Anti-viral activities of *Ficus benjamina* and *Lilium candidum.*

Thesis submitted in partial fulfillment of the requirements for the degree of "DOCTOR OF PHILOSOPHY"

b**y**

Ludmila Yarmolinsky

Submitted to the Senate of Ben-Gurion University of the Negev

Date…20.11.2012……

Beer-Sheva

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Research-Student's Affidavit when Submitting the Doctoral Thesis for Judgment

I___Yarmolinsky Ludmila_____, whose signature appears below, hereby declare that
(Please mark the appropriate statements):

_V__ I have written this Thesis by myself, except for the help and guidance offered by my Thesis Advisors.

_V__ The scientific materials included in this Thesis are products of my own research, culled from the period during which I was a research student.

___ This Thesis incorporates research materials produced in cooperation with others, excluding the technical help commonly received during experimental work. Therefore, I am attaching another affidavit stating the contributions made by myself and the other participants in this research, which has been approved by them and submitted with their approval.

Date: 20.11.2011 Student's name: Yarmolinsky L. _ Signature:_____

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Abbreviations and terms

ACV- acyclovir

ALT - alanine transaminase

ATCC - the American Type Culture Collection

CC_{50} is the concentration found to cause 50% toxicity

CMC – carboxymethyl cellulose

CMV – Cytomegalovirus

CoxB3 – Coxsackievirus B3

CPE - cytopathic effect

EBV - the Epstein-Barr virus

EC_{50} is concentration found to provide 50% prevention of plaque forming units

FFU- focus forming units

FluA – Influenza Virus Type A

HHV-6 -Human herpesvirus 6

HHV-7 - Human herpesvirus 7

HHV-8 - Human herpesvirus 8

HPLC – High performance liquid chromatography

HSV-1- Herpes Simplex Virus -1

HSV-2- Herpes Simplex Virus -2

HTLV-1- Human T-lymphotic Virus

LC- MS - Liquid chromatography-mass spectrometry

m.o.i. - multiplicity of infection

MS - mass spectrometry

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

MuLV - Moloney Murine Leukemia Virus

MuSV- Murine Sarcoma Virus

NBCS- Newborn calf serum

NIH/3T3 cells -mouse fibroblast cells

NMR -Nucleic Magnetic Resonance

p.i. – post infection

PUF - plaque forming units

Rpm – revolutions per minute

RPMI – a medium was developed by Moore *et al*, at Roswell Park Memorial Institute

RSV- Respiratory syncytial Virus

SDS –Sodium Dodecyl Sulfate

SI- Selectivity index

VZV - Varicella-Zoster Virus

Abstract

Medicinal plants have been used in medicine since ancient times and are well known for their antiviral activity. Although several hundreds of plants have been studied as novel antiviral agents, many others still need investigation.

Viral infections remain one of the most important public health problems worldwide. For example, about 80% of the adult population in the USA are infected by or are sero-positive for HSV-1. In addition, despite the availability of effective antiviral drugs such as acyclovir(ACV), a nucleoside derivative which is effective against various members of the herpes family viruses, still there is an urgent need for novel anti-viral agents with high efficacy and low toxicity. In the case of herpes viruses there is a need for anti-viral agents with a different mode of action than ACV and other nucleoside derivatives due to the rapid development of resistant mutants to these existing treatments. Isolation and identification of plant antiviral agents is always a challenging task because bioactive compounds often occur as complex mixtures with other secondary metabolites.

Although viral infections are linked to at least 15% of all malignant tumors in humans, very few publications are devoted to the activity of higher plants against viruses causing cancer.

The main objective of this study is to evaluate and identify plant substances with potent antiviral activity. The specific aims are:

1. Screening and evaluation of antiviral activities of different extracts from various potential plants against members of the herpes virus family and retroviruses.
2. Isolation and identification of antiviral agents from selected plant species.
3. Elucidation of the antiviral mechanisms of the plant extracts.

In this study, several different plant extracts from various parts (leaves, stems, fruits, bulbs, petals) of *Ficus benjamina*, *Lilium candidum*, *Callissia fragrans* and *Simnondsia chinensis* were tested in vitro in order to estimate their antiviral activity against Herpes Simplex Virus -1 and 2 (HSV-1, HSV-2), and Varicella-Zoster Virus (VZV). We hypothesize that plants which are resistant to various plant viruses produce antiviral compounds, which in turn could also inhibit animal and human viruses. *F. benjamina* is not susceptible to various plant viruses unlike other decorative *species of Ficus.* Other plants (*L. candidum, C. fragrans* and *S. chinensis*) were

chosen for estimation because of their rich folkloric reputation as antiviral plants, and to the best of our knowledge, no previous scientific reports are available which deal with their antiviral properties.

F. benjamina and *L. candidum* ethanol leaf extracts were the most effective, so further study was focused on them. These extracts and their fractions were tested *in vitro* against HSV-1, HSV-2, VZV, Murine Sarcoma Virus (MuSV) and Moloney Murine Leukemia Virus (MuLV).

Ethanol leaf extracts of *F. benjamina* inhibited all studied viruses, whereas those of *L. candidum* inhibited only HSV-1 and HSV-2. Bioassay guided fractionation of the ethanolic extract of *F. benjamina* leaves led to the isolation of three flavone glycosides from the 80 %-MeOH fraction ; (1) quercetin 3-O-rutinoside , (2) kaempferol 3-O-rutinoside and (3) kaempferol 3-O-robinobioside with high antiviral activity against HSV-1and HSV-2. These flavones were highly effective against Herpes Simplex Virus 1 (HSV-1) with selectivity indexes (SI‘s) of 266, 100 and 666 respectively, in comparison with their aglycons, quercetin (with SI of 7.1) or kaempferol (with SI of 3.2). Kaempferol 3-O-robinobioside (3) exhibited a similar SI to that of ACV. The structure of the compounds was determined by spectroscopic analyses including NMR and MS.

The fraction eluted with 20%- MeOH (polysaccharide fraction) significantly inhibited VZV, MuSV and MuLV, whereas the fraction eluted with 60%- MeOH (polyphenol fraction) significantly inhibited only MuSV and MuLV.

It was determined that antiviral properties of *L. candidum* against herpes viruses are explained by the presence of kaempferol.

The high efficacy and low toxicity of *F. benjamina* extract and its effective fractions and compounds may suggest that this plant and probably other plants can be applied as a potential natural resource for production of antiviral and anticancer compounds.

Keywords*: Ficus benjamina, Lilium candidum,* HSV-1, HSV-2, VZV,. retroviruses, flavonoids.

1. Introduction

There are innumerable potentially useful medicinal plants waiting to be evaluated and exploited for therapeutic applications against different viruses. A wide variety of various bioactive constituents of different structures, including flavonoids, terpenoids, lignans, sulphides, polyphenolics, coumarins, saponins, furil compounds, alkaloids, polyines, thiophenes, different sugars, proteins and peptides and more have been identified from plants (Jassim and Naji, 2003, Saladino *et al,* 2008, Haque *et al,* 2011).

Although several hundreds of plants considered to contain potentially novel antiviral agents have been studied, many others still need investigation. According to Brower (2008), 50.000-70.000 plant species are thought to have medicinal qualities. However, few plant species have been scientifically evaluated for their possible medical application. Safety and efficacy data supported by experimental or clinical research are available for even fewer plants, their extracts and active ingredients (Rosenbloom *et al,* 2011).

Viral infection remains an important health problem worldwide and the control of viral diseases is the subject of constant scientific endeavor. Appearance of viral strains resistant to antiviral agents is an emerging problem (Stranks *et al,* 2005). As a consequence, there are only few antiviral drugs available for the treatment of viral diseases. Therefore, the search for more effective antiviral agents is a necessary and highly desirable task (De Clereq, 2004).

Although acyclovir (ACV) and other nucleoside derivatives have been approved for therapeutic use against Herpes Simplex Virus -1 (HSV-1), Herpes Simplex Virus -2 (HSV-2} and Varicella-Zoster Virus (VZV) worldwide (Brady and Bernstein, 2004), still the search for new effective anti-herpetic drugs is very important due to the following reasons: (a) The development of anti-acyclovir resistant herpes viruses mutants (Field and Biron, 1994). (b) These drugs have many side effects, such as nausea, vomiting, headaches, skin rash and diarrhea. (c) ACV is not highly effective in recurrent HSV attacks (Devrim *et al*, 2008).

Although viral infections are linked to at least 15% of all malignant tumors in humans (Butel, 2006), very few publications are devoted to the activity of higher plants against viruses causing cancer (Hankumar *et al*, 2008, Hankumar *et al*, 2009). No previous scientific reports about the

antiviral activity of higher plants against transforming retroviruses such as Murine Sarcoma Virus (MuSV) are available.

1.1. Herpes viruses (HSV-1, HSV-2 and VZV)

Herpes infection remains one of the most important public health problems. In the USA, about 80 of the adult population are infected by or sero-positive for HSV-1 and 35% for HSV-2 (Cassady and Whiteley, 1997). Over 90 % of adults in the USA have a serologic evidence of prior VZV infection (Whiteley and Gnann, 1999). In addition, the incidence and

severity of infections caused by HSV have increased over the last decade. The increase is due to a growing number of immunocomprised patients produced by aggressive chemotherapy regimens, a growing number of organ transplantations and a greater occurrence of human immunodeficiency to virus infections. Many people carry a latent herpes simplex virus in their nervous system. Herpes viruses can infect most if not all vertebrate animals and even lower organisms (Davison *et al*, 2005). Herpes zoster is a considerable cause of morbidity, especially in elderly patients, and can be fatal in immunosuppressed or critically ill patients (Wareham and Breuer, 2007).

Herpesviridae constitute a diverse family of viruses characterized by a large dsDNA genome containing between 70 and 150 genes, and posess the ability to enter a latent phase of infection of specific host cells (Schaffer, 1981, Subac-Sharpe and Dargan, 1998). Species of Herpes are classified into three different evolutionary subfamilies: 1) Alphaherpesvirinae (HSV-1, HSV-2, VZV), 2) Betaherpesvirinae (Cytomegalovirus (CMV), Human herpesvirus 6 (HHV-6), Human herpesvirus 7 (HHV-7)) and 3) Gammaherpesvirinae (the Epstein-Barr virus (EBV) and Human herpesvirus 8 (HHV 8)) (Field *et al,* 2006, Wyrwicz and Rychlewski, 2007).

The two types of herpes simplex virus (HSV-1 and HSV-2) share many characteristics, including DNA homology, antigenic determinants, tissue tropism and diseases symptoms. They infect their hosts both by lytic and latent infections, with, a replication cycle of about 15 hours (Jenkins and Turner, 1996). Both types infect epithelial cells in the lythic cycle. Type 1 is classically associated with cold sores, keratitis and encephalitis and caused recurrent attacks of «fever blisters". Type 2 primarily infects the genital mucosa and is mainly responsible for genital

herpes. Both viruses also cause neurologic disease, and can cause neonatal infections which are often severe. For example, infection of the brain leads to herpes encephalitis, a dangerous condition that causes permanent neurological damage with high mortality (Lachmann, 2003). HSV-1 was primarily responsible for infections "above the belt" (including brain disease in adults), whereas HSV-2 was responsible primarily for infections "below the belt" and brain disease in neonates (Rawls and Campione-Piccardo, 1981). This classical epidemiological paradigm has changed significantly in 21st century as HSV-1 became an increasingly frequent cause of genital herpes. Notably, recent studies indicate that either virus can infect the mouth or genital tract (Whitley, 2006).

The first critical step in primary HSV-1 infection is viral entry into the orolabial epithelial cells. Virus entry into the cells is mediated by the viral glycoproteins gB, gC, gD, gH and gL. After successful entry into the host cell, viral gene expression and replication ensues, followed by rapid cell-to-cell spread within the epithelium (Spear, 2004).

In order to establish latency, the virus enters non-dividing sensory neurons and replicates infrequently (Eshleman *et al*, 2011). Latency-associated transcripts are the only mRNAs that are expressed during this period, and these proteins serve to help the neuron survive initial infection. Latency-associated transcripts are also required to establish and maintain viral latency and for the virus to be reactivated from latency (Bystricka and Russ, 2005).

Although the adaptive immune response is present, the virus is occasionally reactivated and when this occurs, it anterogradely travels back to epithelial cells to form herpetic lesions (Mark *et al*, 2008, Hsia *et al,* 2011). Reactivation can be caused by numerous factors, including stress, ultraviolet light, heat, fever, hormonal changes, menstruation and physical trauma to the neuron (Jenkins and Turner 1996).

Primarily varicella-zoster virus (VZV) infection occurs when a susceptible individual, usually a child, is exposed to airborne virus via the respiratory route (Whiteley and Gnann, 1999). Varicella is spread via the respiratory route and by contact with skin lesions during the first week after onset of diseases. The incubation period is 10-20 days. Varicella is highly contagious, and ninety per cents of the population have antibodies before the age of 20 years (Haram, 1998).

Varicella and zoster (chickenpox and shingles) are both caused by VZV. Herpes zoster, or shingles is the painful eruption of a rash, usually unilateral, which usually persists asymptomatically in the dorsal root ganglia of anyone who has had chickenpox, reactivating from its dormant state in about 25% of people to travel along the sensory nerve fibers and cause vesicular lesions in the skin area supplied by that nerve. The rash is accompanied by severe pain which in some people does not subside after healing but persists for months or years. This prolonged zoster associated pain is usually defined as pain persisting for more than four months after rash has healed is known as postherpetic neuralgia. It is the most common complication of herpes zoster (Wareham, 2005).

ACV is the most prescribed anti-HSV drug. Phosphorylation of ACV by the viral thymidine kinase and cellular enzymes activates the drug as a substrate for the viral DNA polymerase. The drug is then incorporated into the synthesized viral genomic DNA and prevents its elongation (Murray *et al,* 2005).

The most prevalent form of resistance to these drugs results from mutations that inactivate the thymidine kinase, thereby preventing conversion of the drug to its active form. Mutations in the viral DNA polymerase also produce resistance (Murray *et al,* 2005).

ACV and other nucleoside derivatives are also used for VZV treatment. ACV is the most effective if administered within 72 hours of onset of rash (Wareham and Breuer, 2007).

The ability of the HSV-1, HSV-2 and VZV to establish a latent infection is due to an effective survival mechanism, as neither vaccines nor anti-viral drugs can attack the virus in its latent form.

No vaccine is currently available for HSV (Murray *et al,* 2005). Numerous efforts have been made to develop vaccines against genital herpes infection; several candidates have reached advanced phase clinical trials. However, intramuscular vaccination with recombinant HSV-2 glycoprotein D (gD) in combination with different adjuvants, conferred no (Straus *et al,* 1997) or only limited protection against genital herpes (Stanberry *et al,* 2002). Although a rectal route has a large potential for the development of novel immunization strategies to elicit immunity in the female genital tract (Tengvall *et al,* 2008), this approach still requires much development.

A vaccine derived from the oka- strain of VZV has been shown to be highly effective in preventing primary varicella in children and was introduced into the USA vaccination program in 1996 (Wareham and Breuer, 2007).

Thus, there is an urgent need for novel anti-HSV agents with high efficacy, low toxicity, especially those with a different mode of action than ACV and other nucleoside derivatives.

1.2. Retroviruses (Murine leukemia (MuLV) and sarcoma viruses (MuSV))

About 15 % of human cancers can be attributed to virus infection and viruses are considered as a second risk factor for cancer after tobacco.

There are two major mechanisms by which oncogenic viruses induce tumors: 1) direct oncogenesis, by which the virus infects a progenitor of the clonal tumor cell population and usually persists in the tumor cells: 2) indirect oncogenesis by which the virus does not necessarily infect the tumor progenitor cell but exerts an indirect effect on cell and tissue turnover or on the immune system predisposing to tumor development (Talbot and Crawford, 2005).

The association between viruses and human cancer was decisively demonstrated some 50 years ago from studies involving rodent tumorigenic viruses (Sarid and Gao, 2011). Retroviruses belong to the group of RNA non-lytic viruses, some of which were found to be implicated in different kinds of human and animal leukemia and other tumors. These viruses contain reverse transcriptase, an RNA-directed DNA-polymerase (Galo, 1991, Nagavi and Goff, 2007) which is essential for viral genomic DNA synthesis, required as a first step for its integration into the host cell genome. Most oncogenes (genes with potential properties for the induction of neoplastic transformation in either natural or experimental conditions) have been isolated from acute transforming retroviruses acting as oncogene transducers (Merlin *et al*, 2009). Moloney (1966) isolated a sarcoma virus (Mo-MuSV) from an intramuscular tumor induced in BALB/c mice. This virus was highly effective at transforming cells in culture, due to the “mos” oncogene elaborated in its genome (Perk, *et al*, 1967).

Mo-MuSV is a defective virus and requires the aid of a helper virus for its assembly and release from the infected cells. When MuLV is injected intravenously or intraperitoneally, it induces lymphoid leukemia in adult mice within a period 6-7 months (Ball *et al*, 1973, Huai, *et al*, 1992).

The genome of the murine leukemia virus (MuLV) is a single stranded, linear, positive-sense RNA molecule of about 8000 nucleotides. It is a prototypical simple retrovirus and replicates efficiently in most dividing cells that express the virus receptor (Sliva *et al*, 2004).

1.3. Antiviral effect of medicinal plants against herpes viruses and retroviruses

Many studies have been undertaken for estimation of the antiviral potential of different plants against herpes viruses. Different kinds of extracts (aqueous, ethanolic, methanolic, hexane and so on) from various plant organs (roots, stems, leaves, buds, flowers, fruits and so on) were examined. The main findings related to antiviral plant extracts can be summarized as follow: aqueous extracts of *Boussingaultia gracilis* , *Serissa japonica* (Chiang *et al*, 2003), *Hypericum mysorense, Hypericum hookerianum, Usnea complanta* (Vijayan *et al*, 2004) and *Ficus carica* (Wang *et al*, 2004) exhibited significant antiviral activity against HSV-1; aqueous extracts of *Carissa edulis* showed antiviral activity against HSV-1 and HSV-2(Tolo *et al*, 2005); aqueous extracts of *Artemisia capillaries, Rheum officinale, Gardenia jasminoids* inhibited HSV-2 infection more effectively (EC_{50} values were in the range of 19.6-29.4 µg/ml) than HSV-1 (EC_{50} values were in the range of 142.5-150.14 µg/ml) *in vitro* (Cheng *et al*, 2008); ethanolic and aqueous extracts of *Satureja boliviana* and *Baccharis genistelloides* were active against HSV-1 (Abad *et al*, 1999); ethanol and aqueous extracts from *Euphorbia canifolia* and *Euphorbia tirucalli* were effective against HSV-2 (Betancur-Galvis *et al*, 2002) ; leaf ethanol extracts of *Aglaia odorata, Moringa oleifera*, and *Ventilago denticulate* were effective against thymidine kinase-deficient HSV-1 and phosphonoacetate-resistant HSV-1 strains(Lipipun *et al*, 2003); methanol extracts from Ethiopian medicinal plants, *Euclea schimperi* and *Innula confertiflora* showed the most potent anti-HSV-1 activity (Gebre-Miriam *et al*, 2006); the acetone, ethanol and methanol extracts of *Phyllanthus urinaria* inhibited HSV-2 infection through disturbing the early stage of the virus infection and through diminishing the virus infectivity (Yang et al, 2005); hexane extracts from *Lannea schweinfurthil, Combretum odenogonium, Ficus sycomorus* and *Terminalia mollis* showed strong antiviral activity against HSV-1 (Maregesi *et al*, 2008.);

dichloromethane-methanol of *Dunbaria bella* was reported as effective against HSV-1 and HSV-2 (Akanitapichat *et al,* 2006); HSV and VZV were inhibited by various extracts from some *Glycyrrhiza* species (Asl and Hosseinzadeh , 2008).

Essential oils from aromatic plants of central west Argentina were tested for their antiviral activity against HSV-1. Only essential oil from *Lanthana grisebachii* showed antiviral activity, its selectivity index was 26.1 (Garcia *et al,* 2010). The antiviral effect of the essential oil of *Melissa officinalis* was also examined. EC_{50} was at high dilutions of 0.0004% and 0.00008% for HSV-1 and HSV-2 respectively. Both herpesviruses were significantly inhibited by preatreatment with the oil prior to infection of cells. These results indicate that *M. officinalis* oil affected the virus before adsorption, but not after penetration into host cells, thus this oil is capable of exerting a direct antiviral effect on herpesviruses (Schnitzler *et al,* 2008).

Flavonoids (compounds were not identified) from *Capparis spinosa* buds were active against HSV-2, but did not show any activity against HSV-1 (Arena *et al,* 2008).

The following compounds with antiviral activities were isolated from various plants.

Many kinds of biflavonoids were isolated from *Rhus succedanea* and *Garcinia multiflora.* Their antiviral activities were estimated against many viruses including HSV-1, HSV-2, and VZV. Amenthoflavone and robustflavone demonstrated moderate activities against HSV-1 and HSV-2 but they did not affect VZV. Amenthoflavone was active at concentrations 5.6 and 2.1 µg/ml against HSV-1 and HSV-2 respectively. Robustaflavone was also more effective against HSV-1 (at concentration17.9 µg/ml) than against HSV-2 (at concentration 48 µg/ml). Rhustflavanone inhibited only HSV-2 whereas succedaneaflavanone didn't affect HSV-1 and HSV-2 but it did inhibit VZV (Lin *et al,* 1999).

Pure compounds isolated from *Plantago major* exhibiting anti-HSV activities are mainly derived from phenolic compounds, especially caffeic acid (Neyts *et al,,* 1992, Bourne *et al,* 1999). The EC_{50} values of chlorogenic acid, caffeic and ferulic acid on HSV-2 were 20, 54 and 8000 µg/ml respectively (Neyts *et al,* 1992).

19-(2-furyl)nonadeca-5,7-diynoic acid and 19-(2-furyl)nonadeca-5-yonic acid were isolated from roots of *Polyathia evecta*. These 2-substited furans were active against HSV-1 (Kanokmedhakul et al, 2006).

Polysaccharide fractions from *Arthrospira platensis* exibited strong inhibition of HSV-1 in vitro (Rechter *et al*, 2006).

Schefflera heptaphylla (L) Frodin is a popular medicinal plant in southern China. Li et al, 2005 isolated three caffeoylquinic derivatives from this plant and investigated them for their antiviral activities against RSV (Respiratory syncytial Virus), FluA (Influenza Virus Type A), CoxB3 (Coxsackievirus B3) and HSV-1. The antiviral action of these substances was specific against RSV, and they had no obvious antiviral activity against any of the other viruses tested.

Zhang et al (2007) demonstrated anti-herpetic properties of *Prunella vulgaris* both *in vivo* and *in vitro*. A lignine-polysaccharide complex with potent activity against HSV-1 and HSV-2 was isolated from this plant.

Emodin was found to inhibit the replication of HSV-1 and HSV-2 in cell culture at the concentration of 50 μg/ml with selectivity index of 2.07 and 3.53 respectively (Xiong *et al*, 2011).

Oxyresveratol is a major compound purified from another Thai medicinal plant, *Artocarpus lakoocha.* It inhibited the replication with pretreatment in one-step growth assay of HSV-1 and HSV-2 in mice (Chuanasa *et al*, 2008).

Excoecarianin Cheng *et al*, 2011) and hippomanin A (Yang *et al*, 2007) were isolated from *Phyllanthus urinaria.* Both compounds inhibited HSV-2 but not HSV-1 infection *in vitro*.

Anionic polysaccharides from *Achyrocline flaccida* are able to block binding HSV-1 (Garcio *et al*, 1999).

The mechanism of antiviral action of the natural products is seldom considered and still poorly understood. Many previous investigations suggested that these products exert their antiviral activity by preventing virus entry into the host cell, by blocking viral attachment to the cell surface like Proanthocyanidin-enriched extract from *Myrothamnus flabellifolia which* exerts

antiviral activity against HSV-1 with selectivity index of 120 (Gescher *et al*, 2011). Others such as Polyphenol compounds (they haven't been identified yet) from *M. flabellifolia* were found to interact directly with viral particles, leading to the oligomerization of envelope proteins as demonstrated for the essential viral glycoprotein D (gD). Using organotypic full thickness tissue cultures, it was shown that treatment of HSV-1 infected cultures with *M. flabellifolia* resulted in reduced viral spread (Gescher *et al*, 2011). Some anti-viral properties of plant compounds may be explained on the basis of antioxidant activity of phytochemicals, scavenging capacities, and inhibition of viral entry or inhibiting the viral reproduction possibly through inhibiting the viral DNA or RNA synthesis (Naithani *et al,* 2008). In the majority of cases the antiviral mechanisms vary depending on the antiviral agent (Chattopadhyay and Khan, 2008, Mukhtar *et al*, 2008, Naithani *et al*, 2008).

Anti-herpetic activities of plant extracts were reported in many reviews (Namba *et.al,* 1998, Jassim and Naji, 2003,, Khan *et al*, 2005, Chattopadhyay and Khan, 2008, Mukhtar *et al*, 2008, Naithani *et al*, 2008). Although many antiviral compounds were identified, like flavonoids, terpenoids, lignans, sulphides, polyphenolics, coumarins, saponins, furil compounds, alkoloids, polyines, thiophenes and different sugars, their antiviral mechanism of action is still poorly understood.

To the best of our knowledge, no scientific reports regarding the antiviral activity of higher plant extracts against transforming retroviruses, such as MuSV are available. Several natural products, including propolis (Huleihel and Ishano, 2001) and red microalgal polysaccharides (Talyshinsky *et al*, 2002) were shown to significantly inhibit malignant transformation of cells by MuSV in culture. Substances as such may provide an excellent alternative to synthetic compounds, like acyclic nucleoside phosphonates and 6-[2-(phosphonomethoxy)alkoxy]-2,4-diaminopyrimidines which were also found to be active against MuSV, but at low efficiency and with serious adverse effects (De Clerq *et al,* 2005).

Polyphenols from various plants such as green tea (Li *et al,* 2000, Sonoda *et al*, 2004) and Scutellaria baicalensis georgi- baicalin (Baylor *et al,* 1992) were found to have inhibitory effect on HTLV-1 (Human T-lymphotic Virus) replication as well as on ALT (alanine transaminase) growth.

1.4. Plants whose antiviral properties were investigated in the present study

1.4.1. White lily *(Lilium candidum* L.)

Madonna Lily, meadow or white lily *(Lilium candidum* L.) is a monocotyledonous plant of the family Liliaceae. *L. candidum* is a beautiful plant with large white and sweet scented flowers. It has been cultivated for centuries in the Middle East for ornamental purposes (figure 1).

Figure 1. White lily *(Lilium candidum* L.)

It is a rare species growing wild in only a few locations in Israel, for example, on Mountain Carmel and Upper Galilee (Zaccai *et al*, 2009). White lily is highly susceptible to plant viruses and fungi (Prosceivicius et al, 2007).

This plant is known in folk medicine as an anti-inflammatory and remedy, and is also used in cosmetics. Bulbs and flowers of the white lily have traditionally been used for treatment of ulcers, boils, finger ulcers, reddened skin, burns and injuries (Eisenreichová et al, 2000).During last few years antifungal and anti-yeast activities of this plant were described. Mucaji et al (2002) presented anti-yeast activity of ethanolic extracts from the flowers and bulbs of *L.*

candidum L., as well as some compounds isolated from these extracts. The extract from the bulbs was shown to be more active than the extract from the flowers, while isolated compounds were inactive against the tested yeasts.

Anticancer properties of several compounds (the spirostanol saponins, jatropham and jatropham glucoside) isolated from the ethanolic extract of *L. candidum* were also demonstrated (Vachalkova *et al*, 2000).

The use of *L. candidum* bulbs as an antiviral agent to treat shingles (Herpes zoster) was first tested in Garfagnana, a part of the province Lucca (Italy). The bulbs of the cultivated *L. candidum* were used in the form of a poultice prepared by frying the bulb in olive oil and applying this externally against shingles (Pieroni, 2000).

1.4.2. Weeping fig (*Ficus benjamina*)

Weeping fig (*Ficus benjamina or Ficus binjamina,* figure 2) belongs to the family *Moraceae* . It is **a** popular park tree in tropical climates, reaching 30 m in natural conditions. It is well-known as a decorative indoor plant in countries with moderate climate.

F. benjamina is a plant whose phytochemicals and biological properties have only been moderately investigated so far. Its fruit extracts have shown antitumor and antibacterial activity (Mousa *et al,* 1994), while aqueous and alcoholic leaf extracts had significant antinociceptive activity (reducing sensitivity to painful stimuli) in analgesiome test (Parveen *et al,* 2009).The presence of polyphenols in *F. benjamina* grandular epithelium has been reported(Pennisi *et al,* 1999) . A new triterpene, named serrate-3-one, along with phytoconstituents pentacontanyl decanoate, friedelin and beta-sitosterol have been detected in *F. benjamina* (var. comosa) benzenoid extracts (Parveen *et al,* 2009).

The weeping fig is not susceptible to different plant viruses unlike other decorative species of *Ficus* (Petrov *et al*, 1974).

Figure 2 . Weeping fig (*Ficus benjamina*).

1.4.3. Inch plant (*Callisia fragrans* (Lindl.) Woodson)

Inch plant (*Callisia fragrans* (Lindl.) Woodson) is an herbaceous plant with small white fragrant flowers and waxy leaves which grow wild in Mexico (figure 3). It has been cultivated as an indoor plant in many countries for over a century. This plant is currently drawing a lot of attention with respect to its medicinal properties in Russia, Bulgaria and Romania. Inch plant leaves are used for treatment of various skin diseases, burns and joint disorders. It also has a very good folkloric reputation as an antiviral and anti-cancer plant (Chernenko *et al*, 2007).

Figure 3. Inch plant (*Callisia fragrans).*

There is insufficient knowledge about this plant. *C. fragrans* leaves contain biologically active flavonoids, neutral glycol-and phospholipids and their fatty-acid compositions (Chernenko *et al,* 2007). Mishkin and Shipovsky (2007) investigated sugar content of *C. fragrans* but their medicinal properties were not considered.

1.4.4. Jojoba (*Simmondsia chinensis*)

Jojoba (*Simmondsia chinensis,* figure 4) is the sole species of the *Simmondsiaceae* family . It is an arid perennial shrub indigenous to Arizona, California and Northwestern Mexico. *S. chinensis* is also grown in Australia, Brazil, Argentina and some Middle East countries. Its flowers are small, greenish-yellow, with 5-6 sepals and no petals. Jojoba is a dioecious plant. This plant is grown in many countries for liquid wax, commonly mistaken for jojoba oil, extracted from its

seeds. This liquid wax was used in folk remedies for renal colic, sunburn, chaffed skin, hair loss, headache, wounds and sore throats. It is also often used in cosmetics. The effectiveness of jojoba liquid wax in combating inflammation in different experimental models was reported (Habashy *et al*, 2005).

Antiviral properties of jojoba are not mentioned in available literature.

Figure 4. Jojoba (*Simmondsia chinensis).*

1.5. Contribution of the present study to the field

Viral infections remain one of the most important public health problems worldwide. For example, about 80% of the adult population are infected by or are sero-positive for HSV-1 in the USA. In addition, despite the availability of effective antiviral drugs against some of the viruses such as acyclovir against various members of the herpes family viruses, still there is an urgent need for novel anti-viral agents with high efficacy and low toxicity. In the case of herpes viruses there is a need for anti-viral agents with a different mode of action than acyclovir and other nucleoside derivatives due to the rapid development of resistant mutants against ACV and its derivatives.

Although previous works have highlighted the antiviral activity of traditional medicinal plants, many plants were never investigated as antiviral agents. *L. candidum, C. fragrans* and *S. chinensis* were chosen for our research because of their folkloric reputation as antiviral plants, and to the best of our knowledge, no previous scientific reports about their antiviral properties were available. The above mentioned extracts and their effective fractions had significant antiviral properties, so they may provide a potential source of anti-herpetic agents.

In addition, *L. candidum*, has a high susceptibility to plant viruses and fungi (Prosceivicius *et al*, 2007). We hypothesized that this plant might produce antiviral compounds against plant viruses that could be effective against human and animal viruses. We hypothesized that plants which were stable to plant viruses might produce much variety of antiviral compounds. To test this hypothesis, we reasoned that a suitable plant would be a weeping fig (*F. benjamina*) which was resistant to various plant viruses (Petrov et al, 1974). *F. benjamina* has not been mentioned as a medicinal plant. Our results showed that antiviral activity of the crude ethanol leave extract of *F. benjamina* was more enhanced than that of other plants that we tested and that were mentioned in current literature. Thus, the innovation of our research is in selection of antiviral plants on the basis of their abilities to protect themselves against plant viruses. Leaf extracts of *F. benjamina* inhibited all studied viruses, whereas those of *L. candidum* inhibited only HSV-1, HSV-2.

A bioassay guided fractionation of the ethanolic extract of *F. benjamina* leaves led to the isolation of three flavone glycosides; (1) quercetin 3-O-rutinoside , (2) kaempferol 3-O-

rutinoside and (3) kaempferol 3-O-robinobioside, all with high antiviral activity. The structure of the compounds was determined by spectroscopic analyses including NMR and MS. Although the flavone glycosides from *F. benjamina* are present in other plants and they are well-known, their antiviral properties were never mentioned in literature. Only quercetin 3-O-rutinoside was described as component in mixture of glycosides which was effective against HIV (Goo *et al*, 2009).

In addition, no previous scientific reports about antiviral activity of higher plants against MuSV-124 and MuLV are available. The present study is considered as the first report about plant products which successfully inhibited both cell transformation and viral replication/production of MuSV-124 and MuLV in cell culture.

1.6. Research objectives and hypotheses

As mentioned above there is an urgent need for new antiviral agents with higher efficacy and lower toxicity than acyclovir and other nucleoside derivatives. Plants are one of the most important sources of antiviral drugs. It is expected that plants *L. candidum*, *C. fragrans* and *S. chinensis* whose antiviral properties were known in folk medicine would will demonstrate antiviral activity.

F. benjamina has not been mentioned as a medicinal plant but this plant was found to be resistant to various plant viruses (Petrov et al, 1974). Our other hypothesis predicts that a plant which is resistant to various plant viruses would produce antiviral compounds which would be effective against animal and human viruses.

The main objective of this study is to evaluate and identify plant substances with potent antiviral activity. The specific aims are:

1. Screening and evaluation of antiviral activities of different extracts from various potential plants against members of the herpes viruses' family and retroviruses.

2. Isolation and identification of antiviral agents from selected plant species.

3. Elucidation of the antiviral mechanisms of the plant extracts.

2. Methods

2.1. Plant material

F. benjamina, C. fragrans , S. chinensis plants and *L. candidum* bulbs were obtained from nurseries and grown in a controlled greenhouse at the Ben Gurion University, Beer-Sheva, Israel.

2.2. Preparation of plant extracts

Ethanolic and aqueous extracts were prepared from stems, leaves and fruit of *F. benjamina*, bulbs, petals and leaves of *L. candidum* and leaves of *C. fragrans* and *S. chinensis*. Plant tissues were destroyed, incubated at room temperature for 48 hours in appropriate solvent, centrifuged at 2000 rpm for 10 min and the supernatant was evaporated by lyophilization. The pellet was dissolved in minimal amount of 95% ethanol (0.5 ml) or water and diluted with water to final concentration of 10mg/ml.The extracts were sterilized by filtration and diluted with medium containing 2% Newborn calf serum (NBCS) to the appropriate concentrations.

Extracts were separated into different fractions using reverse phase column with rising methanol gradient: 0% (v/v), 20%(v/v), 40%(v/v), 60 %(v/v), 80 %(v/v) and 100 % (v/v) ,(RP-C18 Sepack (Supelco, St. Louis, MO, USA)).

2.3. Cells and viruses

The following cell lines were used to cultivate the different viruses: African green monkey kidney (Vero) cells for HSV-1, HSV-2 and VZV; NIH/3T3 cells (mouse fibroblast cells) for MuSV and MuLV. All cell lines were incubated at 37 ^{o}C in a humidified air containing 5% CO_2.

African green monkey kidney (Vero) cells were purchased from the American Type Culture Collection (ATCC), Rockville, MD, USA. Cells were grown in RPMI (a medium was developed by Moore *et al,* at Roswell Park Memorial Institute)containing 10% Newborn calf serum (NBCS) and antibiotics mixture including penicillin, streptomycin and nystatin. HSV-1, HSV-2 and VZV were propagated in Vero cells and their concentration was estimated by a standard plaque assay (Huleihel *et al,* 2001).

NIH/3T3 cells (mouse fibroblast cells) were grown at 37 ºC in RPMI medium supplemented with 10% new born calf serum and antibiotics penicillin, streptomycin and nystatin. Clone 124 of TB cells chronically releasing Moloney MuSV -124 was used to prepare a virus stock that contained an approximately 30-fold excess of MuSV particles over Moloney murine leukemia virus (MuLV) particles (Huleihel and Aboud, 1982). MuSV was grown on NIH/3T3 cells and virus concentration was determined by counting the number of foci (FFU- focus forming units).

2.3.1. Toxicity examination

The normal uninfected cells were treated with various concentrations of plant extracts , their fractions or their isolated compounds and the toxicity was estimated by three different methods:

1. Direct count. The cells were counted daily (for 3 days) using Neubauer hemacytometer indicating their replication rate.

2. Morphological changes were observed daily by optical inverted microscope.

3. MTT assay was performed as previously described (Shi *et al*, 1993). Briefly, Vero cells were incubated with 50µg/ml MTT solution at 37°C for 5h. This solution was converted by mitochondrial succinate dehydrogenase enzyme into purple crystal called formazan. Then, MTT solution was removed and replaced with SDS (Sodium Dodecyl Sulfate) solution which dissolved this crystal. After overnight incubation at 37°C absorbance was measured by a spectrophotometer at 570 nm, indicating the metabolic activity of the cells.

2.3.2. Herpes viruses

2.3.2.1. Cell infection and infection development evaluation

Vero cells were seeded at 0.15×10^6 cells/well in 24-well culture plates, in RPMI with 10% NBCS and antibiotics. Following overnight incubation, medium was removed and each well was infected at a multiplicity of infection (m.o.i.) of 1 plaque forming units (PFU)/cell for two hours at 37°C. The unabsorbed viruses were removed and cells were overlaid with either a layer of CMC, carboxymethyl cellulose , (for plaque assay) or RPMI containing 2% NBCS and antibiotics for cytopathic effect (CPE) development evaluation. The infection development was evaluated by plaque assay and CPE development as previously described (Huleihel *et al,* 2001).

Two to three days post-infection (p.i.) the CMC overlay was removed, cell monolayers were fixed with 10% formalin in saline, stained with crystal violet and plaques were counted. The development of CPE was evaluated daily by microscopic observation and expressed as the percentage of damaged cells.

2.3.3. Retroviruses

2.3.3.1. Cell infection and infection development evaluation

A monolayer of NIH/3T3 cells was grown in 9-cm^2 tissue culture plates and treated with 0.8 µg/ml of polybrene (a cationic polymer required for neutralizing the negative charge of the cell membrane) for 24 h before infection with the virus. After removal of free polybrene the cells were incubated at 37 ºC for 2 h with the infecting virus at various concentrations in RPMI medium containing 2% NBCS. The unabsorbed virus particles were removed, fresh medium containing 2% of NBCS was added, and the monolayers were incubated at 37 ºC. At different timepoints p.i, the cell culture was examined for malignant cells transformation by morphological observation, foci formation and growth on soft agar. The amount of malignant transformed cells was expressed as a number of foci in the infected culture at 7 days p.i. The foci were counted under microscopy observation.

In addition, the infected cell cultures were examined for malignant transformation of the cells by growth on soft agar. The soft agar assay is consisted of bottom and top layers of agar. The bottom layer was prepared on the basis of NBCS (20 %), RPMI (30 %), complete RPMI (25%) and bacterial Agar Agar (25 %). 2.5 ml of this mixture was added immediately after preparation into 5cm^2 plates and left for at least 30 minutes in the biohazard hood for solidation. The NIH/3T3 cells were removed from the wells after treatment with trypsin and counted. The same number of the cells (about 100 cells in every experiment) was added to 0.5 ml to the top layer (NBCS -20%, RPMI-44%, complete RPMI -18% and bacterial Agar Agar- 18 %), mixed together and added on the top of the first layer. Malignant transformed cells are able to grow and replicate on this soft agar and producing visual foci, whereas, normal cells are not able to grow and will die at several days after placing in soft agar.

2.3.3.2. Elucidation of the antiviral mechanism of action of tested products

To elucidate the antiviral mechanism of the plant antiviral agents, the NIH/3T3 cells were treated with increasing concentrations of the tested agents at various periods of time before, during or after infection. NIH/3T3 cells were treated with10 µg/ml of the 60%-MeOH fraction

immediately after 2h of infection with 0.1FFU of MuSV. The treatment was removed at different periods of time p.i. (I, 2, 4, 6, 24 hours).

2.4. Analytical methods

Chromatography (HPLC) and spectroscopic analyses.

The flavonoid fraction was analyzed by HPLC. Gradient elution was performed with solution A, composed of water-acetic acid (97 : 3 V/V) and solution B – methanol, flow rate 1.0ml/min, as described previously (Chen et. al., 2001). UV detector at 360 nm with reverse phase column (Betasil C-18, 5 μm, 250 × 0.46 mm; Thermo-Hypersil, UK) was used.

Liquid chromatography-mass spectrometry (LC-MS) instrumentation and conditions

LC-MS Agilent 1100LC series (Waldbronn, Germany) and Bruker Esquire 3000plus MS (Bremen, Germany) instrument consisting of a C18 column (Betasil C18, 5 _m, 250 × 4.6 mm; Thermo- Hypersil, UK) and methanol-water as the mobile phase (see HPLC in the method above) were used. The UV detector was set at 360 nm, the flow rate at 1 ml/min, and injection volume of 10 μl. The MS conditions were optimized as follows: API electron spray interface, negative mode polarity, a drying gas flow of 10 L/min, a nebulizer gas pressure of 60 psi, a drying gas temperature of 335°C, a fragmentor voltage of 0.4 V, a capillary voltage of 4451 V, and a scan range of *m*/*z* 25–1000, at 1.15 s/scan**.**

MALDI-TOF-MS

High Resolution-Mass spectrometric analysis was performed on a Bruker Reflex IV time-of-flight (TOF) mass spectrometer (Bruker Daltonics, Bremen, Germany). Ions were formed using a pulsed nitrogen laser operating at 337 nm. Mass spectra, obtained from the average of up to 350 single-shot spectra, were collected using an acceleration voltage of 25 kV and a delay of 150 ns.

Nucleic Magnetic Resonance (NMR)

All the isolated compounds were submitted to NMR spectroscopic measurements with Brucker AC 500 (500 MHz) apparatus using CD_3OD as solvent.

2.5. Statistical analysis.

All data were analyzed using Statistica for Windows software (StatSoft, Inc., Tulsa, Oklahoma), and $P<0.05$ was chosen as the minimal acceptable level of significance.

3.Results

Part A. Herpes viruses.

3A1. Cytotoxicity examination

As a first step for evaluating the antiviral activity of the various plant extracts, the cytotoxicity of these extracts on uninfected Vero cells was examined. Vero cells were treated with the plant extracts at different concentrations for four days. The cytotoxicity of these extracts was evaluated by different assays as described in the Methods section. Our results (table 1) demonstrated that no cytotoxicity was observed at concentrations below 95 µg/ml for all examined extracts. The ethanol fruit extract of *F. benjamina* (CC_{50} > 1000) and the aqueous leave extract of C. *fragrans (*CC_{50} =1550) showed the lowest toxicity. The most toxic extracts were ethanol stem extract of *F. benjamina* (CC_{50} =120) and aqueous leaf extract of *S. chinensis* (CC_{50} =95).

Table 1.Toxicity of plant extracts at different concentrations

Plant	**Extract type, CC_{50} (µg/ml)**	**Concentration. (µg/ml)**	**Cell number[1] (% of control)**	**Viability[2] (% of control)**
F. benjamina	Ethanol leaf extract, 700	1	102.5±1.87	104.8±2.17
		10	98.7 ±1.43	97.5 ±0.98
		100	91.0 ±1.93	94.9 ±2.56
		500	66.9±1.89	64.9±4.98
	Aqueous leaf extract, 900	1	101.9±1.34	101.6±1.09
		10	101.2±1.32	102.1±2.11
		100	100.1±1.13	100.7±1.32

		500	72.2±3.09	70.9±2.55
	Ethanol stem extract, 120	1	101.5±1.45	100.5±1.60
		10	98.9 ±1.75	99.2 ±1.53
		50	87.1±3.12	88.0±2.19
		100	55.3±3.06	54.8±2.54
		500	11.4±1.11	11.9±1.56
	Aqueous stem extract , 160	1	102.2±1.54	102.7±1.85
		10	97.3 ±1.22	97.6 ±1.45
		50	84.2±2.53	84.7±2.27
		100	59.7±1.15	59.2±1.43
		500	14.3±1.09	14.1±1.64
	Ethanol fruit extract, > 1000	1	103.2±1.87	103.6±1.23
		10	102.2±1.23	101.9±1.65
		100	100.4±1.47	100.7±1.71
		500	100.7±1.30	100.4±1.98
		1000	100.1±2.21	100.2±1.75
L. candidum	Ethanol bulb extract, >500	1	100.8±1.74	100.4±1.64
		10	100.7±1.53	100.9±1.82
		100	100.2±1.99	100.6±1.06
		500	100.1±1.31	100.0±1.83

	Ethanol petal extract, >500	1	103.5±1.21	102.5±1.74
		10	102.8±0.87	102.3±1.32
		100	102.0±1.54	102.2±1.69
		500	100.8±1.86	100.5±2.15
	Ethanol leaf extract, 700	1	100.3±1.23	100.8±1.84
		10	95.79±1.67	97.98±1.98
		100	83.99±2.14	86.18±1.07
		500	77.98±1.65	76.98±4.76
		1000	11.43±1.27	13.89±3.09
C. fragrans	Ethanol leaf extract, 1100	10	102.5±0.36	99.9±3.09
		100	100.2±1.21	98.8±3.01
		500	100.6±0.46	97.1±3.07
		1000	65.3±3.81	72.6±4.90
		1500	34.3±3.18	33. 9±2.98
	Aqueous leaf extract, 1550	10	101.1±2.45	101. 8±3.55
		100	101.1±1.78	101. 5±3.48
		500	100.2±2.36	100.7±3.45
		1000	100.7±2.61	100.8±2.78
		1500	54.3±2.28	53.3±3.25

S. chinensis	Ethanol leaf extract, 250	10	96.0±0.81	99.5±2.09
		100	98.3 ±2.63	98.7 ±3.13
		500	36.9±1.27	33.6±1.27
	Aqueous leaf extract, 95	10	101.1±2.15	102.6±4.56
		50	100.5 ±2.98	101.2 ±3.78
		100	46.8±2.17	43.6±3.45

Vero cells were treated with the plant extracts at different concentrations for 4 days. [1]Cells were counted by Neubauer hemacytometer indicating their replication rate. [2]Viability of cells was evaluated by MTT assay. Data are presented as mean ± SD of three independent experiments.

3A2. Antiviral activity of plant extracts

The antiviral activity of the various extracts obtained from different parts of the examined plants was investigated against the tested herpes viruses. Vero cell monolayers were treated with increasing concentrations of the appropriate extract at the time and after infection with 1m.o.i. of HSV-1, HSV-2 or VZV. Antiviral activity was evaluated by plaque assay as detailed in Methods section. Our results show that extract of *F. benjamina* fruit had no effect on HSV-1 and HSV-2 infection, while significantly inhibiting VZV infection with an EC_{50} of 10 µg/ml ($p<0.001$) (not shown results). Stems of *F. benjamina* didn't show significant antiviral properties, but had high toxicity (table 1). Neither bulbs nor petals of *L. candidum* had any significant anti-herpetic activity (not shown results). However, leaf extracts of both *F. benjamina* and *L. candidum* demonstrated high and promising antiviral activity against the tested herpetic viruses (table 2).

The *C. fragrans* ethanol extract effectively inhibited the infection of Vero cells by HSV-1, HSV-2 *in vitro*, while its aqueous extract inhibited only VZV. *S. chinensis* leaf extracts strongly inhibited all studied viruses, but their high cytotoxicity led to lower SI values.

C. fragrans, fraction 60%-MeOH of the ethanol extract strongly inhibited HSV-1 and HSV-2 but only slightly VZV, whereas fraction 20%-MeOH of the aqueous extract significantly inhibited all tested viruses. All fractions of both ethanol and aqueous extracts of *S. chinensis* showed relatively high SI but the 60%-MeOH fraction of the ethanol extract had the highest SI values (227.3 against HSV-1, 200 against HSV-2 and 142.9 against VZV), (Yarmolinsky *et al*, 2010).

Table 2 summarizes the antiviral activity of effective plant extracts and ACV. Only the SI of ethanol leaf extract of *F. benjamina* was higher than that of ACV. Although the remainder plants had lower SI's than that of ACV, they were effective against the HSV-2 mutant strain which is resistant to ACV (table 2). *F. benjamina* and *L. candidum* ethanol leaf extracts were the most effective against herpes viruses (table 2), so further study was focused on them.

Table 2. Antiviral activity of effective crude extracts.

Crude extract	**Selectivity index (SI)**			
	HSV-1	**HSV-2**	**HSV-2 ,mutant**	**VZV**
F. benjamina, ethanol leaf extract	**980.0**	**816.7**	**816.3**	**612.5**
F. benjamina, aqueous leaf extract	200.0	180.0	182.5	350.0
L. candidum, ethanol leaf extract	70.0	35.0	35.0	No effect
L. candidum, aqueous leaf extract	54.6	25.0	25.0	2.4
C. fragrans, ethanol leaf extract	66.6	73.3	73.8	1.4
C. fragrans, aqueous leaf extract	3.1	2.4	4.1	91.2
S. chinensis , ethanol leaf extract	5	5.5	5.2	4.5
S. chinensis aqueous leaf extract	9.5	9.5	9.5	6.78
ACV	**700.0**	**280.0**	No effect	466.7

Vero cells monolayers were treated with different doses of the plant extracts at the time of infection. Antiviral activity was evaluated by plaque assay. SI was determined as CC_{50}/EC_{50} on the basis of results of five independent experiments.

3A3. Antiviral activity of ethanol extracts of *F. benjamina* and *L. candidum*

Vero cell monolayers were treated with increasing concentrations of the appropriate extract at the time of infection with 1 m.o.i. of HSV-1, HSV-2 or VZV. The treatment with the extract was terminated immediately post-infection. Antiviral activity was evaluated by plaque assay as detailed in Methods section. The results demonstrate significant and reproducible antiviral activity of *F. benjamina* leaf extracts against HSV-1, HSV-2 and VZV with the respective EC_{50s}=0.5 µg/ml, 1.7 µg/ml and 35 µg/ml (figure 5, A). *L. candidum* leaf extracts had no effect on VZV, while they strongly inhibited HSV-1 and HSV-2 with EC_{50} of 8 and 20 µg/ml respectively (figure 5,
B).

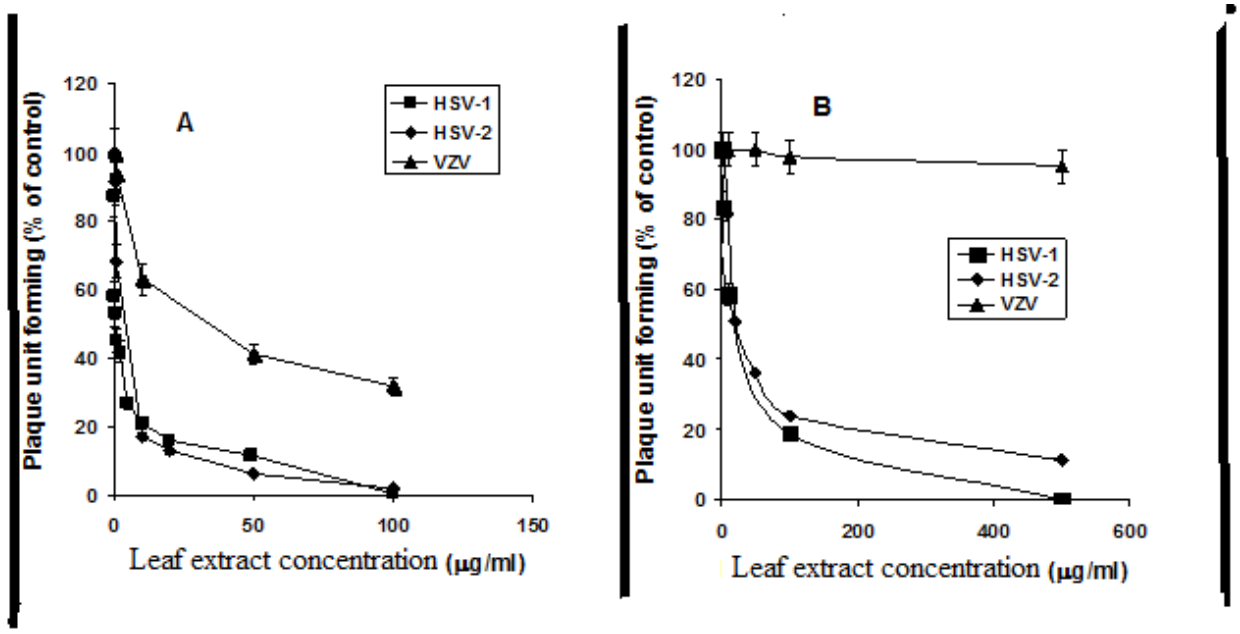


Figure 5. Antiviral activity of ethanol leaf extracts from *F. benjamina* (A) and *L. candidum* (B) against herpes viruses. Vero cells were infected with 1moi of the various tested viruses in the presence or absence of increasing doses of leaf extracts obtained from *F. benjamina* (A) or *L.*

candidum (B). The treatment with the extracts was terminated immediately post infection. Plaques number of the treated cultures was presented as a percentage of the positive control (infected but untreated cell cultures). Values are presented as mean ± SD (n=5).

3A3. 1. Effect of time of leaf extract addition on herpes virus infection.

Vero cells were infected with 1 m.o.i. of the different examined herpes viruses and treated with 100 µg/ml of the tested leaf extracts at various timepoints before, at or after infection. When the cells were treated with the leaf extracts only at the time of infection or both at the time and post-infection, the inhibition of all viruses tested was the highest ($p<0.01$) (table 3). We also found that *F. benjamina* leaf extract was significantly more effective against HSV-1 and HSV-2 than against VZV.

According to these results it seems that these extracts may inhibit early steps of the viral infection such as blocking its adsorption to the host cells, and if so, it remained to determine whether the extract acts through direct interaction with the virus particles, with the host cells or both. To test leaf extract–cell interaction, Vero cells were first incubated in medium containing the extract and then infected with the virus without adding any more of the extract. As detailed in table 3, preincubation of the cells with the leaf extracts had no significant effect on the viral infection of all the tested viruses, the best result was for HSV-1, ca. 84 % of control PFU, when *F. benjamina* extract was added before infection. Vero cells were treated with *F. benjamina* extract at a concentration of 100 µg/ml for two hours. The tested extract was removed with or without washing with 0.9 % NaCl, and then the cells were infected with 1 moi of HSV-1. When the extract wasn't washed, the result was similar to that described above (table 3), and when cells were washed once or twice before adding the virus, no inhibition effect on HSV-1 was observed.

To examine possible interaction between viral particles and leaf extracts, the appropriate viruses were preincubated with the extracts for different periods of time as detailed in Methods section. These mixtures were then diluted with fresh medium and used to infect Vero cell monolayers. This resulted in considerable inhibition of plaque formation even by the highly diluted virus–leaf extracts mixtures (figure 6). It is worthwhile to mention that at these the high dilutions, the extracts had no significant antiviral effect when they were added at the time of infection. In the case of HSV-1an incubation period of 15 min was sufficient to inhibit about 90 or 75% of plaque formation by *F. benjamina* and *L. candidum* leaf extracts, respectively (figure 6, A, B) . Incubation for 30 min was sufficient for complete inhibition of plaque formation by both plant extracts. In the case of HSV-2, a 30 min incubation period with *F. benjamina* extract completely inhibited plaque formation while 90 min with the *L. candidum* extract was required for such inhibition (figure 6, B). Similar results were obtained at both incubation temperatures tested (4°C or 22°C).

Interestingly, when the infected cells were treated with the extract only p.i., there was a partial reduction of the plaque number induced by the different examined viruses (table 3).

At concentration of 100 µg/ml *F. benjamina extract* completely inhibited the development of HSV-1 and HSV-2 infections as opposed to about 70% VZV inhibition (table 3). By contrast, *L. candidum* leaf extract had no significant effect on VZV ($p>0.1$), but strongly inhibited HSV-1 and HSV-2 at concentration of 100 µg/ml ($p< 0.01$). At concentration of 500 µg/ml *L.candidum* leave extract almost completely inhibited HSV-1 (0±0%) and HSV-2 (1.08±0.07%).

Table 3. **Effect of time of leaf extract addition on herpes virus infection.**

Leaf extract (100 µg/ml)	Virus (1 m.o.i.)	Plaque Forming Units (% of control)			
		Before infection only	During infection only	After infection only	During and after infection
F. benjamina	HSV-1	84.2±6.30	0.5±0.04	37.1±2.01	0±0.08
	HSV-2	94.6±5.27	1.8±0.28	41.1±3.29	1.6±1.16
	VZV	100.1±0.47	31.6±4.25	29.7±4.16	26.8±3.64
L.candidum	HSV-1	99.3±3.11	18.7±1.75	42.5±2.11	13.4±0.91
	HSV-2	100.0±0.62	23.6±2.19	56.3±3.18	20.1±3.02
	VZV	100.0±0.09	97.5±1.29	100.2±1.14	96.1±2.14

Vero cell monolayers were treated with 100 µg/ml of the appropriate extract for different periods of time before, at or after infection with 1m.o.i. of HSV-1, HSV-2 or VZV. Antiviral activity was evaluated by plaque assay as detailed in Methods section. Values are presented as mean ± SD (n=5).

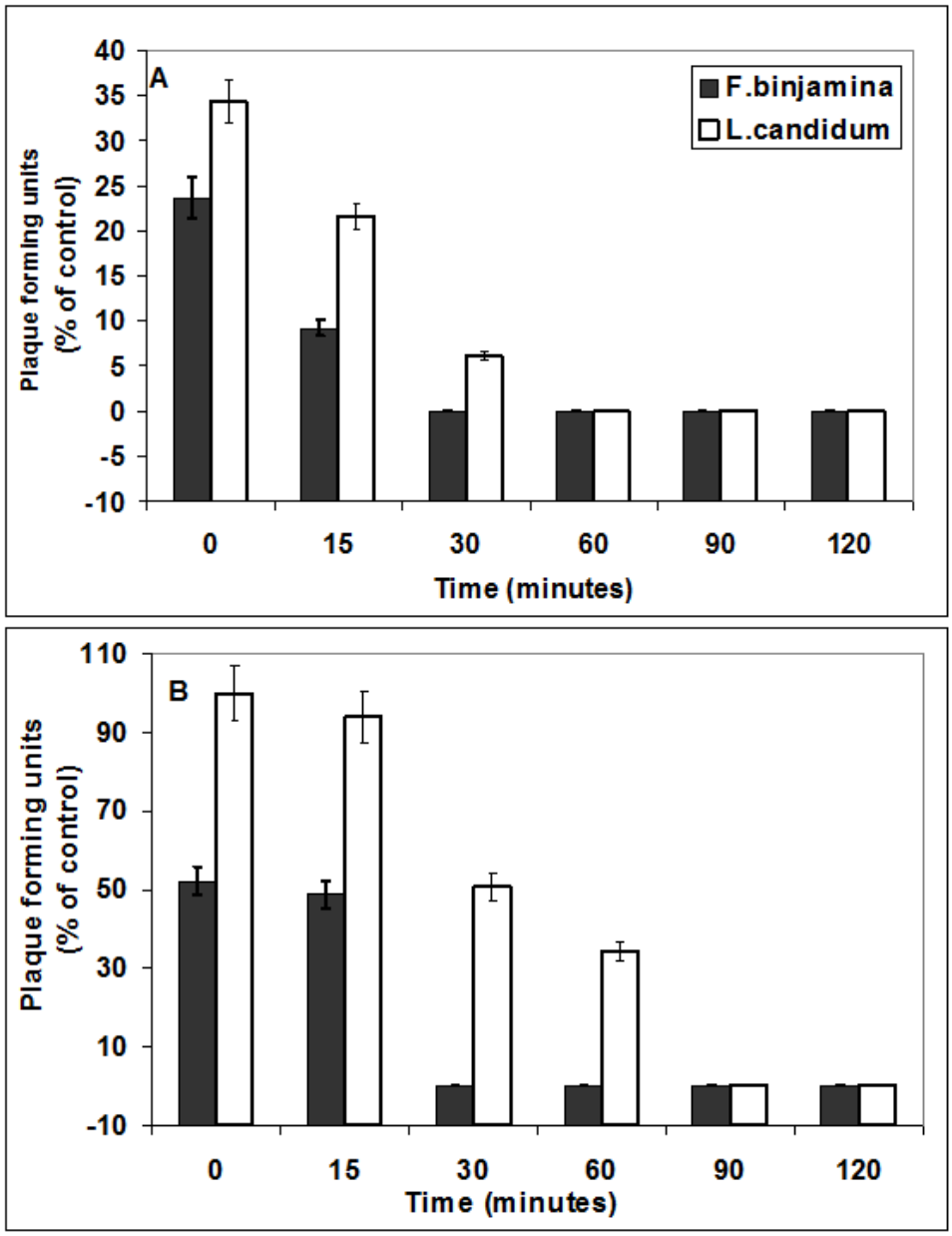


Figure 6. Effect on virus infectivity of preincubation of leaf extracts with HSV-1 (A) and HSV-2 (B). 1000 µg/ml of leaf extracts was incubated with the infecting virus (5 m.o.i.) at 4°C for different periods of time (15, 30, 60, 90 and 120 minutes). This mixture was diluted 10^4 times with fresh medium and the cells were infected with the diluted mixture. Plaque forming units were evaluated by the standard plaque assay and presented as mean ± SD (n=5).

3A3.2. Antiviral activity of *F. benjamina* and *L. candidum* ethanol extract fractions.

Trying to isolate components with effective antiviral activity, the extracts were, as a first step, separated into different fractions by reverse phase column with rising methanol gradient: 0%,

(v/v), 20%(v/v), 40%(v/v), 60 %(v/v), 80 %(v/v) and 100 %(v/v), (RP-C18 Sep-Pack, Supelco, St. Louis, MO).

Vero cells were treated with 10 µg/ml of leaf extract fractions of *F. benjamina* or *L.candidum* at the time of infection with 0.1 m.o.i. of the herpes viruses. The results (figure 7) showed that fraction eluted with 80%-MeOH of *F. benjamina* leaf extract completely blocked viral infection (by HSV-1 and -2) but only slightly inhibited VZV, whereas fractions 0 and 20% MeOH significantly inhibited VZV.

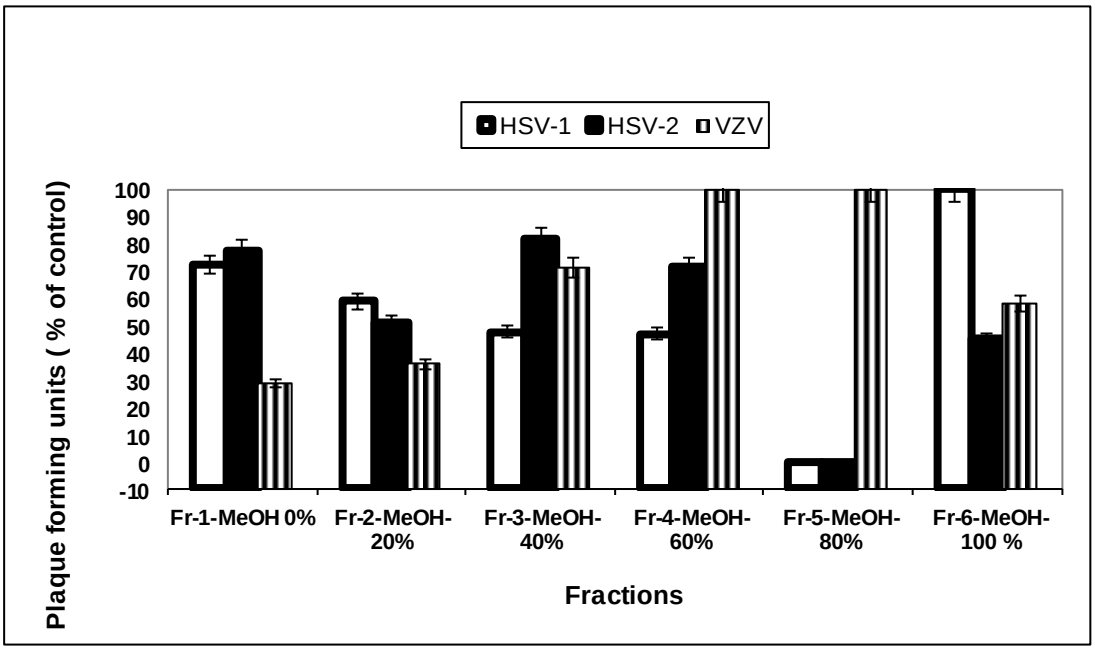


Figure 7. Antiviral activity of fractionated *F. benjamina* extract on HSV-1, HSV-2 and VZV infection. Vero cells were infected with 1 m.o.i. of the herpes viruses.in the presence of 10 µg/ml of the appropriate fractions of *F. benjamina* leaf extract. Plaque forming units of the treated culture are presented as a percentage of positive control (infected but untreated cell cultures). Values are mean ± SD (n=5).

The results presented in figure 8 further demonstrate that the fraction of *L. candidum* leaf extract eluted with 80%-MeOH completely blocked viral infection (by HSV-1 and -2) while the crude extract inhibited HSV-1 much better than HSV-2 (figure 5, B). Although the crude extract of *L. candidum* did not inhibit VZV(figure 5, B), the following fractions (0%-MeOH, 20%-MeOH and 100%-MeOH) significantly inhibited this viral infection (figure 8).

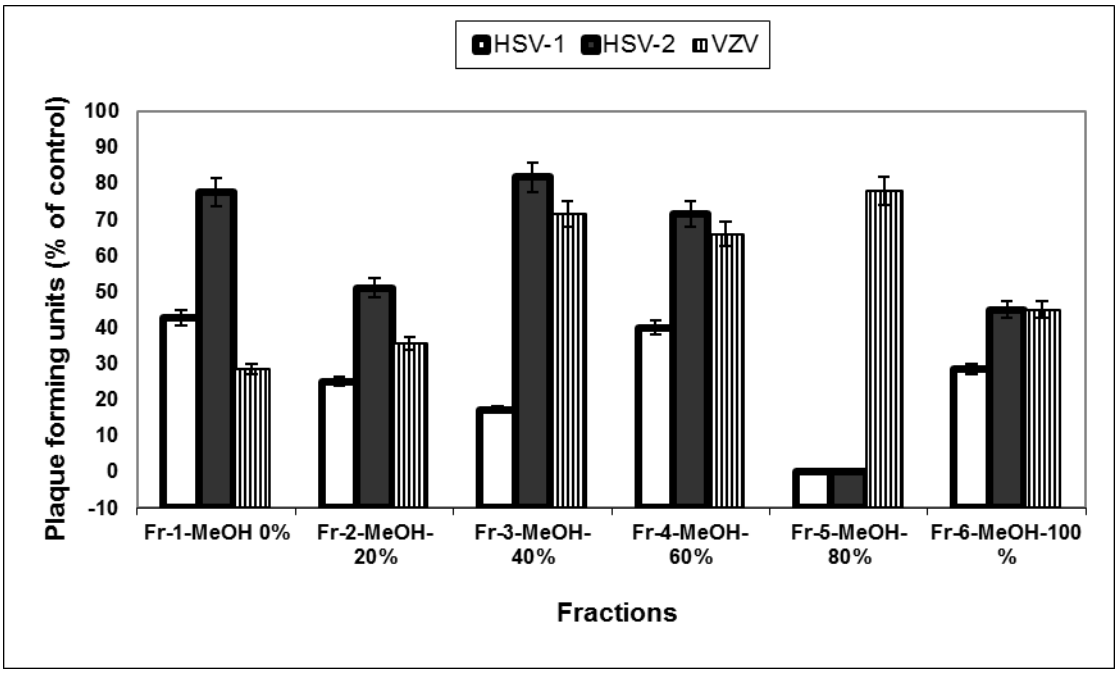


Figure 8. Antiviral activity of fractionated *L. candidum* extract on HSV-1, HSV-2 and VZV infection. Vero cells were infected with 1 m.o.i. of the HSV-1, HSV-2 and VZV in the presence of 10 µg/ml of the appropriate fractions of *L. candidum* leaf extract. Plaque forming units were evaluated by the standard plaque assay and presented as mean ± SD (n=5).

3A4. Isolation and identification of the antivirally active components of the 80%-MeOH fraction of *F. benjamina* leaf extract.

The most active fraction of *F. benjamina* ethanolic extract was the 80%-MeOH fraction as shown in figure 7. This fraction was subjected to repeated purification and HPLC to afford several flavonoids. In order to investigate which of these substances have promising antiviral activity, the antiviral effect of the separated and purified components was evaluated on HSV-1

in cell culture. Only three compounds demonstrated significant antiviral activity; their retention times were 32.34; 33.65 and 34.39 respectively (figure 9).

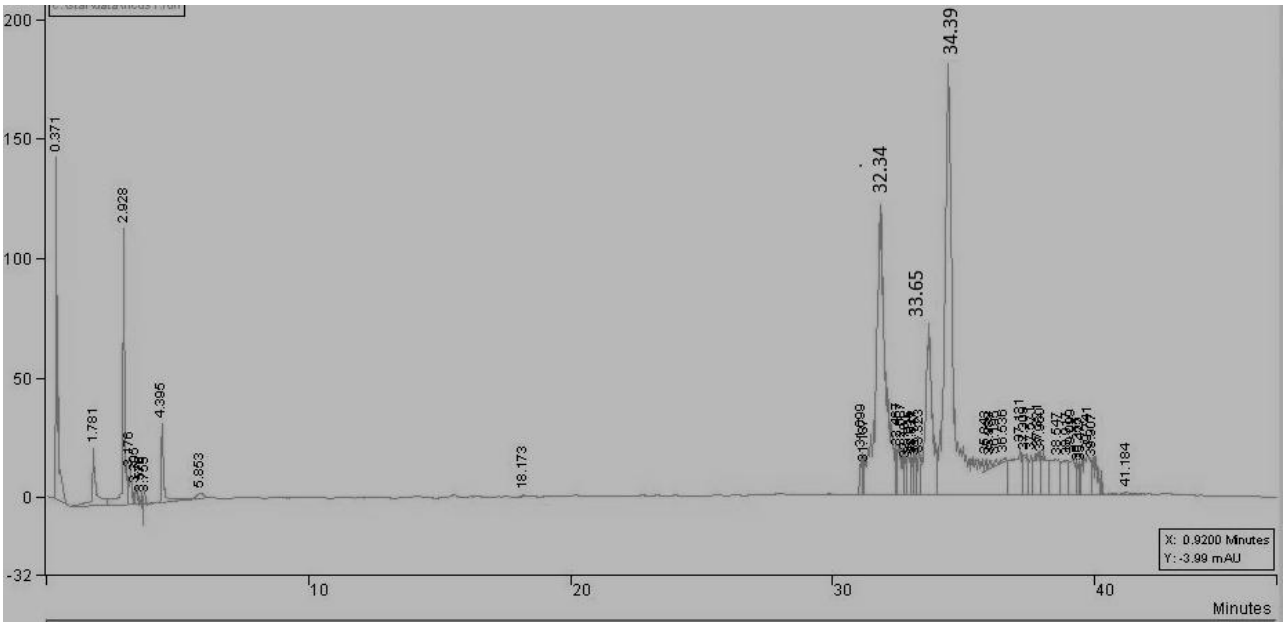


Figure 9. HPLC chromatograms of flavonoids in the extract of *F. benjamina.* The flavonoid fraction (80%-MeOH) was analyzed by HPLC. Gradient elution was performed with solution A, composed of water-acetic acid (97 : 3 V/V) and solution B – methanol. Leaf ethanol extracts of *F. benjamina* were subjected to repeated purification and HPLC for flavonoid collection . In order to investigate which substances most efficiently inhibited HSV-1, the antiviral activities of the separated and purified components were evaluated in cell culture. Three compounds, with retention times of 32.34; 33.65 and 34.39, with best antiviral activity were further purified and analysed.

3A4.1. Determination of the structure of antivirally active compounds of *F. benjamina.*

The structure of the above mentioned components of *F. benjamina* that exhibited enanced antiviral activity was determined by spectroscopic analyses including NMR and MS. The obtained results of these analyses proved the following composition of the different compounds.

Compound 1 (Quarcetin 3-O-α-rhamnopyranosyl(1--- 6)-β-glucopyranoside)

^{1}H-NMR (CD3OD, 500 MHz): σ7.6 (1H, dd, J=8.6 and 2 Hz, H-6'), 7.6 (1H. d, J=2.2 Hz, H-2'), 6.9 (1H, d, J=8.6 Hz, H-5'), 6.4 (1H, d, J=1.8 Hz, H-8), 6.3 (1H, d, J=1.7 Hz, H-6), 5.4 (1H, d, J=7.8 Hz, glucosyl H-1), 4.5 (1H, rhamnosyl H-1), 3.4 (m, sugar protons), 1.1 (3H, d, J=6 Hz, rhamnosyl-Me). ^{13}C-NMR (CD3OD, 125 MHz): σ 177.4 (C-4), 163.9 (C-7), 160.8 (C-5), 156.2 (C-2, C-9), 148.4 (C-4'), 145.0 (C-3'), 133.4 (C-3), 120.9 (C-1'), 120.1 (C-6'), 116 (C-5'), 155.4 (C-2'), 104.2 (C-10), 102.2 (C-1''), 100.0 (C-1'''), 98.8 (C-6), 93.8 (C-8), 73.4 (C-5''), 73.2 (C-3 ''), 72 (C-4'''), 71.1 (C-2''), 70.7, 70.5 (C-2''', C-3'''), 68.5 (C- 5'''), 68.2 (C-4''), 65.4 (C-6''), 17.9 (C-6'''). LC-ESI-MS/MS (ion trap) of m/z 609 [M-H]-, m/z: 301.0 [(Quarcetin-H)]- ; HR-MS *m/z* [M+Na]+ 633.1467 (calcd for $C_{27}H_{30}O_{16}Na$, 633.1426)

Compound 2 (Kaempferol 3-O-β-rhamnopyranosyl(1--- 6)-β-glucopyranoside)

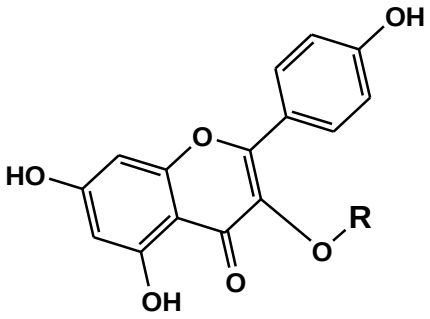
^{1}H-NMR (CD3OD, 500 MHz): σ 8.08 (2H, d, J=8.6Hz, H-2', H-6'), 6.92 (2H, d, J=8.6Hz, H-3', H-5'), 6.40 (1H, s, H-8), 6.23 (1H, s, H-6), 5.12 (1H, d, J=7.4Hz, H-1''), 4.54 (1H, broad s, H-1'''), 3.82-3.42 (10H, rhamnose and glucose). ^{13}C-NMR (CD3OD, 125 MHz): σ 179.2 (C-4), 166.4 (C-7), 162.7 (C-5), 159.53 (C-9), 158.66 (C-2), 135.7 (C-3), 133.0 (C-2', C-6') 122.6 (C-1'), 165.2 (C-3', C-5'), 104.6 (C-10), 102.5 (C-1'', C-1'''), 100.2 (C-6), 95.14 (C-8), 77.6 (C-5''), 77.4 (C-3 ''), 75.7 (C-4'''), 73.7 (C-2''), 72.4 (C-2''', C-3'''), 69.9 (C- 5'''), 68.3 (C-4''), 68.9 (C-6''), 17.9 (C-6'''). LC-ESI-MS/MS (ion trap) of m/z 593 [M-H]-, m/z : 447 [(M-H)-Rha]- ; 326.7 [(M-H)-Rha-120]- ; 284.7 [(kaempferol-H)]- ; 256.6 ; 228.6 ; 212.7. HR-MS *m/z* [M+Na]+ 617.1497 (calcd for $C_{27}H_{30}O_{15}Na$, 617.1478)

Compound 3 (Kaempferol 3-O-α-rhamnopyranosyl(1--- 6)-β-galactopyranoside)

^{1}H-NMR (CD3OD, 500 MHz): σ 8.06 (2H, d, J=8 Hz, H-2' and H-6'), 6.87 (2H, d, J=8Hz, H-3' and H-5'), 6.6 (1H, s, H-8), 6.2 (1H, s, H-6), 5.33 (1H, d, J=7Hz, galactosyl H-1), 4.41 (1H, s, rhamnosyl H-1), 1.07 (3H, d, J=6Hz, rhamnosyl-Me). ^{13}C-NMR (CD3OD, 125 MHz): σ177.7 (C-4), 164.2 (C-7), 161.4 (C-5), 160.1 (C-4'), 156.7 (C-9, C-2), 133.5 (C-3), 131.1 (C-6') 121.1 (C-1'), 115.2 (C-3', C-5'), 104.0 (C-10), 102.1 (C-1''), 100.1 (C-1'''), 98.8 (C-6), 94.2 (C-8), 73.8 (C-5''), 73.0 (C-3 ''), 72 (C-4'''), 71.2 (C-2''), 70.4 (C-2''', C-3'''), 68.5 (C- 5'''), 68.2 (C-4''), 65.6 (C-6''), 17.9 (C-6''').

LC-ESI-MS/MS (ion trap) of m/z 593 [M-H]-, m/z : 447 [(M-H)-Rha]- ; 326.8 [(M-H)-Rha-120]- ; 284.7 [(kaempferol-H)]- ; 254.6 ; 228.7 ; 210.6. HR-MS *m/z* [M+Na]+ 617.1498 (calcd for $C_{27}H_{30}O_{15}Na$, 617.1477).

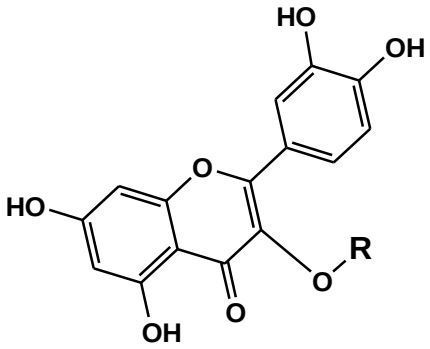
Nuclear magnetic resonance (NMR) and mass spectrometry (LC-ESI-MS, and MALDI) analyses established the structure of these three compounds as flavone glycosides (figure 10). Compound 1 was isolated as a yellow amorphous powder. Its molecular formula was $C_{27}H_{30}O_{16}$, which was determined on the basis of positive/negative ion m/z and supported by MALDI data and HR-MS. Quercetin 3-O-rutinoside with the same molecular formula was used as a standard. A comparison with this standard confirmed that Compound 1 was indeed Quercetin 3-O-rutinoside (rutine). The molecular formula $C_{27}H_{30}O_{15}$ was assigned to both Compound 2 and 3.



Kaempferol ; R = H

Compound 2; R = Glu-Rha

Compound 3; R = Gala-Rha



Quercetin ; R = H

Compound 1; R = Glu-Rha

Glu- glucose ; Gala-galactose ; Rha-Rhamnose

Figure 10. Chemical structure of compounds 1-3 and their aglycons, quercetine and kaempferol. The structure of the compounds was determined by spectroscopic analyses including NMR and MS.

3A4.2. Antiviral activity of flavonol glycosides from *F. benjamina* leaves and their aglycons, quercetin and kaempferol.

We evaluated the antiherpetic activity of the flavonoid glycosides that we have isolated from *F. benjamina* and compared their activity with those of the aglycons, quercetin and kaempferol.

Vero cell monolayers were treated with increasing concentrations of the appropriate compound at the time of infection with 1 m.o.i. of HSV-1. The treatment with the compound was terminated immediately post-infection and the antiviral activity was evaluated by plaque assay. The obtained results demonstrate significant and reproducible antiviral activity of *F. benjamina* compounds against HSV-1 (figures 11, 12). Quercetin 3-O-rutinoside was significantly more effective than quercetin (figure 11), and kaempferol 3-O-rutinoside in paticular, but also kaempferol 3-O-robinobioside were significantly more effective than kaempferol. Full inhibition of HSV-1 was obtained with about 10 μM of kaempferol 3-O-robinobioside, whereas kaempferol didn't manage to inhibit the virus completely because of its high toxicity (figure 12).In order to dertermine their SI values, the CC_{50} and IC_{50} of these compounds were determined and the Sis were calculated. For determining the IC_{50}, Vero cell monolayers were treated with different concentrations of the appropriate compound during and after infection with 1 m.o.i. of HSV-1 and HSV-2. The SI of all antiviral compounds from *F. benjamina* extracts tested in this study was significantly higher than that of quercetin or kaempferol. It should be noted that kaempferol 3-O-robinobioside showed the highest SI value in the case of HSV-1, which was similar to that of ACV (table 4). All these examined *F. benjamina* flavonoids were significantly less toxic than kaempferol ($p<0.001$), but had similar toxicity to that of quercetin (table 4).

Table 4 Cytotoxicity and anti-HSV-1 and HSV-2 activity of flavonol glycosides, quercetin and kaempferol from *F. benjamina* leaf extracts and from quercetin and kaempferol standards.

		HSV-1		HSV-2	
Compound (number)	**CC_{50} (μM)**	**EC_{50}(μM)**	**SI**	**EC_{50}(μM)**	**SI**
* Synthetic rutin	400	20	20		
Quercetin 3-O-rutinoside **(1)**	400±3.56	1.5 ±0.56	266.7	2.2 ±0.76	181.8
Kaempferol 3-O-rutinoside **(2)**	300±2.79	3.0 ±0.97	100.0	5.7±0.63	52.6
Kaempferol 3-O-robinobioside **(3)**	600±10.45	0.9±0.23	666.7	1.4 ±0.43	428.5
Quercetin	425 ±9.08	60±8.96	7.1	70±4.57	6.1
Kaempferol	80±2.58	25±3.29	3.2	34±3.96	2.4
ACV	70±0.65	0.1±0.04	700	0.2±0.06	280

Vero cells were treated with the tested compounds at different concentrations for 4 days in order to determine CC_{50}. The cells were counted by Neubauer hemacytometer indicating their replication rate. Viability of cells was evaluated by MTT assay. For determining their antiviral activity, Vero cells monolayers were treated with different doses of the tested compounds at the time of infection and after infection with 1 m.o.i. of HSV-1 and HSV-2. Antiviral activity was evaluated by plaque assay. SI (Selectivity index) was determined as CC_{50}/EC_{50}. Values are mean ± SD (n=5). * Discrepancy was observed between the SI values obtained from the synthetic rutin and compound 1 (quercetin 3-O-rutinoside). Since the amount of compound 1 obtained from the plant was very little, some imprecisions could have occurred during the weighting process.

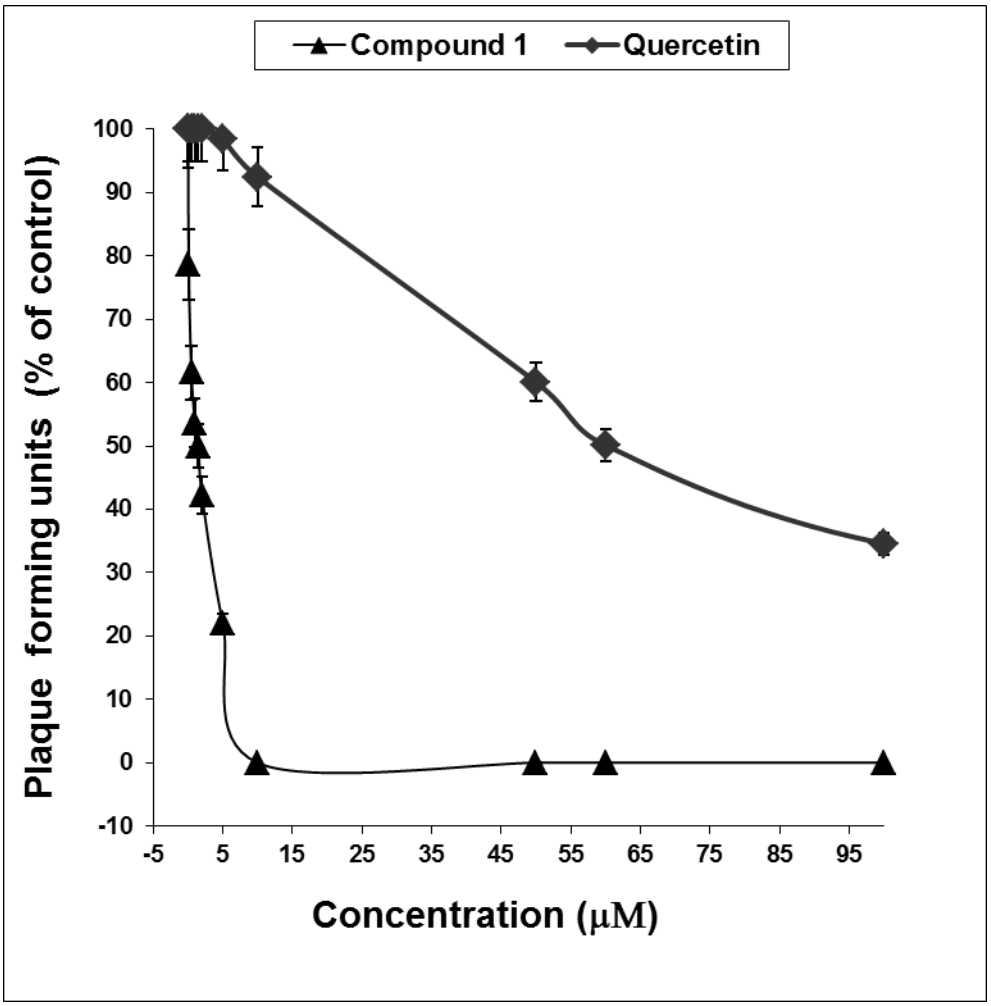


Figure 11. Antiviral activity of Quercetin 3-O-rutinoside (compound 1) and quercetin on HSV-1. Vero cells were infected with 1m.o.i. of HSV-1 in the presence or absence of increasing doses of compound 1 and quercetin. PFU of the treated cultures is presented as a percentage of the positive control (infected but untreated cell cultures). Values are presented as mean ± SD (n=5).

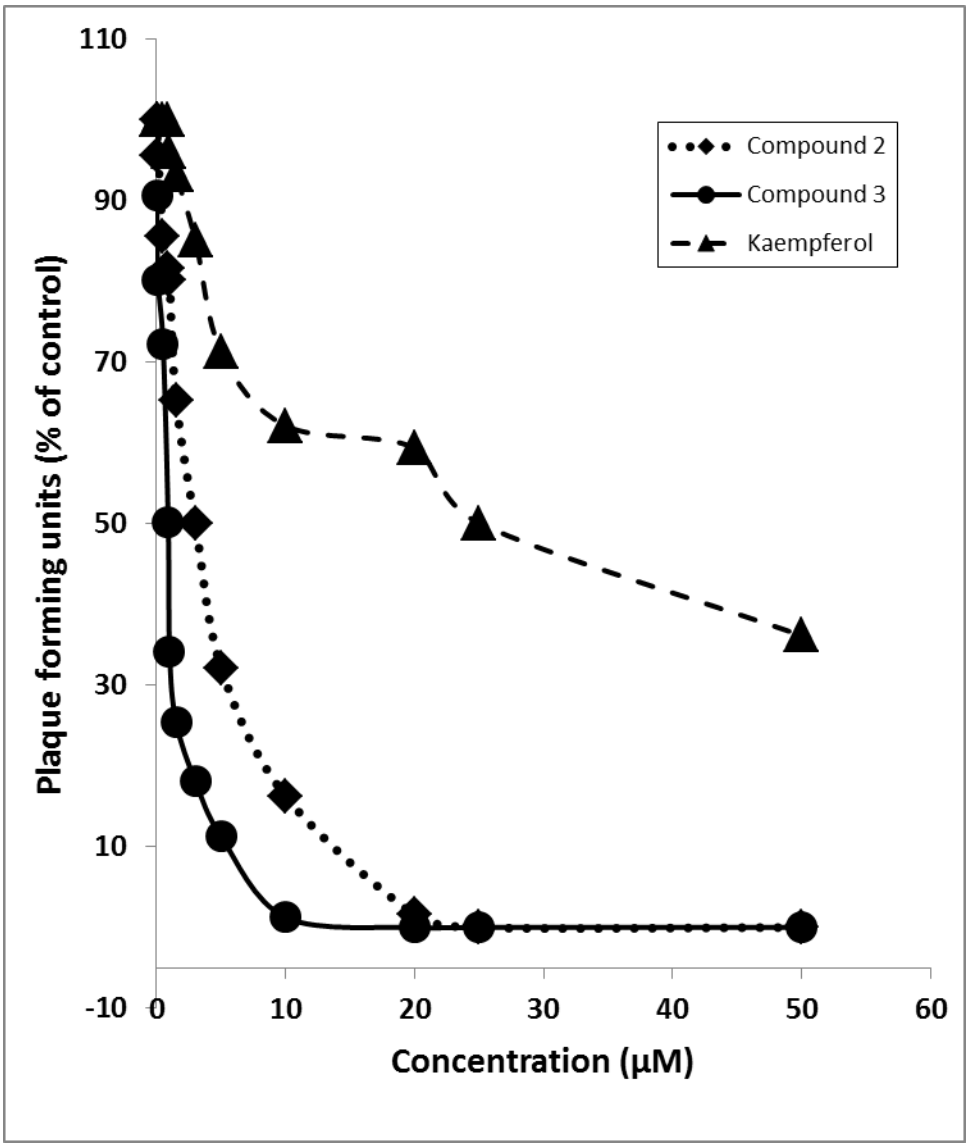


Figure 12. Antiviral activity of kaempferol 3-O-rutinoside (compound 2) , kaempferol 3-O-robinobioside (compound 3) and kaempherol on HSV-1. Vero cells were infected with 1m.o.i. of HSV-1 in the presence or absence of increasing doses of compound 2, compound 3 and kaempherol. PFU of the treated cultures is presented as a percentage of the positive control (infected but untreated cell cultures). Values are presented as mean ± SD (n=5).

3A5. Antiviral compound from *L. candidum* leaves.

The most active fraction of *L. candidum* leaf ethanolic extract was 80%-MeOH one. This fraction was subjected to repeated purification and HPLC to afford several flavonoids (figure 13). Several flavonoids with known molecular formulas were used as a standard.The numbered peaks corresponded to the compounds that were determined, but only compound number 5 demonstrated antiviral properties. A comparison with the available standards revealed that compound 5 is kaempferol (figure 13). Its antiviral properties were tested (figure 12). The antiviral activities of other components (figure 13) were evaluated in cell culture but they did not have any anti-herpetic activity.

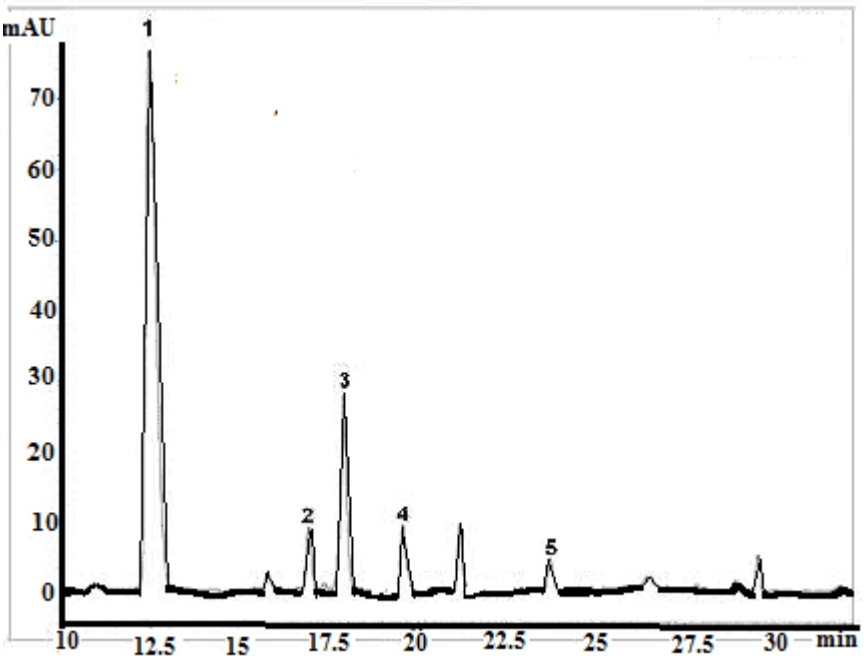


Figure 13. HPLC chromatograms of flavonoids in the extract of *L candidum*. The flavonoid fraction was analyzed by HPLC. Gradient elution was performed with solution A, composed of water-acetic acid (97 : 3 V/V) and solution B – methanol.

Part B. Retroviruses.

3B1. Cytotoxicity examination

Different concentrations of *F. benjamina* leaf ethanolic crude extract and its fractions were added to NIH/3T3 cell monolayers for three days and their cytotoxicity was evaluated as described in Methods section. CC_{50} value of the ethanolic crude extract was about 300µg/ml, whereas the CC_{50} of most of the fractions was higher than 800 µg/ml, except fractions 0%-MeOH and 100%- MeOH, which had much lower CC_{50} values (200 and 350µg/ml, respectively) (figure 14).

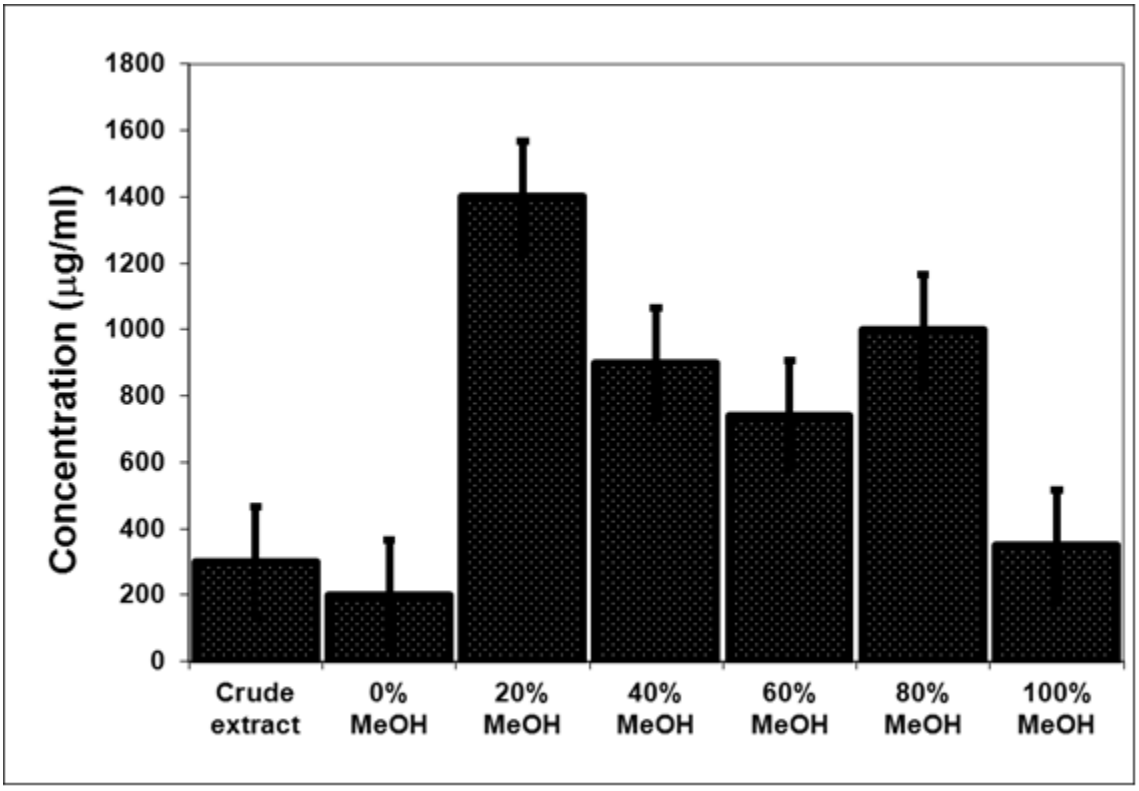


Figure 14. CC_{50} of *F. benjamina* ethanolic crude extract and its fractions . NIH/3T3 cells were treated with crude extract and its fractions at different concentrations (1-1500 µg/ml) for 3 days. Data are presented as mean ± SD of six independent experiments.

3B2. Characterization of cell transformation by MuSV-124

Normal untransformed NIH/3T3 cells which are grown on plastic dishes in RPMI medium with 10% NBCS appear as flat cells (figure 15, A) and are unable to grow in soft agar at all. When these cells were infected with an appropriate dilution of MuSV-124, tiny foci of transformed cells, with a highly refractile spindle shape, growing randomly in a criss-cross fashion, could be detected by microscopic observation within three to five days p.i. (Fig 15, B). With time, these foci gradually increased in size and compactness until they became visible to the naked eye on day 10 to 12 p.i. The actual number of foci in these cultures remained unchanged during the entire observation time. When the cells were infected with a high titer of MuSV-124 (1 FFU/cell), most of the cells were transformed two to three days p.i.

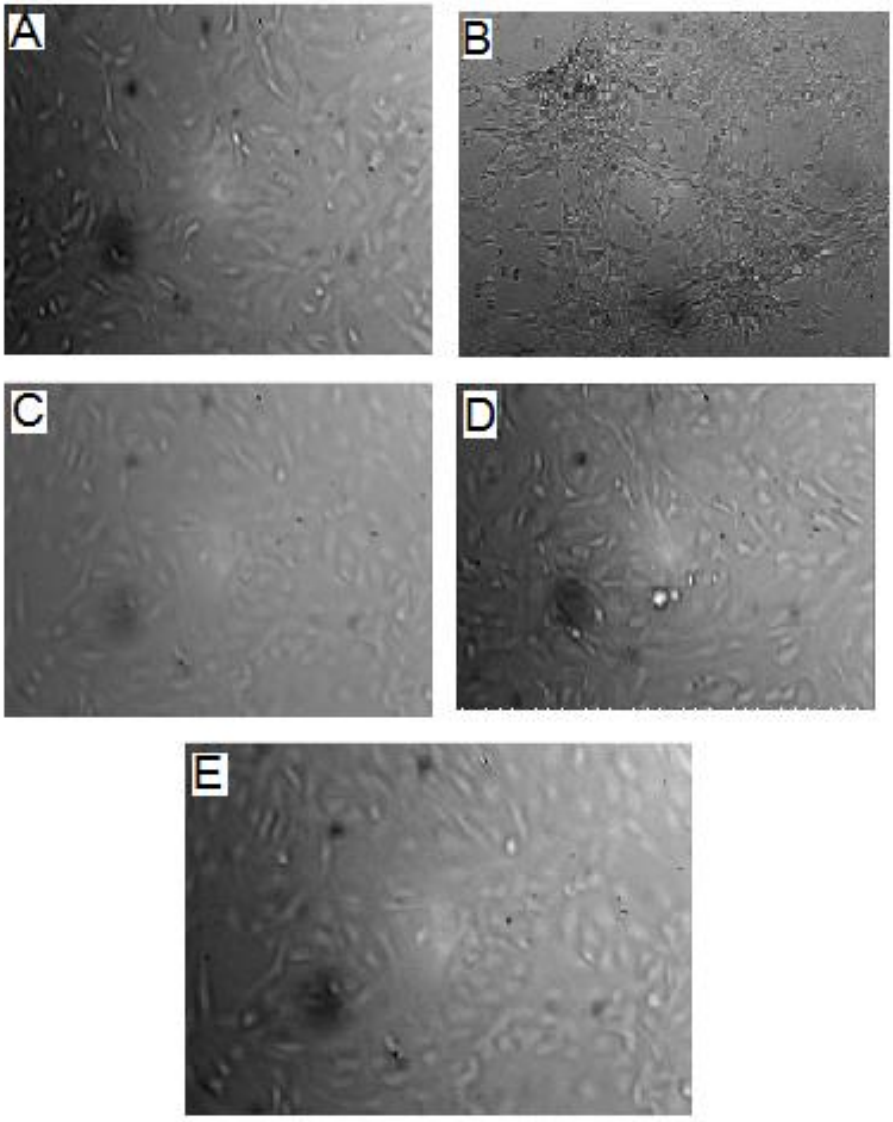


Figure 15. Morphological changes in NIH/3T3 cells as a result of infection with MuSV with or without treatment with the extract or its fractions as observed by optical microscopy. (A) normal untransformed NIH/3T3 cells; (B) infected NIH/3T3 cells with 1FFU/cell of MuSV; (C) infected NIH/3T3 cells with 1FFU/cell of MuSV treated with *F. benjamina* crude extract at the time of infection and p.i.; (D) infected NIH/3T3 cells with 1FFU/cell of MuSV treated with 20%-MeOH fraction at the time of infection and p.i. and (E) infected NIH/3T3 cells with 1FFU/cell of MuSV treated with 60%-MeOH fraction at the time of infection and p.i.

3B3. Effect of product dosage on cell transformation

NIH/3T3 cells were infected with 0.1 FFU/cell of MuSV and treated with the appropriate doses of *F. benjamina* crude extract or its fractions. The treatment was continued until the end of the experiment. At seven days p.i the number of formed foci in each culture was counted.

The results presented in Table 5 demonstrate that *F. benjamina* crude extract was able to totally prevent cell malignant transformation at a concentration of 15 µg/ml (Fig. 15, C). Similarly, the 20%-MeOH and 60%-MeOH fractions fully inhibited malignant cell transformations at a concentration of 10 µg/ml (Table 5, Fig 15, D and E), while the other tested fractions had no effect on cell malignant transformation at this concentration (Table 5) nor at higher concentrations (data not shown).

Table 5. The effect of *F. benjamina* ethanolic crude extract and its fractions on malignant cell transformation by MSV

Extract/fraction	Concentration (µg/ml)	FFU
None	0	74.3±3.25
Crude extract	1	73.5±4.28
	5	53.5±3.49
	10	25.4±9.56
	15	0.5±0.08
	20	0
0%-MeOH	10	75.7±9.07
20%-MeOH	10	0
40%-MeOH	10	78 .2±5.89
60%-MeOH	10	0
80%-MeOH	10	72 .7±5.19
100%-MeOH	10	73.8±2.84

NIH/3T3 monolayers were treated with different concentrations (1-20 µg/ml) of *F. benjamina* ethanolic crude extract and its fractions at a concentration of 10 µg/ml, 2 h before, during and after infection with 0.1 FFU/cell of

MuSV. At seven days p.i the number of formed foci in each culture was counted. Data are presented as mean ± SD of three independent experiments. *F. benjamina* crude extract was effective at concentration 5 µg/ml and was able to totally prevent cell malignant transformation at a concentration of 15 µg/ml. The 20%-MeOH and 60%-MeOH fractions fully inhibited malignant cell transformations at a concentration of 10 µg/ml, while other fractions did not inhibit cell transformations.

3B4. Effect of timing of the crude extract and its effective fractions addition on cell transformation by MuSV

In order to investigate the antiviral mechanism of the *F. benjamina* crude extract and its effective fractions against MuSV, NIH/3T3 cells were treated with 20 µg/ml extract or 10 µg/ml of the appropriate fraction before, during and at various times (2, 24, 48 and 72 h) p.i. Cell reatment with *F. benjamina* crude extract before infection, exclusively during infection or even 2h p.i. yielded almost full prevention of malignant cell transformation by this virus ($p<0.001$, figure 16). When the cells were treated with the crude extract 24h p.i., a partial prevention of cell transformation was obtained. Treatment with the crude extract at 48h or later p.i. provided almost no significant inhibitory effect on cell transformation.

Fraction 20%-MeOH was effective only when the cells were treated before or during infection, while fraction 60% -MeOH fully inhibited malignant cell transformations when it was added during ($p<0.001$) or at 2 h p.i. ($p<0.001$). When the cells were treated with this fraction (60% -MeOH) at 24 p.i. a 65% inhibition of cell transformation was obtained.

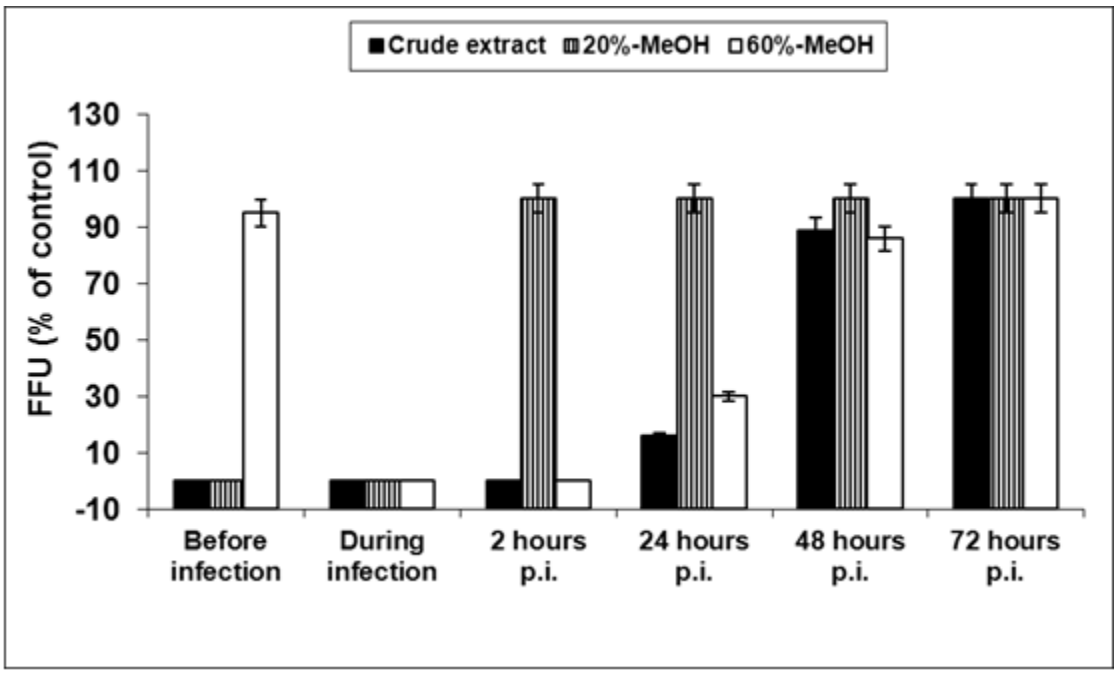


Figure 16.Timing effect of ethanolic crude extract and its fractions addition on cell transformation by MuSV infection. NIH/3T3 cells were treated with 20 µg/ml crude extract or 10 µg/ml of the appropriate fraction at various times before, during or p.i with 0.1moi of MuSV. The treatment was continued to the end of the experiment. Cell transformation was evaluated by counting the number of foci at 7 days p.i. Data are presented as mean ± SD of eight independent experiments

3B5. Effect of 60 %-MeOH fraction removal on cell transformation

NIH/3T3 cells were infected and treated with 10 µg/ml of the 60% -MeOH fraction immediately p.i. The treatment was terminated at different timepoints: 1, 2, 6 and 24 hours p.i.. When the treatment was terminated at 1 or 2 hours p.i., no inhibitory effect of the fraction on cell transformation was obtained (figure 17). Removal of the fraction 6 hours p.i. induced only partial (20%) inhibition of malignant cell transformation. However when the same fraction was removed at 24 h p.i. or later, full prevention of malignant cell transformation was obtained.

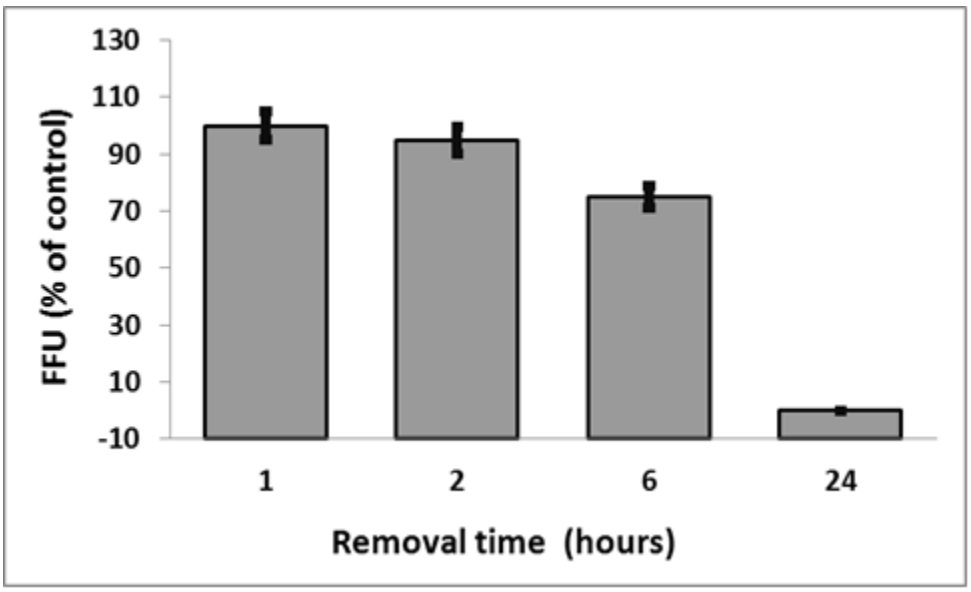


Figure 17. Effect of 60%-MeOH fraction removal on cell transformation by MuSV. NIH/3T3 cells were treated with10 µg/ml of the 60%-MeOH fraction immediately after 2 hours of infection with 0.1FFU of MuSV. The treatment was removed at different timepoints p.i. Data are presented as mean ± SD of three independent experiments.

4. Discussion

This research provides new information on the biological properties of several plants (*F. benjamina, L. candidum, C. fragrans* and *S. chinensis*) against different members of the herpes virus and retrovirus families. As a first step various extracts from different parts of these plants were prepared and examined for their antiviral activity. *F. benjamina* and *L. candidum* ethanol leaf extracts were the most effective against HSV-1, selectivity index of ethanol leaf extract of *F. benjamina* was higher than that of ACV (table 2), so further study was focused on only these extracts.

We demonstrate herein that leaf ethanolic extract of *F. benjamina* effectively inhibited infection of Vero cells by HSV-1, HSV-2 and VZV *in vitro*. The leaf extracts of *L. candidum* strongly inhibited HSV-1 and HSV-2 and had no effect on VZV (figure 5, table 2). Although HSV-1, HSV-2 and VZV belong to the subfamily *Alphaherpesviridae* and have many similar characteristics, they have different clinical manifestations, as well as different biochemical and serological properties (Field *et al.*, 2006) and (Wyrwicz and Rychlewski, 2007). Previous studies have reported also differential antiviral activity of plant extracts on HSV-1, HSV-2 and VZV. For instance, an aqueous extract of Yin Chen Hao Tang (YCHT), a Chinese prescription containing *Artemisia capillaries, Rheum officinale*, and *Gardenia jasminoids* inhibited HSV-2 infection more effectively than HSV-1 *in vitro* (Cheng *et al,* 2008). Flavonoids from *Capparis spinosa* buds were active against HSV-2, but did not show activity against HSV-1 (Arena *et al,* 2008).

Amenthoflavone and robustflavone which were isolated from *Rhus succedanea* demonstrated moderate inhibition of HSV-1 and HSV-2 but did not affect VZV infection. Rhustflavanone inhibited only HSV-2 whereas succedaneaflavanonol inhibited only VZV (Lin *et al.*, 1999). Acetone, ethanol and methanol extracts of *Phyllanthus urinaria* and two compounds isolated from this plant (hippomanin and excoecarianin) inhibited HSV-2 but not HSV-1 infection

(Yang *et al,* 2005, Yang *et al,* 2007, Cheng *et al,* 2011).

While ACV inhibits viral DNA synthesis (Rang *et al.*, 2003), plant extracts are thought to exert their inhibitory action at a very early stage in the viral infection cycle that is virus adsorption

onto and/or penetration into the host cell (Li *et al*, 2005) . In agreement with these findings our results showed that the tested plant extracts exert their antiherpetic effect mainly by direct interaction with the virus particles and by blocking virus access to the host cells. We showed a direct effect of the plant extracts on the herpes viruses, which significantly inhibited the infection (figure 6). It seems probable that this effect is caused either by a strong interaction (maybe irreversible) between the virus and the extract, the effect being maintained after several serial dilutions, or by direct inactivation of the virus. These results are in agreement with those obtained by Cheng *et al*, 2008, who showed that the aqueous extract of YCHT inhibited HSV-1 and HSV-2 infections probably through direct inactivation of the virus infectivity. Our results also showed a weak inhibition of the viral infection in plant extract pretreated cell cultures (table 3). This slight inhibition could be explained by a weak or reversible interaction between the extract and the cell membrane.

Although the mode of action of the plant extract's activity has been partially revealed, the picture is far from clear due to the likely possibility that multiple mechanisms may exist.

The multiple mechanisms may be connected with the presence of various groups of active compounds. The most active compounds in both extracts were flavonoids (figures 7,8), so we focused on the isolation of these compounds; the other fractions showed some antiherpetic effect, for example fraction 40%-MeOH of *L. candidum* was highly effective against HSV-1 (figure 8).

Figures 7 and 8 demonstrate that fractions 20%-MeOH, which are rich with polysaccharides, were effective (at least 50% reduction in plaque forming) in both extracts against HSV-1, 2 and VZV. This activity may be connected with the ability of polysaccharides to inhibit HSV-1 replication prior to onset of the late viral protein synthesis (Naithani et al, 2008).

The antiherpetic properties of plant compounds could be explained, among others, by the antioxidant activity of phytochemicals and scavenging capacities (Naithani et al, 2008). It was also shown that some flavonoids of extracts of *Achyrocline satureioides* are able to interfere with the events occurring between the third and ninth hour of HSV-1 replication cycle, which includes transcription and translation of viral proteins (Bettega *et al*, 2004).

Bioassay guided fractionation of the ethanolic extract of *F. benjamina* leaves led to the isolation of three flavone glycosides; (1) quercetin 3-O-rutinoside , (2) kaempferol 3-O-rutinoside and (3) kaempferol 3-O-robinobioside (figure 10) with high antiviral activity against herpes viruses (figures 11, 12).

The biological activity of Quercetin 3-O-rutinoside has been investigated in many plants showing antioxidant (Adesino *et al,* 2000, Chiang *et al,* 2004, Olivera *et al,* 2009, Nazimeyeh *et al,* 2010), antidiabetic (Olivera *et al,* 2009) and antiobessity properties (Goo *et al,* 2009). In addition, this compound was described as a UV-absorbing substance (Iwashina *et al,* 2004). Its antiviral properties, however, were poorly studied, and only anti-HIV effect has been demonstrated (Goo *et al,* 2009).

The antiviral properties of other flavonol glycosides of *F. benjamina* were never tested, to the best of our knowledge. Compound 2, kaempferol 3-O-rutinoside has been isolated from many plants, its antimicrobial activity has been analyzed and reported (Olivera *et al,* 2009), but no information is available on its antiviral activity. Kaempferol 3-O-rutinoside, isolated from *Alternanthera brasiliana*, has also been shown to inhibit human lymphocyte proliferation *in vitro* (Brochado *et al*, 2004) and to have antioxidant and radical scavenging activities (Fiorentino *et al,* 2007).

It is worthwhile also to mention that the ethanolic crude extract of F. benjamina was highly effective against HSV-1, HSV-2 and VZV reaching significantly higher SIs than that of ACV (table 2). Although the three flavone glycosides (components 1, 2 and 3) from F. benjamina showed lower SIs against both HSV-1 and HSV-2 than the crude extract (table 4), component 3 was the most effective with similar SIs to that obtained with ACV (table 4). In addition, although figure 7 demonstrates that the most effective fraction of F. benjamina ethanolic extract against HSV-1, HSV-2 was the 80%-MeOH but still several other fractions (20%-MeOH, 40%-MeOH and 60%-MeOH) were also effective against these viruses. These results proved possible presence of other antiviral compounds in the crude extract which may contribute to the higher SI of the crude extract compared to the pure compounds. This is in addition to the possibility of probable interactions between different components of the crude extract which also may contribute to its antiviral activity. Further research is required for their identification.

Although antiherpetic activity of the aglycons, quercetin and kaempferol has been previously demonstrated (Khan *et al*, 2005, Lyu *et al*, 2005, Schnitzler *et al*, 2010), their mechanism of action is still unknown. It has been suggested that quercetin intervenes in the events occurring between the third and ninth hour of the HSV-1 cycle, which includes transcription and translation of the viral proteins (Bettega *et al*, 2004). However, this hypothesis requires further investigation since this study was performed with plant extracts containing quercetin and other flavonoids, which could have interfered in the results, as well. In the present study we showed that structural changes in the aglycons, quercetin and kaempferol (addition of two sugars), caused a drastic improvement in their antiviral activity. The potent inhibitory activity of sulphated polysaccharides against different viruses, including HSV-1, has been previously reported and explained by the strong interaction between the negatively charged polysaccharides and the positively charged enveloped virus particles (Huleihel *et al*, 2001).

In addition, previous studies with different viruses showed that flavonoids affect different steps during the replication cycle of these viruses, such as the attachment of the viruses to cell membranes, entry into the cell, genome replication, viral protein translation and formation of certain glycoprotein complexes of the virus envelope (Chattopadhyay and Khan, 2008, Mukhtar et al, 2008, Naithani et al, 2008).

It was demonstrated that antiviral properties of *L. candidum* against herpes viruses are explained by the presence of kaempferol in the leaves (figure 13), which are generally poorly researched. In line with our foundings, kaempherol isolated from *L. candidum* bulbs showed carcinogenic and inhibitory activity (Vachalkova et al, 2011).

We examined the activity of the ethanol crude extract from *F. benjamina* leaves against malignant cell transformation induced by MuSV *in vitro*. Our results demonstrated a potent and promising anti-transforming activity of the crude extract (table 5). Several fractions were isolated from this extract and evaluated for their anti-transforming activity, but only two of them were found highly active: the 20% -MeOH fraction, rich in polysaccharides and the 60% -MeOH fraction, rich in polyphenols (table 5). These fractions also showed very low cytotoxicity (figure 19). We suggested that the anti-transforming mechanism of action of the 20% and 60% -MeOH fractions is different, probably due to the different composition of the fractions. The 20% -

MeOH fraction, which is mainly comprised of polysaccharides, was effective only when added to the cell prior to or at the time of infection. On the other hand, the 60% -MeOH fraction, which is largely composed of polyphenols, was highly effective even when added at 24 h p.i. (figure 16). Apparently, the 20% -MeOH fraction mainly affects the very early steps of the virus replication cycle, such as viral adsorption and penetration into the host cells, whereas the 60% -MeOH fraction affects later steps of the viral infection after its penetration into the host cell.

These results are in agreement with many previously published studies showing that polysaccharides from various origins were able to prevent diverse viral infections, most likely due to inhibiting their attachment and penetration into the host cells (Garcio *et al,* 1999, Huleihel *et al,* 2001, Saha *et al,* 2010, Chen *et al,* 2010, Bandyopadhyay *et al,* 2011).

In addition, it has been demonstrated that sulfated microalgal polysaccharides significantly inhibited cell transformation by MuSV (Talyshinsky *et al,* 2002). It was suggested that these polysaccharides may affect more than one step during retrovirus life cycle, including the prevention of its penetration into the host cells and the inhibition of event/s occurring after the proviral integration into the host cell genome (Rechter *et al,* 2006, Li *et al,* 2010).

Polyphenols obtained from different origins were shown to provide effective antiviral activity against a variety of viruses (Jain *et al,* 2008, Ho *et al,* 2009, Sandararajan *et al,* 2010, Kwon *et al,* 2010). For instance, several polyphenols were isolated from the roots of *Glycyrrhiza uralensis* and showed potent anti-rotavirus activity (Kwon *et al,* 2010). Large amounts of polyphenols were found in extracts of *F. benjamina* grandular epithelium, but their antiviral activity was not examined (Pennisi *et al.,* 1997). The mechanism of action of these polyphenols as antiviral agents is not fully understood, although in some cases, it has been proposed that early stages during the viral infection cycle, such as adsorption and penetration into the host cells, were affected (Kwon *et al,* 2010). In other cases, polyphenols were proven to affect internal steps during virus replication inside the host cells (Ho *et al,* 2009, Kwon *et al,* 2010). Although anticancer activity of polyphenols has been previously demonstrated (Pesakhov *et al,* 2010, Chen *et al,* 2011), there were no reports on their potential activity against retroviruses or against the transforming competence of retroviruses.

Our results showed that treating infected cells with the 60% -MeOH fraction for only 1 or 2h p.i. had no inhibitory effect on the transforming ability of MuSV, while treatment with the same fraction for 6 h p.i. provided partial inhibition about 20%, (figure 17). Treatment with the same fraction for 24h p.i. or more caused a full irreversible prevention of cell transformation by MuSV. The fact that the inhibitory effect of the 60% -MeOH fraction on cell transformation by MuSV was irreversible suggests that this fraction prevents proviral integration into the host cell genome or some other early step before the proviral integration.

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תקציר

כבר בזמנים עתיקים השתמשו ברפואה העממית בצמחי מרפא. צמחים אלו ידועים בתכונותיהם האנטי-ויראליות בנוסף לסגולות ביולוגיות אחרות שיש להם דוגמת פעילות אנטי-סרטנית, אנטי-בקטריאלית ואנטי-דלקתית. עד כה נחקרו צמחים אחדים כדי לבדוק אם הם עשויים להיות מקור של חומרים אנטי-ויראליים, אך עדיין נותרו צמחים רבים שניתן לחקור.

הנגיפים נחשבים לאחד מגורמי המחלות הקשות אצל בני אדם. אמנם יש חיסונים יעילים נגד כמה נגיפים, אך נגד רובם כמו למשל איידס והרפס אין תרופות יעילות וחיסונים זמינים. נגיפי ההרפס אחראים למחלות רבות כגון HSV-1, HSV-2 ו-VZV הגורמות פצעים מכאיבים בעור באזורים שונים בגוף בין היתר בעיניים ובמערכת המין. למרות קיומה של תרופה יעילה נגד נגיפי ההרפס – אציקלוויר, יש צורך למצוא תרופות יעילות נגד המוטנטים הרבים שלהם המפתחים עמידות אליה. חומרים טבעיים בעלי פעילות ביולוגית שמקורם בצמחים הם בסיס מצוין לתרופות נגד מחלות רבות. על פי רוב, אין קושי להפיק חומרים טבעיים מצמחים, כמו כן הם זולים ותופעות הלוואי הלא-רצויות שלהם מועטות מאוד בהשוואה לחומרים כימיים דוגמת אציקלוויר.

אף על פי ש-15% מגידולי סרטן קשורים להדבקה ויראלית, מעט מאוד מאמרים עוסקים ביעילותם של צמחי מרפא בהתמודדות עם המחלה. מטרתו העיקרית של מחקר זה היא לזהות חומרים טבעיים בעלי פעילות אנטי-ויראלית גבוהה נגד נגיפים שונים ובעיקר נגד נגיפי הרפס ורטרו-וירוסים, ולהעריך את מידת פעילותם. מטרותיו הנוספות של המחקר הן: לסקור ולהעריך את פעילותם האנטי-ויראלית של מיצויים מצמחים שונים נגד נציגים ממשפחת ההרפס (HSV-1&2 ו-VZV) וממשפחת הרטרו-וירוסים (MuSV and MuLV); להפריד ולזהות חומרים אנטי-ויראליים מצמחים נבחרים; לחקור את המנגנון האנטי-ויראלי של חומרים אלו.

במחקר זה נבדקו in vitro מיצויים של הצמחים *Ficus benjamina, Lilium candidum, Callissia fragrans* ו-*Simnondsia chinensis*. המיצויים הם מחלקים שונים של הצמחים: בצלים, גבעולים, עלים, פרחים ופֵּרות.

הנחת היסוד היא כי צמחים היציבים לדבקה עם נגיפים של צמחים מסוגלים ליצור חומרים אנטי-ויראליים המשפיעים על נגיפים התוקפים בעלי חיים ובני אדם. ה-*Ficus benjamina* אינו רגיש לנגיפים של צמחים בניגוד לצמחים אחרים ממשפחת ה-*Ficus*.

התוצאות שהתקבלו הראו כי הצמחים *Lilium candidum* ו-*Ficus benjamina* היו היעילים ביותר לעומת השאר ולכן המחקר התמקד בהם לאחר מכן. פעילותם של מיצויים אלכוהוליים מהם ופרקציות שלהם נבדקו in vitro בהתמודדותם עם HSV-1, HSV-2 ו-VZV ועם נציגים ממשפחת הרטרו-וירוסים Murine leukemia (MuLV) and sarcoma viruses (MuSV). המיצויים של *Ficus benjamina* הצליחו לעכב את כל הנגיפים האלו ואילו המיצויים של *Lilium candidum* הצליחו לעכב רק HSV-1 ו-HSV-2. פרקציאנציית מיצוי של *Ficus benjamina* הראתה שפרקציית פלוונוידים הייתה פעילה רק נגד HSV-1 ו-HSV-2. באנליזה ספקטרוסקופית כולל NMR ו-MS הופרדו וזוהו שלושה חומרים: (1) quercetin 3-O-rutinoside, (2) kaempferol 3-O-rutinoside and (3) kaempferol 3-O-robinobioside. התגלה שחומרים אלו יעלים מאוד נגד HSV-1 ו-HSV-2 ואינדקס הסלקטיביות שלהם (SI) גבוה: 266, 100 ו-666 בהתאמה לסדרם שלעיל. בהשוואה לאגליכונים שלהם quercetin (SI=7.1I) ו-kaempferol (SI=3.2) הם הראו פעילות רבה ויעילה יותר לאין שיעור.

הפרקציה Me-OH 20% (פרקציה של פולי-סוכרים) הצליחה לעכב VZV, MuSV, MuLV והפרקציה 60% Me-OH (פרקציה של פוליפנולים) הצליחה לעכב רק MuSV ו-MuLV באופן ניכר.

החומר הפעיל ביותר נגד נגיפי ההרפס שהופק ממיצוי אלכוהולי של *Lilium candidum* הוא kaempherol. פעילותם האנטי-ויראלית הגבוהה והטוקסיות הנמוכה של חומרים שהופקו ממיצוי אלכוהולי של ה- *Ficus benjamina* מגבירים את האפשרות להפיק בהצלחה מצמחים חומרים אנטי-ויראליים ואולי אף אנטי-סרטניים.