

2.6.5. Comparison with acyclovir inhibition

Sub confluent cell cultures, grown in 6 well plates, were inoculated with 3 pfu/cell of HSV-2 incubated at 4 °C for 30 minutes at a plate rocker. After adsorption plates were incubated at 37 °C for 30 minutes and inoculums were removed. Infected cells were then treated with 1.5 mL of 2 % FBS culture medium

with or without extract/ACV (100 µg/mL), and incubated at 37 °C, for 48 hours. Products were present throughout the whole infection, unlike in most experiments, once ACV requires continuous presence to exert maximum inhibitory effect. Produced HSV-2 were subsequently titrated in 24 well plates, as described before, and treated suspensions titers were compared to control titers for the evaluation of yield reduction percentage.

2.7. Visualization of treated and non-treated infected cells and virus suspensions by Transmission Electron Microscopy (TEM)

2.7.1. Infected cells – treated and control – observation by TEM

Sub confluent Vero cell cultures, grown in 6 well plates, were inoculated with 140 pfu/cell of HSV-2 and incubated at 4 °C for 1 hour at a plate rocker. After the adsorption period plates were incubated at 37 °C for 30 minutes, for viral entry. Then, inoculums were removed and each culture was washed with 500 µL of PBS. Infected cells were subsequently treated with 1 mL of 2 % FBS culture medium with or without extract (100 µg/mL) and incubated at 37 °C, in the presence of the extract, for 3 hours. The content of all wells was replaced by 1.4 mL of 2 % FBS culture medium following the treatment period.

Infected cells – treated and control – were collected at 14 hours post infection, by scraping to the medium with a policeman, to 1.5 mL microtubes and centrifuged at 3,000 *g* for 5 minutes. Supernatants were rejected and pellets were resuspended in 800 µL of 3 % glutaraldehyde in sodium cacodylate buffer (0.1 M, pH 7.3), which was replaced, 24 hours later, by cacodylate buffer. Samples were kept at 4 °C until they were sent to Dr. A. P. Alves de Matos (CESAM and Centro de Investigação Interdisciplinar Egas Moniz), being then processed and analyzed as described before ^[52].

2.7.2. Virus suspensions – treated and control – Negative Staining for TEM

Sub confluent cell cultures, grown in T75 flasks, were inoculated with 1 pfu/cell of HSV-2 in 2.5 mL of virus suspension, and incubated at 4 °C for 1 hour at a plate rocker. After the adsorption period flasks were incubated at 37 °C for 30 minutes and inoculums were removed. Infected cells were then treated with 5 mL of 2 % FBS culture medium with or without extract (100 µg/mL), and incubated at 37 °C, in the presence of the extract, for 2.5 hours. The content of both flasks was replaced by 13 mL of 2 % FBS culture medium without extract, following the treatment period. Treated and control suspensions were collected 28 hours post infection.

Virus suspensions – treated and control – were collected at 28 h p.i. to 15 mL Falcon tubes and centrifuged at 3,000 *g* for 5 minutes. Supernatants were collected in centrifuge tubes and centrifuged at 19,000 *g* for 5 hours, for virus concentration. Supernatants were rejected and the pellet (concentrated virus) was resuspended in 100 μ L of PBS.

Samples were kept at 4 °C until they were sent to Dr. A. P. Alves de Matos (CESAM and Centro de Investigação Interdisciplinar Egas Moniz), being then processed and analyzed as described before ^[52].

2.8. Kinetics of HSV-2 infection in treated and non-treated cells

To determine the extract effect on viral DNA replication, sub confluent cultures grown in 12 well plates, were infected with 3 pfu/cell of HSV-2, incubated at 4 °C for 1 hour at a plate rocker and at 37 °C for 30 minutes. Then, the viral inoculums were removed and 1 mL of culture medium with or without 100 μ g/mL of extract was added to each treated or control well.

Each treated and respective control culture was scrapped into the culture medium and collected in a 1.5 mL microtube, at different hours post-infection – 2 to 7 h p.i. Collected suspensions were centrifuged at 3,000 *g* for 5 minutes, supernatants were rejected and kept at 4 °C until DNA purification.

DNA was purified using JETQUICK® protocol for the purification of DNA from up to 1 mL of whole blood ^[53] as per manufacturer's instructions and eluted in 200 μ L of DNase free water. Purified DNA samples were observed thru electrophoresis in a 0.7 % agarose gel in TBE with 0.005 % EtBr (ethidium bromide), to confirm their integrity. DNA concentration was determined for every sample using a NanoDrop® Spectrophotometer (Thermo Scientific ND-1000), and 19.41 ng of each DNA sample was used for polymerase chain reaction (PCR), to detect HSV-2 DNA.

Each sample was amplified with HV/Cons primers ^[54], and reactions were carried out in a total volume of 5 μ L prepared in ultrapure water with 2.5 μ L of 2X MyTaq™ Red Mix (Bioline), 0.5 μ M of each primer and 1 μ L of DNA sample, following manufacturer's instructions. The amplification program consisted of an initial 3 min denaturation at 95 °C followed by 30 cycles of denaturation – 95 °C for 30 seconds, annealing – 45 °C for 30 seconds – and extension – 72 °C for 1 minute. The final extension was carried out at 72 °C for 5 minutes. DNA samples extracted from HSV-2 particles, with JETQUICK® protocol for the purification of DNA from up to 1 mL of whole blood ^[53], were used as PCR positive controls. PCR products were visualized by electrophoresis in a 0.7 % agarose gel in TBE with 0.005 % EtBr using 1Kb Plus DNA Ladder (Gibco) as a molecular weight marker. The expected amplified fragment size should be about 500 bp ^[54].

To further detect and quantify the amount (copy number) of virus DNA, in treated and non-treated cells, 2 ng – in 10 μ of each DNA sample were subjected to real-time PCR (qPCR). qPCR was performed with HSV1 HSV2 VZV R-gene[®] kit from ARGENE[®] [55] in an Applied Biosystems™ 7500 Instrument, according to manufactures instructions - except in the primers addition step, where only HSV-2 amplification premix was added to the reactions.

3. Results

3.1. Cytotoxicity assays

Results shown were obtained through the MTT colorimetric assay.

3.1.1. Determination of the extract CC_{50} and MNCC

Cell viability (Fig. 2, Tab. 1 and Tab. S. 1 in supplementary data) was evaluated by comparison of measured absorbance in wells with treated cells – maintained in 2 % FBS medium with different extract concentrations - and control cells – maintained in 2 % FBS medium without extract. Cytotoxic concentration that destroys 50 % of the cells (CC_{50}) and maximum non-cytotoxic concentration (MNCC) were calculated through linear regression of the dose-response curve.

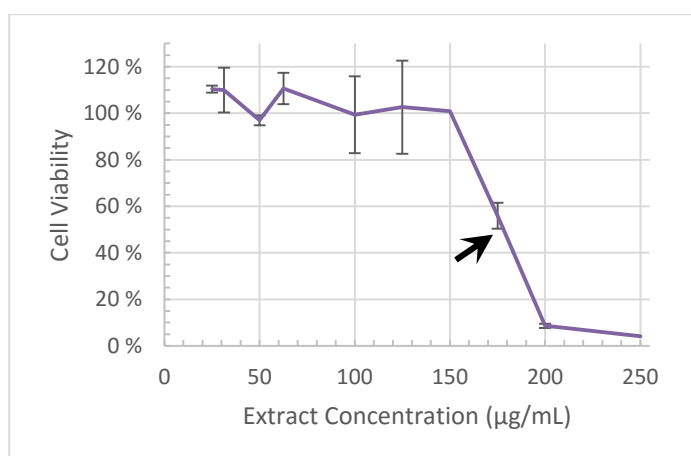


Fig. 2 - Graphic representation of the extract cytotoxicity. Values represent the mean \pm SD of four independent experiments. The black arrow indicates the CC_{50} .

Extract Concentration ($\mu\text{g/mL}$)	% Cell Viability
250	4.4 \pm 1
200	8.1 \pm 2
*175	*55.9
150	100.9 \pm 20
125	102.7 \pm 17
100	99.4 \pm 4
62.50	110.6 \pm 2
50	98.26 \pm 6
31.25	110.0 \pm 1
25	114.8 \pm 19

Tab. 1 – Cell viability percentages obtained in MTT assays post 24 or 48 hours of incubation with the extract. Values represent the mean \pm SD of two independent experiments (except *) with 4 replicates.

According to the calculations the extract CC_{50} and MNCC are 177.3 and 150.3 $\mu\text{g/mL}$, respectively.

3.1.2. Correlation between FBS concentration in the culture medium and cytotoxic effect of the extract

To investigate possible dissimilarities of the extract cytotoxic effect when it is diluted in medium with different serum concentrations, sub-confluent cell cultures were treated with different extract concentrations in medium with different FBS concentrations - 0, 2 or 10 % - and controls (cultures maintained in medium with the same FBS concentrations, without extract). The extract exhibited extremely variable cytotoxicity (Fig. 3, Tab. 2) with variations in the FBS concentration in the culture medium.

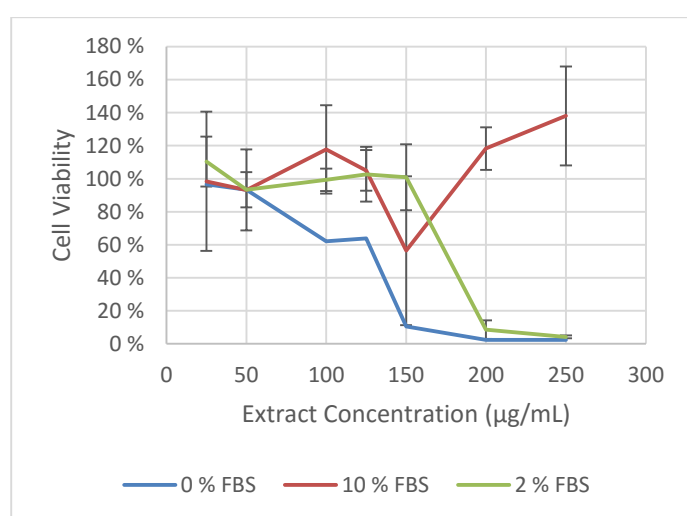


Fig. 3 - Graphic representation of the extract cytotoxicity in different FBS concentrations. Values represent the mean \pm SD of 1, 3 and 4 experiments – respectively to 0, 10 and 2 % FBS culture medium – with 4 replicates.

Extract Concentration ($\mu\text{g/mL}$)	% Cell Viability		
	0 % FBS	10 % FBS	2 % FBS
250	2	138 \pm 30	4 \pm 1
200	3	118 \pm 13	9 \pm 6
150	11	56 \pm 45	101 \pm 20
125	64	105 \pm 12	103 \pm 17
100	62	118 \pm 27	99 \pm 7
50	93	93 \pm 24	93 \pm 11
25	97	98 \pm 42	110 \pm 15

Tab. 2 - Cell viability percentages obtained in MTT assays after 24 or 48 hours of incubation with the extract diluted in culture medium with 0, 2 or 10 % FBS.

Extract CC_{50} and MNCC in medium with 2 % FBS were 177.3 and 150.3 $\mu\text{g/mL}$, respectively, and 123.2 and 21.2 $\mu\text{g/mL}$ in serum free medium. These values could not be determined in the assays with 10 % serum medium.

3.2. Virucidal effect

Virucidal effect results were obtained by titration of previously treated (with 100 $\mu\text{g/mL}$ of extract) and control viral suspensions and comparison of the obtained titers. As noticeable in Fig. 4 and Tab. S. 2 (in supplementary data) the extract revealed no meaningful effect against viral particles outside the host cell, with a 3.44 ± 2.37 % (mean of 3 experiments) reduction on the virus titer of treated suspensions – 7.67×10^6 pfu/mL – relatively to non-treated ones – 7.94×10^6 pfu/mL.

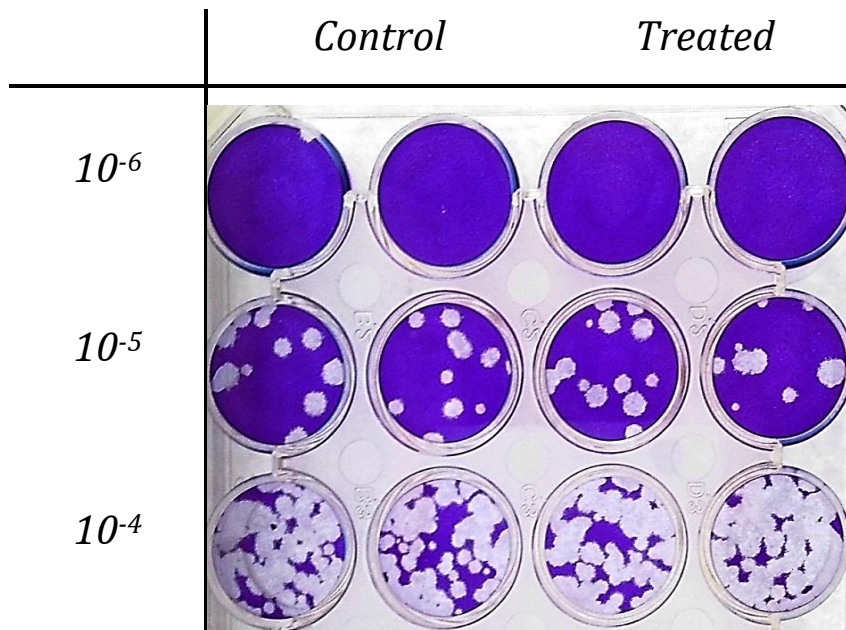


Fig. 4 – HSV-2 suspensions – treated and control - titration results.

3.3. Extract effect on viral plaques formation

In order to test the extract effect on HSV-2 plaque formation, cell cultures, grown in 24 or 6 well plates were infected with serial dilutions of the virus. After the viral adsorption and entry periods, the inoculums were removed and 2 % FBS culture medium – with or without 100 µg/mL of extract – was added to treated and control wells. Following the treatment period, 2 % Sephadex titration medium was added to each well.

The extract inhibitory effect was evaluated by comparison of treated and control titers and plaques' diameters.

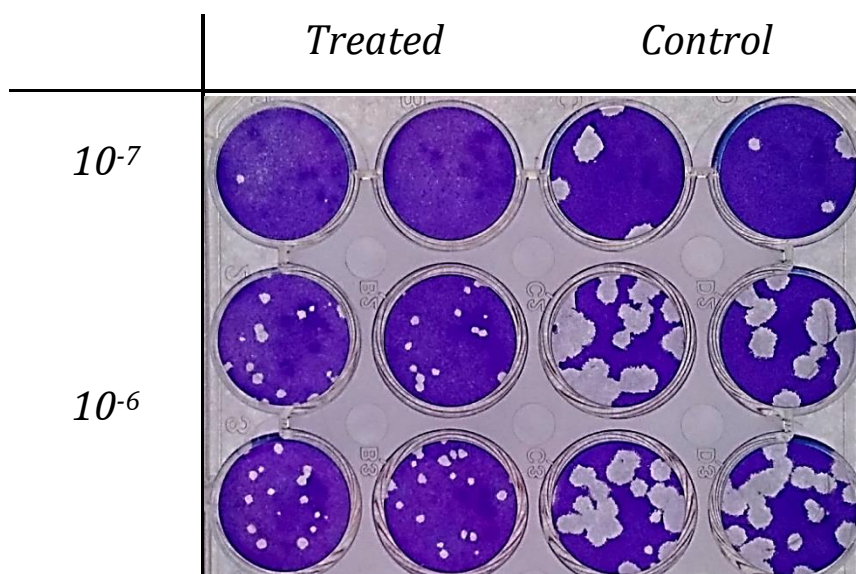


Fig. 5 - HSV-2 suspensions – treated and control - titration results.

When added during titration the extract exhibit a 71.6 ± 26.8 % (mean of 3 experiments, see Tab. S. 3 in supplementary data) reduction in titer and a visible decrease in plaque diameter (Fig. 5) of treated versus control conditions.

3.4. Extract effect on virus yield

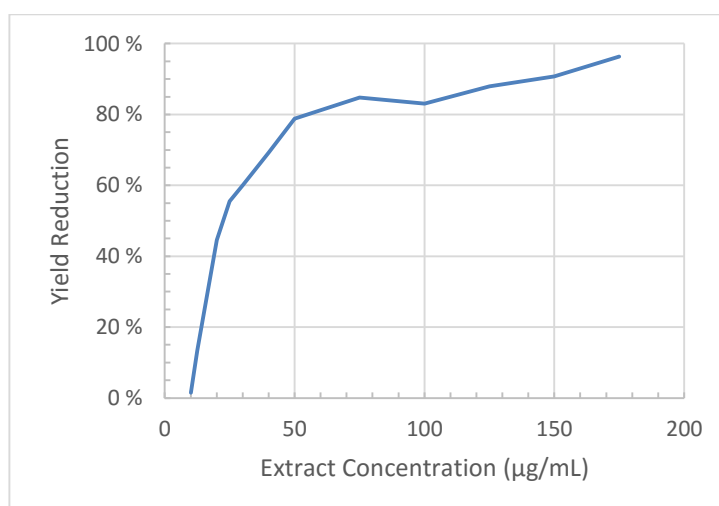
Several experiments, involving HSV-2 production under treatment, were performed, to uncover the extract effect on virus yield and the involved infection stages.

It's noteworthy that a mean yield reduction of 93.65 ± 8 % was calculated using data obtained in 13 experiments (see Tab. S. 4 in supplementary data) performed in "standard" treatment conditions – 2 % FBS medium with 100 µg/mL of extract at 0.5 h p.i. during 2 to 4.5 hours.

3.4.1. IC₅₀ and IC₉₀ determination

To determine the extract IC₅₀ and IC₉₀, HSV-2 was produced in culture medium with different extract concentrations, and controls were produced in culture medium without extract. Produced suspensions were then titrated, and viral titers (in treated and control cultures) were compared to obtain the percentage of yield reduction attributed to each concentration (Tab. 3, Fig. 6). Concentration that reduces viral yield in 50 % (IC₅₀) and concentration that reduces viral yield in 90 % (IC₉₀) were calculated through linear regression of the dose-response curve.

Concentration (µg/mL)	% Yield Reduction
175	96.34
150	90.77
125	87.99
100	83.03
75	84.76
50	78.86
40	69.23
30	60.00
25	55.56
20	44.62
12.5	13.45
10	1.54



Tab. 3 - HSV-2 yield reduction percentage when cell cultures were treated with different extract concentrations.

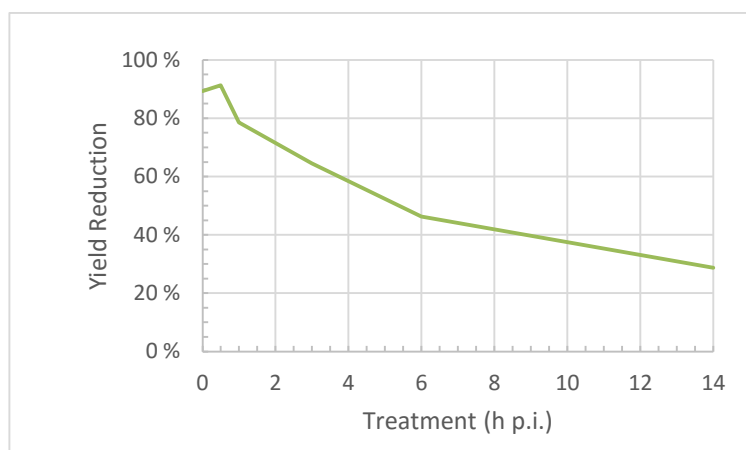
Fig. 6 - Graphic representation of HSV-2 yield reduction when cell cultures were treated with different extract concentrations. Values were obtained in one experiment.

According to the calculations the extract IC₅₀ and IC₉₀ are 35.1 and 128.1 µg/mL, respectively.

3.4.2. Determination of the affected stage of virus replication

HSV-2 was produced under treatment conditions - in culture medium with 100 µg/mL of extract – with different treatment starting times, and a control suspension was produced in culture medium without extract. Yield reduction percentage was determined by titration of the produced virus suspensions and comparison of the titers obtained in treated and control conditions.

Treatment Start (h p.i.)	% Yield Reduction
0.5	91.27
1	78.55
3	64.53
6	46.27
14	28.74



Tab. 4 - HSV-2 yield reduction percentage at different treatment starting times (h p.i.).

Fig. 7 - Graphic representation of HSV-2 yield reduction by the extract (100 µg/mL) in different treatment starting points. Values were obtained in one experiment.

There is a noticeable decrease (Tab. 4, Fig. 7 and Tab. S. 4 in supplementary data) of the extract effectiveness when added between 3 to 6 h p.i. and no effect when the treatment is started at 15 h p.i. or later (data not shown).

3.4.3. Comparison with ACV inhibition

To evaluate the mechanisms which mediate the virus yield reduction by the aqueous extract, HSV-2 was produced under treatment conditions with both compounds – aqueous extract and ACV – at the same concentration – 100 µg/mL. Control suspensions were produced in culture medium without extract. Each compound yield reduction percentage was determined by titration of the produced virus suspensions and comparison of titers obtained in treated and control conditions.

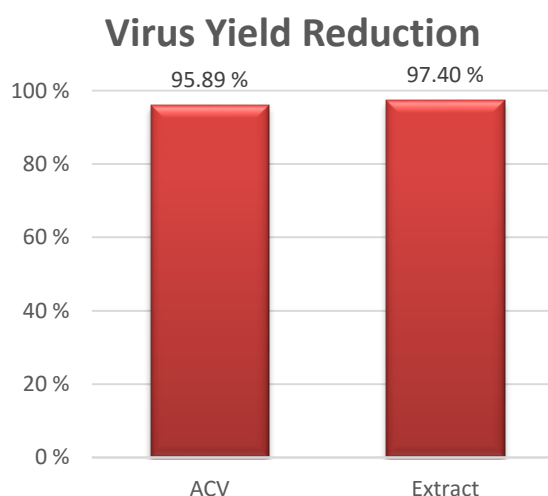


Fig. 8 - Graphic representation of HSV-2 yield reduction by the extract and ACV (100 µg/mL). Values were obtained in one experiment with 2 replicates.

Fig. 8 shows HSV-2 yield reduction percentages in virus produced under treatment with ACV – 95.89 % - or *S. virgaurea* aqueous extract – 97.40 % - , relatively to non-treated virus. The extract exhibited a very similar inhibition to the one observed in ACV treatment.

3.4.4. Visualization of treated and non-treated infected cells and virus suspensions by Transmission Electron Microscopy (TEM)

Cell cultures were infected with HSV-2 and treated with 2 % FBS culture medium with or without 100 µg/mL of extract. Infected cells were then collected at 14 hours post infection and observed by TEM. Treated and control viral suspensions were concentrated and observed by negative staining.

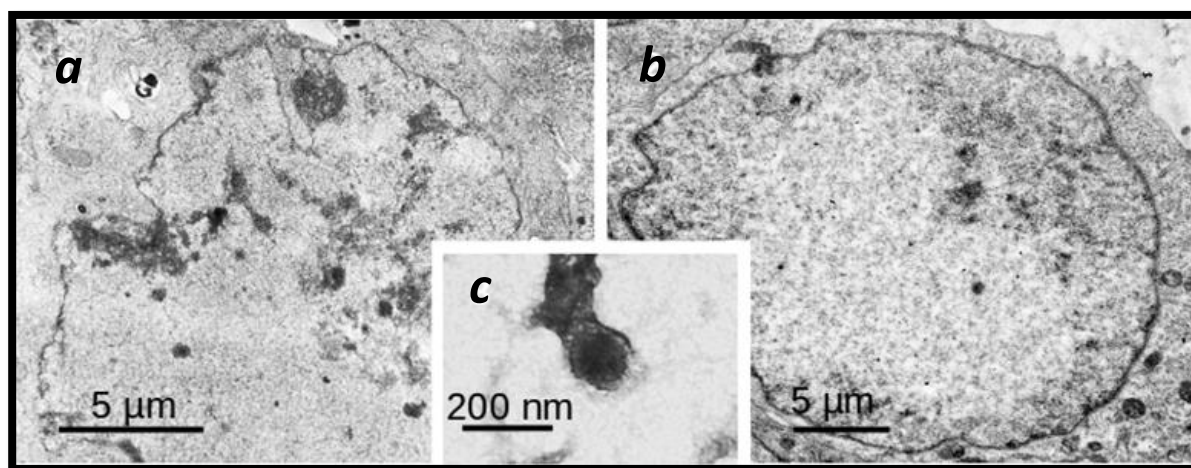


Fig. 9 – a-b) Thin section TEM micrographs of HSV2 infected Vero cells processed for TEM at 14 hours p.i. a) Non-treated (control). b) Cells treated with 100 µg/mL of the extract. c) HSV-2 virus particle negatively stained with 1 % aqueous uranyl acetate. Photos were kindly provided by Dr. A.P. Alves de Matos.

Negative staining showed a highly reduced amount of virus produced under treatment, as compared to controls, with no significant morphogenetic defects (Fig. 9 c), while fine sections with treated infected cells (Fig. 9 b) exhibited fewer morphological deficiencies, due to infection, than control infected cells (Fig. 9 a).

Infection Time (hours)	DNA concentration (ng/µL)	
	Treated	Control
3	26.35	20.34
4	24.18	19.41
5	22.39	25.93
6	22.40	26.74
7	34.24	31.25

Tab. 5 - Infected cells - treated and non-treated - DNA quantification.

3.4.5. HSV-2 Infection Kinetics

DNA was extracted from Vero cell cultures infected with HSV-2 – in treatment and control (non-treatment) conditions – at 14 hours post-infection (h p.i.) and was observed by gel electrophoresis (Fig. 10). DNA concentration of

each sample was determined (Tab. 5) using a NanoDrop® and was equalized to 19.41 ng/μL in every sample, with DNase free water. PCR was performed to 26 ng of each sample, in order to verify the presence of HSV-2 DNA.

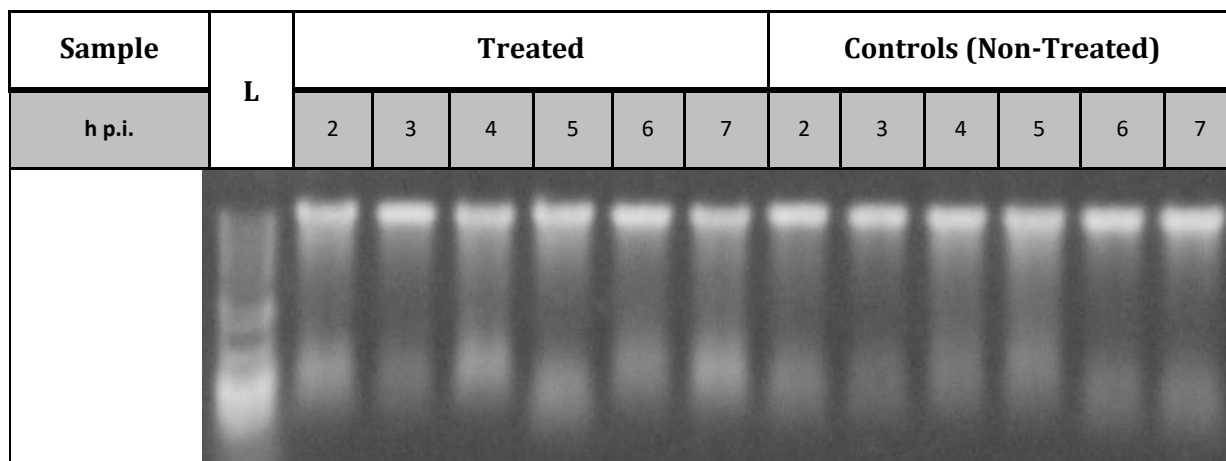


Fig. 10 – DNA from treated and non-treated infected cells (from 2 to 7 h p.i.) DNA observed through gel electrophoresis.

All extracted DNAs showed similar bands when observed in gel electrophoresis (Fig. 10), indicating that only cellular DNA is visible.

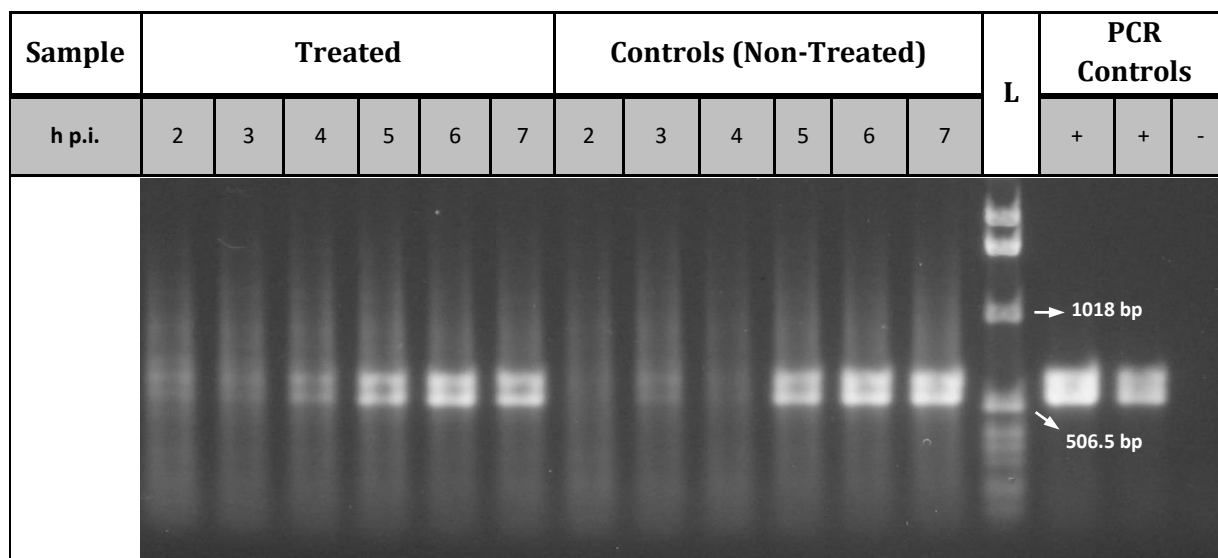


Fig. 11 – Results of PCR for the detection of HSV-2 DNA in infected cells – treated and controls – at different times post infection. White arrows indicate the size of two molecular weight marker bands that surround the PCR product bands.

Although no differences are evident between treated and non-treated PCR products (Fig. 11) this experiment shows that, in the applied experimental conditions, viral DNA replication is started between 4 and 5 h p.i.

The PCR products visualization (Fig. 11) showed that the amplified fragment size was slightly above 506 bp but under 1018 bp, which fits the expected size.

3.4.6. Quantitative real-time PCR (qPCR)

To evaluate possible differences between the amounts of viral DNA in treated and non-treated cell cultures at different hours post infection, qPCR was performed with 2 ng of the extracted DNA samples (Fig. 10).

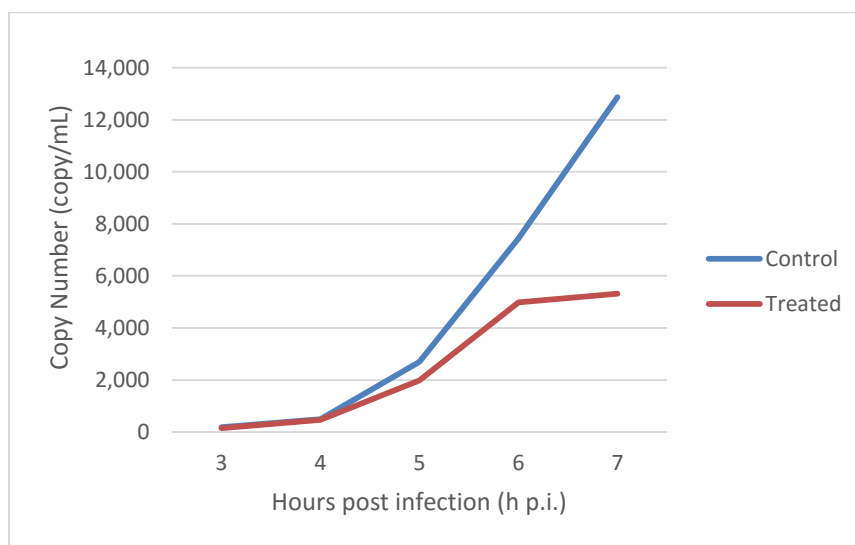


Fig. 12 - Quantification of HSV-2 DNA (copy/mL) in infected cells (treated and non-treated) at different hours post infection (h p.i.).

While at the beginning of infection – 3-5 h p.i. – viral DNA quantities are similar with or without treatment, it's noticeable a slower increase of the copy number in treated relatively to non-treated samples, that gets more evident as the infection progresses.

4. Discussion

4.1. Extract cytotoxic effect

When evaluating a product effect against a species or class of micro-organisms it's important neither to overlook the incidence of false-positives nor to discriminate aspecific cell toxicity ^[56]; this can be solved by inclusion of a parallel evaluation of the product effect on host cell lines (cytotoxicity evaluation) ^[57].

The lyophilized extract was diluted in DMSO, one of the most frequently used solvents to prepare stock solutions, once it eliminates microbial contamination and assures good solubility during the serial dilution procedures. It's important to notice that DMSO is potentially toxic for cells and some microorganisms ^[56]. In fact, DMSO has shown anti-herpetic activity for concentrations above 2 % ^[58]. While previous studies have verified 100 % cell viability for DMSO concentrations under 1 % ^[56, 58] a 2012 ^[19] work showed, through microscopic observation, that DMSO concentrations higher than 0.5 % cause cellular modifications. Considering this, all tested extract concentrations were correspondent to a lower than 0.5 % concentration of DMSO, to prevent false positives in both cell viability and anti-viral activity assays.

Extract effect in Vero cells viability was evaluated by sub-confluent cell cultures exposition to different extract concentrations, at adequate experimental conditions for viral infection – 2 % FBS culture medium and 24 or 48 hours incubation at 37 ° C. The determined CC₅₀ and MNCC were 177.3 and 150.3 µg/mL under these conditions (Fig. 2), but varied critically when differences in the FBS concentration in the culture medium were introduced, showing greater susceptibility in serum free culture medium with a CC₅₀ of 123.2 µg/mL and a MNCC of 21.2 µg/mL (Fig. 3).

This data shows that, when determining an extract or compound effect on the viability of a virus host cell, experimental conditions should be the same as in virus inhibition experiments. However, some antiviral studies lack to refer the serum concentration used in cytotoxicity assays ^[20, 59-61] and others describe the utilization of high FBS concentrations ^[21], what may lead to the employment of cytotoxic concentrations in antiviral activity experiments.

4.2. Virucidal effect

Extract direct effect on virus particles was evaluated through the treatment - in non-cytotoxic concentrations - of HSV-2 suspensions for 1 to 3 hours, at room temperature, with stirring.

While previous studies involving this plant aqueous extract ^[17, 19] concluded that it exhibited strong virucidal effect on HSV-2 particles, in the present study it showed irrelevant virucidal effect, since treated virus titer reduction relatively to non-treated virus was only 3.44 ± 2.37 % and viral plaques' dimensions were similar (Fig. 4). It's important to notice that this dissimilarity is probably due to the utilization of different batches of extract in the different studies, obtained from plants harvested in different years. This shows the discrepancies faced when working with natural products, once the compounds produced by plants may vary considerably, depending on environmental factors.

Further virus production experiments, aiming to analyze the affected stage of the replicative cycle stage during treatment, also support the inexistent virucidal activity of the extract, as when the extract is added at 15 h p.i. or later, and kept until the end of infection, yield reduction is null (data not shown). This suggests that, although viral particles remain several hours in contact with the extract outside host cells, it exerts no effect on them showing no virucidal effect.

4.3. Extract effect on plaque formation

When added during titration - in non-cytotoxic concentrations - the extract showed minor and more dissimilar - 71.6 ± 26.8 % - titer reduction of treated relatively to non-treated infected cells than when treatment was applied during viral production. This is probably due to the fact that a percentage of the cells are still capable of productive infection, yet with lower productions; this may also explain the smaller plaques observed in treatment conditions (Fig. 5): a lower number of produced particles will infect a lower number of neighbor cells in successive replication cycles.

These results are consistent with a 2014 study ^[17] where the extract showed 56 % inhibition and led to the production of smaller plaques when added to HSV-2 titration at 100 µg/mL, and a 2012 work ^[19] where it exhibited 86 % inhibition at 300 µg/mL.

4.4. Extract effect on virus yield

Treatment - in non-cytotoxic concentrations - was extremely effective when performed during virus production, with a mean yield reduction of $93.65 \pm 8\%$ of treated versus non-treated infected cells. Yet, viral plaques were morphologically normal, comparatively to controls.

When evaluating a product antimicrobial proprieties by means of *in vitro* models the activity of extracts or compounds is generally expressed by numeric values - IC_{50} , IC_{90} , etc. – but for a correct interpretation of the results a profound knowledge of the model and used protocol is required. Yet, stringent endpoint criteria can be defined, being relevant antimicrobial activity set for IC_{50} values below 100 $\mu\text{g}/\text{mL}$ for natural extracts ^[56]. *Solidago virgaurea* aqueous extract IC_{50} – in treatment during virus production - was 35.1 $\mu\text{g}/\text{mL}$, which fits the values described as common endpoints in antimicrobial activity.

The selectivity index (SI) is also an intrinsic component of any antiviral testing and refers to the ratio of the maximum drug concentration causing either 50 % or 90 % inhibition of growth of normal cells - CC_{50} , CC_{90} - and the minimum drug concentration at which 50 % or 90 % of the virus is inhibited - IC_{50} , IC_{90} ^[56]. Accordingly, the extract SI is 5.1 and its noteworthy that this result was founded in cytotoxicity experiments performed with 2 % FBS culture medium, while many other studies ^[20, 59-61] use 10 % FBS media, which allows the utilization of much higher product concentrations without cell death, incurring in possible results adulteration.

Furthermore, experiments were performed where treatment was started at different times post infection, in order to investigate at which HSV-2 replicative cycle stage does the extract exert its inhibitory effect. Results (Tab. 4, Fig. 7) revealed a decrease in the inhibition percentage from treatments started at 30 minutes post infection where yield reduction was about 90 %, until treatments started at 14 hours post infection where yield reduction dropped to 28.74 %. When added at 15 h p.i. or later the extract showed no effect on virus yield. This suggests that the extract may apply its inhibitory effect in one or more earlier stages of the infection but after virus adsorption, once the effect drops to about 50 % the treatment is started between 3 and 6 hours post infection (Fig. 7). These stages may include: nucleocapsid transport to host nuclear pores, viral DNA release into the nucleus, *immediate early* and/or *early* genes expression and DNA replication ^[28].

Once acyclovir is known to inhibit HSV DNA replication ^[40-42], an experiment was performed using parallel treatments with ACV and *S. virgaurea* aqueous extract, in order to compare both inhibitory effects in virus progeny yield. Results (Fig. 8) shown a very similar inhibition, even a little higher by the aqueous extract, but it's important to highlight that this result was obtained in only one experiment, although with two replicates for each treatment. A manifest difference between the two products is

the length of the treatment that is required to achieve maximum inhibition; while ACV presence is obligatory throughout the entire infection - once it is removed viral replication may carry on - extract treatment may be as short as 2 hours (data not shown). This may indicate that only earlier HSV-2 replication stages are affected and/or that some components of the extract, namely the ones that display antiviral activity, remain inside host cell when culture medium is removed.

To evaluate possible morphological modifications among treated and non-treated infected cells, an experiment where treated and non-treated infected cell cultures were collected at 14 hours post infection and subsequently observed by TEM was performed. Morphological differences between treated and control infected cells were very evident (Fig. 9 *a* and *b*) as control cells exhibited significantly greater damage, mainly in the nuclear envelope, due to infection than treated cells, suggesting that infection was impaired by the treatment.

Virus particles produced under treatment were also compared with controls by TEM visualization, in an experiment where large amounts of HSV-2 were produced, in T75 flasks, with and without treatment and were subsequently concentrated. The concentrated viruses were then negatively stained with uranyl acetate and counted through TEM. Observations showed that the number of viral particles was severely reduced – about 1 % - in suspensions produced in treatment conditions – 100 µg/mL of aqueous extract - relatively to control suspensions. However, HSV-2 particles produced under treatment displayed no morphological deficiencies (Fig. 9 *c*). This indicates that the extract inhibitory activity leads to an actual reduction in HSV-2 progeny yield, rather than leading to production of deficient particles, or other possible inhibitory situation.

In order to further evaluate the extract action mechanism, a HSV-2 infection kinetics was executed, where infected cell cultures - treated and non-treated – were collected from 2 to 7 hours post infection. Each infected cell culture DNA was then extracted and observed in gel electrophoresis (Fig. 10), all DNA samples presented similar bands, even in later times into the infection, hence only cellular DNA was visible in total DNA gel electrophoresis.

Extracted DNAs were then quantified and equalized to the same DNA concentration – 19.41 ng/µL. A PCR for the detection of HSV-2 DNA was then performed with about 25 ng of each total DNA extracted and PCR products were observed through gel electrophoresis. While PCR results (Fig. 11) did not supply any informative data about the extract inhibitory activity on viral DNA replication - once results were quite similar in treated and non-treated samples - they allowed the determination of the time post infection at which viral DNA replication occurs, in the applied experimental conditions. This information is very relevant to future experiments planning, and interpretation of the obtained results. For example, when the beginning of the treatment is delayed to 6 h p.i. the yield reduction drops to

less than 50 %, which is consistent with HSV-2 infection kinetics results, as at 5 h .p.i. it's noticeable a major increase in the amount of viral DNA present in the infected cells, which reveals that HSV-2 DNA replication stage is already in an advanced phase at that time.

Accordingly when the treatment is started after viral DNA replication it loses much of its efficiency, suggesting that this could be the affected process by the aqueous extract when exerting its antiviral activity. To further investigate possible divergences between the HSV-2 DNA replication in treated and non-treated cells the same DNA samples were used for real-time PCR to detect and measure the amount of viral DNA present in infected cells at the several times post infection – 3 to 7 h p.i. The results (Fig. 12) revealed that in shorter infection times – 3 to 5 h p.i. – no major differences can be observed in the amount of viral DNA present in treated and control cells but, as the infection progresses, its noticeable that the amount of DNA increases in a severely slower rate in treated relatively to non-treated cells. This may indicate that HSV-2 DNA replication may be impaired when *S. virgaurea* aqueous extract is present in the culture medium during infection.

The overall conclusion of this work is that when infected Vero cells, with HSV-2, are treated with *S. virgaurea* aqueous extract during early infection stages - 0.5 to 5 hours post infection - the virus progeny yield is significantly reduced. Moreover, it was evident that viral DNA replication is severely impaired when HSV-2 infection occurs under treatment conditions. This antiviral effect may result from the inhibition of viral DNA polymerase activity by one or more extract components, leading to the termination of HSV-2 DNA replication.

4.5. Further Perspectives

Further works should focus on the investigation of possible differences in the processes that are part of the infection early stage, in HSV-2 macromolecules (RNA, proteins, or DNA) produced under treatment comparatively to non-treated suspensions, to assess early genes expression and viral DNA replication.

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6. Supplementary Data

6.1. Extract Cytotoxic Effect

Concentration ($\mu\text{g/mL}$)	% Cell Viability			Standard Deviation
	Experiment 1 ^a	Experiment 2 ^b	Mean	
250	5.15	3.68	4.42	1
200	6.66	9.60	8.13	2
175	-	55.92	55.92	-
150	86.71	115.04	100.87	20
125	114.36	90.93	102.65	17
100	102.08	96.63	99.36	4
62.5	109.13	112.15	110.64	2
50	102.16	94.37	98.26	6
31.25	111.04	108.93	109.98	1
25	127.92	101.65	114.79	19

Tab. S. 1 – Cell viability percentages obtained in MTT assays post 24 (a) or 48 (b) hours of incubation with the extract and standard deviation. Cell viability percentages were calculated relatively to controls (non-treated). Each value is the mean of 4 replicates. The mean of the two experiments and respective standard deviation is shown.

6.2. Extract Virucidal Effect

		Experiment 1	Experiment 2	Experiment 3	Mean	SD
Treated	pfu/mL	1.00×10^7	1.04×10^7	2.64×10^6	7.67×10^6	-
Control	pfu/mL	1.05×10^7	1.05×10^7	2.82×10^6	7.94×10^6	-
Inactivation	%	4.35	1.72	6.45	3.44	2.37

Tab. S. 2 - Treated - with 100 $\mu\text{g/mL}$ of aqueous extract – and control (non-treated) HSV-2 suspensions titers. Inactivation percentages were calculated relatively to controls.

6.3. Extract effect on viral plaques formation

		Experiment 1	Experiment 2	Experiment 3	Mean	SD
Treated	pfu/mL	8.03×10^7	1.43×10^8	5.36×10^8	2.53×10^8	-
Control	pfu/mL	1.06×10^9	7.00×10^8	9.09×10^8	8.90×10^8	-
Inhibition	%	92.4	79.6	41.0	71.6	26.8

Tab. S. 3 – Results of three titration experiments where infected cultures were treated - with 100 µg/mL of aqueous extract - at 0.5 h p.i. and 2 % Sephadex medium was added 4 h p.i.. Inhibition percentages were calculated relatively to controls.

6.4. Extract effect on virus yield production along infection period

Experiment	% Yield Reduction	Treatment Period (h p.i.)
1	98.86	0.5 - 24.0
2	77.78	0.5 - 3.5
3	97.67	0.5 - 3.0
4	79.35	0.5 - 2.0
5	78.55	1.0 - 24.0
6	76.32	0.5 - 2.0
7	91.71	0.5 - 2.5
8	46.27	6.0 - 24.0
9	81.54	0.5 - 16.0
10	28.74	14.0 - 24.0
11	89.88	0.5 - 5.0
12	91.27	0.5 - 24.0
13	97.40	0.5 - 24.0
14	64.53	3.0 - 24.0
15	83.90	0.5 - 2.5
16	93.97	0.5 - 2.5
17	98.93	0.5 - 2.0

Tab. S. 4 – HSV-2 yield reduction percentage of treated - with 100 µg/mL of aqueous extract – relatively to non-treated virus suspensions at different treatment periods (h p.i.).