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„Antiviral activity of selected essential oils and terpenes“

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For my mother

Abstract

Essential oils, originating from medicinal plants are known for their antiviral properties and have been used in the traditional medicine for many thousand years. Many studies report the in vitro antiviral activity of essential oils and their components. Most of them are tested on herpes simplex virus type 1 and type 2 and influenza virus and exhibit a high antiviral effect. Especially, essential oils from *Artemisia Arborescens*, *Eucalyptus globulus*, *Leptospermum scoparum*, *Melaleuca alternifolia*, *Melissa officinalis*, *Mentha piperita, Salvia officinalis* and *Santolina insularis* are featured with these properties. Some essential oils, which are mainly used as spices demonstrate a high level of activity against herpes simplex virus type 1. These are essential oils from *Allium cepa, Allium sativum, Corriandrum sativum*, *Ocimum basilicum*, *Oreganum vulgare* and *Petroselinum sativum*. Essential oils from *Cynanchum stautonii* and *Melaleuca alternifolia* demonstrate a significant activity against influenza virus. Monosubstances like isoborneol, eugenol, α-terpinene, γ-terpinene and α-pinene are effective against herpes simplex virus. In addition, essential oils from *Hyossopus officinalis, Leptospermum scoparum*, *Santalum insularis*, *Thymus vulgare* and *Zingiber officinale* are also effective against acyclovir resistant viruses. These are only some examples of potential antiviral agents, among the essential oils and their main components which are promising and therefore further evaluations in this field seem to be promising.

Zusammenfassung

Ätherische Öle, welche aus Heilpflanzen gewonnen werden, sind für ihre antiviralen Eigenschaften bekannt und wurden seit vielen tausend Jahren in der traditionellen Medizin angewendet. Viele in vitro Studien bestätigen die antivirale Aktivität von ätherischen Ölen und deren Inhaltsstoffen. Die meisten ätherischen Öle und Einzelsubstanzen wurden gegen Herpes Simplex Virus Typ 1 und Typ 2 ausgewertet und zeigen eine hohe antivirale Aktivität: Ätherische Öle aus *Artemisia Arborescens, Eucalyptus globulus, Leptospermum scoparum, Melaleuca alternifolia, Melissa officinalis, Mentha piperita, Salvia officinalis* und *Santolina insularis.* Einige ätherische Öle aus *Allium cepa, Allium sativum, Corriandrum sativum, Ocimum basilicum, Oreganum vulgare* und *Petroselinum sativum*, die hauptsächlich als Gewürz verwendet werden zeigen eine starke Aktivität gegen Herpes simplex virus typ 1. Ätherische Öle aus *Cynanchum stautonii* und *Melaleuca alternifolia* zeigen eine signifikante Wirkung gegen das Influenzavirus. Monosubstanzen wie Isoborneol, Eugenol, α-Terpinene, γ-Terpinene und α-Pinene sind wirksam gegen Herpes Simplex Virus. Darüber hinaus wurde eine Wirksamkeit gegen Acyclovirresistente Viren nachgewiesen, diese sind ätherische Öle *aus Hyossopus officinalis, Leptospermum scoparum, Santalum insularis, Thymus vulgare* und *Zingiber officinale*. Dies umfasst nur einige Beispiele für potenzielle antivirale Wirkstoffe, darunter ätherischen Öle und ihre Hauptinhaltsstoffe, die vielversprechend sind und daher weitere Forschung benötigen.

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1. Introduction

1.1. Essential oils

Essential oils are komplexe mixtures with a distinct odor. They are extracted from different parts of plants, such as leaves, steams and flowers by destillation. They can also be obtained through pressing of fruit skin of several citrus fruits. Almost 87 families contain essential oils, such as *Apiaciae*, *Lamiaceae*, *Myrtaceae*, *Pinaceae*, *Piperaceae, Rutacae* and *Zingiberaceae.* Essential oils are characterized by a high refractive index and are optically active. They are not water soluble but have a high solubility in organic solvents. Their main constituents are terpenes (mono- and sesquiterpenes) and phenylpropanes. Polyketides and compounds containing nitrogen and sulfur are present as well. Due to their lipophil character, essential oils are easily absorbable in the gastrointestinal system and can be absorbed through the skin as well. In suitable solutions essential oils can be used as aerosole (medicinal spray) for its antiseptic effect. For example *Salvia officinalis* and *Melaleuca alternifolia* produce antiseptic essential oils. Further properties are hyperemisating effects and the promotion of inflammation, which are applied for therapeutic uses. Examples for these properties are essential oils from *Rosmarinus*, *Lavendula officinalis*, *Laurus nobilis*, *Juniperus communis*, *Pinus* or components like camphor. Essential oils can also be inhaled affecting the mucous membrane. The weak dose can stimulate the bronchial secretions, and therefore can be applied as expectorans like essential oil from *Foeniculus vulgare, Pimpinella anisum*, *Thymus vulgaris*, *Eucalyptus* and also some components such as anethole and cineole. Besides, essential oils also stimulates the appetite and the digestion. They have an effect in the mucous membran in the

mouth and in the gastrointestinal system. Essential oils from *Carum carvi*, *Foeniculum vulgaris* or *Mentha* serve as stomachicums. In addition, essential oils are also applied as carminatives (*Pimpinella anisum*, *Foeniculum vulgaris*, *Mentha*) diuretics (*Juniperus*, *Levisticum officinale*) and sedatives (*Melissae officinalis*, *Lavendula angustifolia*) (Samuelsson, 2004, p. 162-164; Hänsl and Hölzl, 1996, p. 126-128; Teuscher et al. 2004, p. 383-399).

Essential oils have a wide medicinal application and one of the properties which have been evaluated recently in many studies is the aniviral activity. The first part of this work describes antiviral drugs and various methods of testing in particular for new drug targets. The main part focuses on the essential oils of plants which are commonly used in the tradional medicine with promising antiviral properties.

1.2. Viruses

Viruses are infective agents, which can only replicate themselves in a host cell. A virus owns either DNA or RNA strands, which can be surrounded by a lipoprotein envelope. They are able to utilze host cells to multiply more viruses and have a very unique strategy for cell invasion.

Most of the antiviral drugs are used for the treatment of human immunodeficiency virus (HIV) infection. Other antivirals which are currently available are primaly used for the treatment of hepatitis B virus (HBV), herpes simplex virus (HSV), varicella-zoster virus (VZV), cytomegalovirus (CMV), influenza virus, respiratory syncytial virus (RSV) and hepatitis C virus (HVC) infections.

Basically, the aim of the antiviral drugs is the prevention of the virial repilication. These drugs for example interfere during the attachment of the virus or on the virion. The virion is the infected host cell. The intracellular targets are usually the

protein synthesis and DNA replication. Additionally, the assembly of new infected cells or the uncoating are also viral targets. For some viruses like influenza, measles and mumps virus vaccines are administered. Many antiviral drugs are safe and effective but also have a lot of side effects (see page 4).

1.2.1. Antiviral drugs

Acyclovir is a nucleoside analoga which is similar to guanosine with a acyclic sugar group. It is a prodrug which is converted by the thymidine kinase to a monophosphate and it is efficacious against HSV-1, HSV-2 and varicella-zoster virus (chickenpox and shingles). The enzyme thymidine kinase only exits in the virion, therefore in noninfected cells no phosphorylation takes place. It is indicated also for recurrent HSV infections and for supressive treatments of genital herpes. Besides, it is also frequently used for prophylaxis of HSV infections of immunocompromised patients. For topic applications it is used for keratitis and additionally for the treatment of herpes labialis. Acyclovir can cause local irritation and gastrointestinal effects. Besides it is nephrotoxic, mutagenic, cancerous and teratongenic.

Ganciclovir is a derivate of acyclovir and it is used for the treatment of cytomegalovirus. In the infected cell, the viral kinase phosphorylate gancyclovir. Ganciclovir is applied intravenously and it is used only for infected AIDS patients and immunosuppressed transplant patients. An oral formulation is also avaliable for prophylaxis and longtime use. Compared to the intravenous formulation the oral formulation is less toxic.

Foscarnet inhibits the DNA polymerase of herpesviruses and the reverese transcriptase of HIV. Side effects are kidney and bone toxicity, therefore it is only

applied as last option. Another toxic example is Ribavirin, which inhibits the cellular inosine monophosphate dehydrogenase. It interferes with the capping of mRNA. Ribavirin is applied against respiratory syncytial virus infection, against Lassa fever virus infection and hantavirus infection.

Lamivudine is a nucleoside analoga and blocks competitively the reverse transcriptase of Hepatitis B virus and HIV. This drug is low toxic and has a high bioactivitiy and therefore is appropriate for long time use.

For the HIV treatment the HAART (highly active antiretroviral therapy) is used. HAART is a combinationtherapy, which consists of 2 reverse transcriptase inhibitors and 1 protease inhibitor.

Cidofovir is a broad spectrum drug and is effecious against herpesvirus, papillomavirus, adenovirus and poxyvirus infections. It is an acyclic nucleoside phosphonate, which is converted to di- and triphosphate derivatives by host enzymes.

Amantadine inhibits the influenza A virus by blocking the M_2 ion channel activity. It has to be applied in the first 24 to 48 hours of infection for at least 10 days. In addition it is also used against symptomps of parkinson. At higher concentation (100mM or higher), it acts as weak base and influences the pH level of the endosomes. Therefore the pH dependent level of the membrane is inhibited, so the virus is affected.

Zanamivir and Oseltamivir are neuroaminidase blocker and are used against influenza A and B. They reduces the symptoms of influenza infections and reduces the time period of one day of the disease. Zanamivir is orally applied and Oseltamivir in form of inhalation (Flint et al. 2009, p. 289-293).

1.2.2. Resistance developement

Besides toxic side effects that occur during the antiviral treatment, a resistancy has to be considered when administered for short or a long time period. In the study of Stranska et al. (2005) the prevalence of acyclovir-resistant herpes simplex virus in the Netherlands is determined. In total 542 ACV-resistant HSV isolates from 496 patients are screened in susceptibility assay. As a result ACV-resistant HSV infection take place more frequently in immunocompromised patients (7%; $p > 0,0001$) compared to immuncompetent patients (0,27%). This outcome indicates a continoues monitoring of HSV infections in particular in immunocompromised patients with persisting infection during the antiviral theraphy. In the study of Christophers et al. (1998) the resistancy of herpes simplex virus to acyclovir in Northwest England is investigated. 2000 HSV isolates from immunocompetent and immunocompromised patients are tested for sensitivity against acyclovir. The outcome shows a resistancy of 0,1-0,6 % from immunocompetent patients. In the immunocompromised group the prevalence of resistance was about 6%. These studies demonstrate a resistancy against acyclovir, which mostly happens to immunocompromised patients.

1.3. Methods

1.3.1. Cell cultures, Cytotoxcity

Cell cultures testings are the most common method for multiplication of viruses. Mainly, tissues are dilluted in single cell suspension and afterwards are treated with proteolytic enzymes. The cells are suspended in cell medium and are grown in plastic flasks or plates. During cell division, a monolayer is formed, which contains epithelial and fibroblastic cells, only lyphoscytes are left and do not adhere to the plastic surface. The incubating period is about 24-48h, where the cell growth is optimaly in a defined and buffered medium.

There are 3 kinds of cell cultures. One cell culture is the primary cell culture, which are prepared from animal origins, like monkey kidneys, human embryonic amnion, human embryonic kidneys, human foreskins and chicken or mouse embryos. In addition continuous cell lines, which cells are from tumor tissue or cells with chemical or tumor virus are used. Examples are HeLa (Henrietta Lacks) cells and L and 3T3 cells. HeLa cell lines, the most widely used cell line in virology is derived from human carcinomas, L and 3T3 cells are from mice. Another type of cell cultures are suspension cultures. The cells are continuously stired and a large number of cells can be bred.

One of the tests that have to be carried out is the cytotocxity test of cell cultures. It is an important test to determine the toxicity of a test compound in an organism. It is a complementary method, aiming to reduce animal experiments. In the work of Halle and Göres (1987) a prediction of LD_{50} in cell culture is described, where the correlations of in vitro and in vivo toxicity have been evaluated. These values are referred to 28 chemical structures from different substance classes. In many

studies the cytotoxicity test is based on this work.

	IC_{50} mmol/l	LD_{50} oral mg/kg (rat/mouse)
highest level of toxicity	<0,0001	>5
extremely toxic	0,0001-0,001	5-50
highly toxic	0,001-0,01	50-500
moderately toxic	0,01-0,1	500-5000
little toxic	0,1-1,0	5000-15000
very little toxic	1,0-10	>15000
non-toxic	>10	-

Table 1. Scale of Cytotoxicity on cell lines, IC_{50}= concentration of testcompound that kills 50% of the cell lines.; LD_{50}= concentration that causes the death of 50% of the test animals (Halle and Göres,1987)

1.3.2. Virus growth, cytopahtic effects

Viruses are intracellular parasites, they attach to the host cell and infect them and finally replicate themselves. This developement becomes visibly as more and more cells are infected. This morphologic changes are the cytopathic effects. The cytopathic effects vary from virus to virus, basically a morphologic difference can be observed such as breaking of chromosomes, rounding up and detachment of cultured cells, cell lysis and swelling of nuclei (Herpes virus). Some of them can be detected with a light microscope or phase contrast microscope and no fixing or straning is necessary. Other cytopathic effects, which contain the virions or other components in the nucleus or cytoplasma are more complex and require a high power microscopy. Also the time period has an influence, for example herpes virus can cause cytopathic effects in maximum 2 days. On day 3 the cell monolayer is fully destroyed. Wheras some adenoviruses or cytomegalovirus do not produce

such effects for weeks. Many members of *Arenaviridae*, *Paramycoviridae* and *Retroviridae* do not cause obvious cytopathic effects. For this viruses other methods are used like the transformation assay (see page 10).

1.3.3. Virus assays

There are two types of assays, biological and physical ones. Biological assays are the plaque assays and end-point titration methods. In these assays only the infectious particles are detected, whereas physical assays, for example electron microscopy or immonological assays measure noninfectious particles.

1.3.3.1. Plaque assay

In the plaque assay monolayers of cultured cells are incubated with virus titer (virus concentration) and after an incubation period the inoculum is removed and another medium, mostly agar is added. A gel is resulted and when the infected cells produce new viruses, a plaque is observed. Plaques are rounded zones, which are produced of each infectious particle. They grow larger in time and can be seen. The plaque develepment is described in a dose-response curve. Most viruses expose a linear relationship. The number of plaques is directly proportional to the relative virus concentration. The PFU[1] (plaque performing unit) per milliliter calculates the virus titer.

[1] PFU (Plaque performing unit) is a concentration which causes plaque per unit volume.

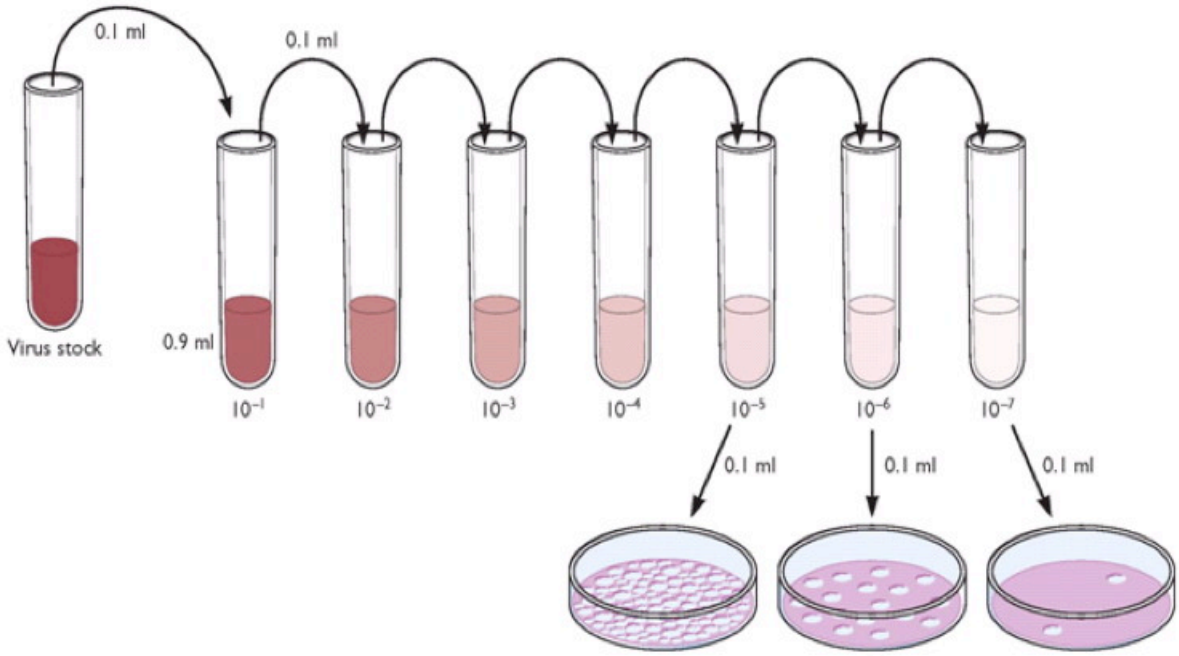

Figure 1. From virus titer to plaque assay (Flint 2004)

For the calculation of PFU 10-fold serial dilution of virus stock is used, and 0.1 ml aliqouts are inoculated onto cell medium. After inoculation period the monolayers are stained and the plaques are counted (Fig.1).

1.3.3.2. Flourescent-focus assay

One of the modifications of plaque assaies, is the flourescent-focus assay, which is used when the virus do not kill cells. At first the cells are inoculated with one antibody against the viral protein and a flourescent indicator like flourescein. Afterwards the cells are examined microscopically and the virus titer is calculated in flourescent-focus-forming units per milliliter.

1.3.3.3. Infectius-center assay, Transformation assay

The infectious-center assay defines the infected cell fraction in a culture. This method is usually applied for the measurement of resistantly infected culture. Some viruses such as some retroviruses do not form plaques but foci (small piles). In this case the transformation assay is exercised. For example the Rous sarcoma virus can transform chicken embryo cells. During cell growth the cells accumulate and can pile up and is measured in focus-forming units per milliliter.

1.3.3.4. Endpoint dilution assay

One method which is also usually used is the end-point dilution assay. The virus titer is detected before the plaque assay is performed. It is especially suited for viruses that do not form plaques or for examining viral action in animals. Mediums can be cell cultures, eggs or animals. Basically, 8 or 10 test units are prepared, and in cell culture the cytopathic effect is determined, wheras in eggs or animals the infection is expressed by death or disease. The cytopathic effect is scored with a +. The end point describes the virus dilution that affects 50% of the test units, which is the 50% infectious dose (ID_{50}[2]) per mililiter. In the viral activity in eggs or animals the LD_{50}[3] (lethal dose) or PD_{50}[4] (paralytic dose) per mililiter is calculated.

[2] ID_{50} (infectious dose) is a concentration of pathogenic microorganism that will cause infection in 50% of the test subjects.
[3] LD_{50} (lethal dose) is a concentration which causes death of 50% of the test subjects.
[4] PD_{50} (paralytic dose) is a concentration which causes the paralysis of 50% of the test subjects.

1.3.3.5. Serological methods

1.3.3.5.1. Hamagglutination

For some viruses especially from the *Adenoviridae*, *Orthomyxoviridae* and *Paramyxoviridae*, the principle of hemagglutionation is used. For example influenza virus contains hemagglutinin that binds to erythrocytes (N-acetylneuraminic acid). Two fold dilutions of the virus titer are mixed with red blood cells and the agglutinated red blood cells can be distinguished. This assay only takes 30 min., but small virus particles are not detected. In the hemagglutination inhibition antibodies against viral proteins are added, which blocks the virus from binding to erythrocytes. This assay has a lot of advantages, it is sensitiv, simple, rapid and inexpensive. In addition, the viral enzyme activity can be measured. Nucleic acid polymerase are mixed with particles that are radioactive labeled and finally the radioactivity is measured. Examples are retroviruses, which do not form plaques. In this type of assay, the reverse transcriptase is mixed into the virus with a particular detergent, template, primer and a radioactive nucleosid triphosphate. When a reverse transcriptase occurs, a radioactive product can be detected, which is produced by the primer.

1.3.3.5.2. Virus neutralization assay

Another serological method is the virus neutralization. This method is based on the antibodies which are produced by an animal, and through the virus-antibody binding the infection is neutralized (inhibited). In this assay dilutions of antibodies are mixed with the virus titer, afterwards assayed in cultured cells, eggs or

animals, in which the infectivity is analyzed. The highest dillution of antibody which inhibits the viral replication is the end point solution.

1.3.4. Light microscopy, Flourescence microscopy

In the light microscopy virus particles can be visuallized with few exceptions, when the virus size is too small, an electron microscopy can be an option. The flourescence microscopy enables to capture virus particles in living cells. Moreover entry, uncoating, repilcation and assembly can be observed (Flint, 2004 p. 34-44).

2. Main part

2.1. Artemisia arborescens

Artemisia arborescens is a plant of the *Asteracea* familiy. It is a perennial shrub and is widely spread in mediterraniean regions. The plant grows up to about 60 to 150 centimeters. A characteristic colour of this plant is the white to grey-green silver. Artemisia essential oil has been used in the folk medicine and also for flavorings, fragrances and repellents (Sacco et al. 1983; Presti et al. 2007).

In the paper of Sacco et al. (1983) the composition of the essential oil of *Artemisia arborescenes* is evaluated. Leaves and flowers from Sardinia are steam destillated and the obtained oil is investigated by capillary GLC (gas liquid chromatography) and capillary GLC-MS (gas liquid chromatography-mass spectrometry). From a hundred minor components 44 compounds are identified. The principal components are sabinene, thujone, camphor, terpinen-4-ol, ß-cubebene and chamazulene. In table1. the main compounds of *Artemisia Arborecens* essential oil from different locations are listed.

	El Beyrouthy et al. (2011) Algerian oil	Abderrahim et al. (2010) Lebanese oil	Presti et al. (2007) Silicy oil	Presti et al. (2007) Calabria oil	Presti et al. (2007) Lipari oil
ß-thujone	68,5	27,8	-	-	-
chamazulene	12,3	30,2	37,6	27,1	34,6
terpinen-4-ol	1,5	1,8	-	-	-
camphor	-	-	21,4	39,5	20,0
α-thujone	1,2	-	-	-	-

Table 2. *Artemisia arborescens* essential oil components (% v/v)

In the work of Sinico et al. (2005) the antiviral activity of liposomal essential oil from *Artemisia arborescens L*. is studied. The main components as determined by GC (Gaschromatography) and GC-ITMS (gas chromatography-ion trap mass spectrometry) are α-pinene (3,17% area), ß-thujone (23,97% area), camphor (35,73% area), terpinen-4-ol (2,20% area) and chamazulene (7,66% area). For the experiments three different vesicular formulations are prepared, such as Enriched soya phosphatidylcholine (Phospholipon 90, P90), hydrogenated soya phosphatidylcholine (P90H), stearylamine and polyoxyethylene and laurylether (Brij 30). Each vesicle contains a concentration of 5mg/ml essential oil. To avoid any emulsion droplets, the vesicles are purified by ultracetrifugation and also a morphological screeing was completed. Each formulation consists of MLV [5] (Multilamellar vesicles) (empty/essential oil-loaded) and SUV[6] (small unilamellar vesicles) (empty/essential oil-loaded). Brij 30 vesicle incorporating essential oil

[5] MLV (multilamellar vesicle) is a liposome which consist of more than one layer of phospholipides.
[6] SUV (small lamellar vesicle) is a liposome with only one phospholipide layer, with a diameter <0,1μm.

indicates to be toxic on vero cells. This is tested in vitro by a colormetric cell viability assay. Consequently the Brij 30 vesicles are excluded from the antiviral assay. Only the liposomal carriers are evaluated for in vitro antiviral activity against HSV-1 (Herpes simplex virus type 1). To study the antiviral activities of the free and liposomal *Artemisia arborescens* essential oils against HSV-1 (Herpes simplex virus type 1), a MTT (3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide) colormetric method is used. As a result, the free essential oil shows a very low activity against HSV-1 (22.86% inhibtion at 100µg/ml). The essential oil in P90H SUVs values 21.1% inhibition at the concentration of 100µg/ml, whereas the essential oil in P90 SUVs has a higher inhibition (8.1% at 100µg/ml). In comparison, the essential oil in MLVs shows a significant increase of the antiviral activity (EC_{50} [7]= 18.5), 43.6 µg/ml are determined for PH MLV). P90 MLV (100µg/ml) has a 100% viral reduction and P90H MLV (50µg/ml) a reduction of 22,86%.

The study of Saddi et al. (2007) investigates the antiviral activities of the essential oil obtained from the leaves of *Artemisia arborescens* against HSV-1 and HSV-2. The leaves collected from Sardinia are distilled in a Clevenger-type apparatus for 5 h, afterwards the essential oil is dried. The cellular toxicity on vero cells are evaluated morphologically after 24, 48 and 72 hours. The essential oil shows a CC_{50} [8]of 132 µg/ml, which is determined by the MTT assay, the MNTD (maximum non toxic dose) values 100mg/ml. In the first experiment 250 PFU (plaque perfoming unit) of the viruses and dilutions of the essential oil are incubated for 1h at 37 °C and afterwards are adsorbed to the vero cells. The assays demonstrate a

[7] EC_{50} decribes the half maximal effective concentration.
[8] CC_{50} (cytotoxic concentration) is as a concentration, that cause a 50% reduction in the number of cells.

concentration dependent inhibition of the plaque formation (for HSV-1: IC_{50} [9] = 2,4µg/ml, $IC_{80=}$ 5,6µg/ml; for HSV-2: IC_{50}=4,1µg/ml, IC_{80}=7,3µg/ml). Furthermore, when the HSV-1 virus is preincubated for 2h at 37°C, a higher inhibition is observed for HSV-1 (IC_{50}=1,14µg/ml; IC_{80}=2,6µg/ml). This shows the dependency of HSV-1 inhibition when the incubation time is doubled. In addition, when the temperature is lowered to 4°C before virus adsorption the ICs of HSV-1 are increased (IC_{50}=19,4µg/ml; IC_{80}=32,2µg/ml). In the experiment where the cells are incubated with untreated viruses, no inhibition is observed, as well when the cells are pre-treated with the essential oil and afterwards incubated with untreated viruses. In another experiment the developement of plaque inhibition in infected cells is evaluated. Firstly, the vero cells are infected with 100 PFU of HSV-1 and HSV-2, after incubation for 3h at 37°C, nutreint agar is used for a medium. Various concentrations of the essential oil and also 10µg/ml of HSV-1 and HSV-2 neutralizing antibody are added. The neutralizing antibody is important to gurantee that the cell to cell spread is only based on that plaque developement. As a result, the assay demonstrate a concentrate-dependent plaque reduction for both virus types. When 100µg/ml of the essential oil is added, an inhibition value of 68,3% for HSV-1 resulted after 96h of incubation, and after 48h with a 50µg/ml concentration an inhibition of 67,1% is observed. Also by lower concentration the plaque reduction developement is significantly reduced. For HSV-2 a significant reduction (55,9%) is shown after 48h of incubation at a concentration of 100µg/ml. In the attachment assay, the virus is inhibited at a concentration of 50µg/ml. This remarkable concentration, which is much more higher than the control dose of HSV-1 and the dose resulted in the experiment when HSV-2 has been pre-incubated for 2h, shows that the virus attachment is not affected and indicates an

[9] IC_{50} (inhibitory concentration) is a concentration of an inhibitor wherein a half-maximal inhibition is observed.

effect on the virion directly. In the penetration assay and post attachment virus neutralization assay no inhibition was observed.

In general, these studies demonstrate the antiviral effect of the essential oil from *Artemisia arborescens*. In particular, when the essential oil is incorporated in a liposomal carrier, such as MLVs, the antiviral effect is significantly increased compared to the free essential oil (Sinico et al. 2005). The intracellular antiherpetic effect is considerably enhanced in multilayerd liposomes, in which the essential oils are more stable. An interesting outcome of the study of Saddi et al. (2007) shows the ability of *Arthemisia Arborescens* in the mode of action, in particular the antiherpetic ability to inhibit the virus diffusion from cell-to-cell in infected cells. In addtion, the essential oil inhibited the HSV-2 replication at higher concentration. This indicates that other mechanisms are involved. *Artemisa Arboresences* seems to be a very promising target and thus further researches has to be carried out, especially in the detailed antiviral action.

2.2. Cynanchum stautonii

Cynanchum stautonii belongs to *Asclepiadeaceae* family and is most commonly used in the traditional chinese medicine. This herbal drug called ''Bai Qian'' is widley applied to the treatment of the respiratory tract, especially the extracts of the roots are used as antitussives and expectorants (Wang et al. 2004).

In the work of Zai-Chang et al. in 2005 the antiviral activities of the volatile oil from *Cynanchum stauntonii* against influenza virus is investigated. For the chemical analysis, roots are collected and distilled for 3h. The volatile oil is examined by GC-MS (Gas chromatography-mass spectrometry) and thirty-eight components representing 63,53% are detected. The main components with more than 3% of

the total peak area are (E,E)-2,4-Decadienal (23,030%), 3-Ethyl-4-methylpentanol (3,535%), 5-pentyl-3H-furan-2-one (3,847%), (E,Z)-2,4-decadienal (3,002%) and 2(3H)-Furanone,dihydro-5-pentyl (4,176%). For the experiments Madin-Darby canine kidney (MDCK) in Eagle's minimum essential medium (EMEM) with 10% fetal calf serum and 75µg of kanamycin are used. For invitro experiments influenza virus A/NWS/33 (H_1N_1) are used and for in vivo experiments Kung ming male mice are used. In the cytotoxicity assay, the volatile oil at a concentration of 512µg/ml has no cytotoxic effect against MDCK cells. In the antiviral assay the IC_{50} values 64µg/ml. In the in vivo experiment, mice are anesthetized with methyl ether and 20µl influenza virus is intranasally administered for each mouse. Up to the concentration of 600 PFU/mouse is lethal, therefore serial 10-fold dilutions of the virus are used. The volatile oil is diluted in distilled dimethyl sulphoxide (DMSO), with the final concentration of 0,2%. The infected mice are separated into 4 groups containing 10 mice each group. A dose of 50, 150 and 300mg/kg of body weight are injected intraperitoneal twice a day for 6 days with 12h interval, starting 1h after infection. To evaluate the anti-influenza activities of the volatile oil, weight loss, mean day to death (MDD) and survival rate are observed. The highest dose (300mg/kg) saves all the mice from death ($P<0,01$), the survival rate is 70% ($p<0,01$) at the dose of 150mg/kg per day, and the MDD 11,7± 0,3 ($P<0,01$). In the group with 50mg/kg per day treatment, the survival rates are 40%, the MDD was lower (9,5±0,5). The third parameter is the body weight, which is determined every other day. On day 2 the weight of each group is lowered in a continoues period. All mice in the placebo group died completely on day 5. On day 8 the weight is at its lowest value, then from this point on the weight of the mice raises up till day 22. This data demonstrates a dose dependent manner of the investigated volatile oil

by inhibiting the influenza-induced weight loss.

To sum up, this study shows the aniviral effect of the volatile oil from *Cynanchum stauntonii* against influenza virus both in in vitro and in vivo experiments. This drug has been used for thousand of years in China, and is a safe agent as well. Many of the anaylized compounds of the oil are unknown before, but still the antviral agent has not been found yet. To investigate the viral agent further studies, which may contribute to the developement of new and effective agents, have to be carried out.

2.3. Houttuynia cordata

Houttuynia cordata is a plant from the Saururaceae family, which is traditionally used in Japan and China. The essential oil has a characteristic coriander odor. The main components are methyl n-nonyl ketone, lauryl aldehyde and capryl aldehyde (Hayashi et al. 1994).

In the study of Hayashi et al. (1994) the antiviral effect of *Houttuynia cordata* essential oil and its components is tested against HSV-1, influenza virus and HIV virus. Lauryl aldehyde and capryl aldehyde show a moderate effect against HSV-1. When the HeLa cells are pretreated with the essential oil before infecting with HSV-1 or influenza, no significant effect is observed. A complete inactivation is demonstrated after incubating the HSV-1 and the essential oil for 3 hours. Different dilutions (2- to 32-fold) of the essential oil are tested and as a result HSV-1 and influenza virus are inactivated after 3h whithin 24h of extension. At the 2-fold dilution the infectivity of both viruses are >1% after 3h, from 6-9h no viruses are detected. The concentration of 0,0083% capryl aldehyde inactivates 62% of the HSV-1, and 0,0083 % of methyl n-nonyl ketone, 2,5%. In testing against HIV-1 2-fold dilution of the essential oil 20-40% of the virus is inactivated when pretreated with the essential oil for 2h and 6h. At the concentration of 0,0083% the components methyl n-nonyl ketone, lauryl aldehyde and capryl aldehyde demonstrate a virucidal activity. Among them, the highest amount is observed by lauryl aldehyde.

This study demonstrate the antiviral effect of *Houttuynia cordata* essential oil and their components. There is an indication that this essential oil and the main components interfere with the envelope of the virus.

2.4. Melissa Officinalis

Melissa officinalis belongs to the Lamiaceae family which has been used in folk medicine for many years. It is a perennial plant and can grow up to 30-75 cm. The components are the essential oil (lemon balm), tannins, flavanoids and triterpenoids. The percentage value of lemon balm is 93,51% and the hydrocarbons and oxygenated compounds make about 6.49% (Sarer and Kökdil, 1991). The main components of the Melissa essential oil are citral a (20.13% v/v), caryophyllen (17,31% v/v), citral b (13.58% v/v), citronellal (3.86% v/v), ß-cubeben (3.75% v/v), menthylheptenon (2.31% v/v), caryophyllenoxid (1.13% v/v) and ocimen (0.7% v/v). A characteristic feature is the ratio of 4,4:3 for citral a and citral b (Schnitzler et al. 2008).

The study of Allahverdiyev et al. (2004) deals with the antiviral activity of the voltile oils of *Melissa officinalis L*. against herpes simplex virus type-2. For the experiments HEp-2 cells (line nr. ATCC CCL23) are used. Four concentrations (25, 50, 100, 150 and 200 µ/ml) of the volatile oils of *Melissa officinalis* are evaluated. A non-toxic concentration amounts up to 100µg/ml, slightly toxic >100µg/ml. The microscopic examination shows a ++ CPE [10] on the lowest concentration (25µg/ml), a + CPE at 50µg/ml, no cytopathic effect is observed at 100µg/ml, the virus control has a ++++ CPE. To examine the effects of the voltile oil on the replication of HSV-2, uninfected HEp-2[11] cells (control) and HEp-2 cell infected with HSV-2 were observed. The cell cultures were inoculated for 72h and the highest non-cytocidal concentration (100µm/ml) showed no cytophatical changes. In addition, no microscopic changes are observed and in terms of cell

[10] CPE (Cytophathic effect) are structural changes in a host cell resulting from viral infection.
[11] Hep-2 is a human lyrngal carcinoma cell.

counts no significant differences are determined. In higher concentrations (150µg/ml and 200µg/ml), significant differences in cell counts are observed in comparison to the control group, concentrations above 100µg/ml of the voltile oil are observed to be toxic for the HEp-2 cells. In comparison to acyclovir, the voltile oils reduce the viral activity significantly (78,15% decrease for 1 $TCID_{50}$[12] value). The study indicates that the antiviral activity could be due to the components of the volatile oil or of the monoterpenoid citral and citronellal.

The study by Schnitzler et al. (2008) examines the antiviral activity of *Melissa officinalis* oil, too. The inhibitory activity against HSV-1 and HSV-2 is tested on monkey kidney cells. On the plaque reduction assay the inhibition of HSV repilication is examined. The TC_{50}[13] is determined at 0,003%, the maximum noncytotoxic concentration at 0,002% in the standard neutral red assay after 3 days of incubation. The non cytotoxic concentration of acyclovir is 1.25mg/ml which is used in all assays. Time-on-addition experiments during the herpes simplex virus replication cycle are performed. Serial dilutions of lemon balm in ethanol have been incubated with HSV-1 and HSV-2 prior to the infection of cells, for control virus mixed with 1% ethanol is used. As a result, both types of herpes virus showed a clearly concentration-dependent activity in the dose-response curves. The IC_{50} of balm oil is at 0.0004% and 0.00008% for HSV-1 and HSV-2, respectively (both amount as a percentage of virus control). The plaque formation of the virus is inhibited in a dose-dependent manner. The study also shows that at a concentration of 0,002% balm oil, the titres of HSV-1 and HSV-2 are reduced by 98,8% and 97,2%, respectively, and even at higher concentration the viral activity are completely abolished. The plaque formation is not influenced while adding balm oil prior the infection, a reduction of 64,8% and 39,9% is observed, when

[12] $TCID_{50}$ expresses the 50% tissue culture infectious doses per milliliter.
[13] TC_{50} (toxic concentration) is a concentration that produces a toxic effect in 50% of a cell medium.

HSV-1 and HSV-2 are treated with balm oil during adsorption period. In comparison, when the virus is pretreated with maximum noncytotoxic concentrations of balm oil, the plaque assay is significantly reduced.

All in all, this study shows the antiviral activity of balm essential oil against free herpes simlpex virus particles. The result shows that the viruses were influenced by the essential oil before adsorption, which in comparsion to acyclovir, has a different mechanism.

Another study by Nolkemper et al. (2006) also deals with the antiviral activity against HSV-1, HSV-2 and an acyclovir-resistant strain of HSV-1 (ACV^{res})[14]. This study examines the activity of aqueos extract from species of the Lamiaceae family, specially extracts from lemon balm (*Melissa officinalis*), peppermint (*Mentha x piperita*), prunella (*Prunella vulgaris*), rosemary (*Rosmarinus officinalis*), sage (*Salvia officinalis*) and thyme (*Thymus vulgaris*) in vitro on RC-37 cells in a plaque reduction assay. In order to investigate the antiviral action, the extracts are added at different stages of infection. The plaque perfoming unit shows a reduction of 36-87% for HSV-1 and 56-88% for HSV-2, respectively. On pretreated host cells lemon balm extract had a plaque formation reduction of 35% for HSV-1 and of 47% for HSV-2, peppermint and prunella show a 22% and 25% plaque reduction for HSV-2, and rosemary decreased HSV-1 for 24%. On the contrary, when the virus were pretreated with the extracts prior the infection, the infectivity is reduced by >95% for HSV-1 and >90% for HSV-2. There is no significantly diminution when extracts are added after penetration of the viruses into the cells. The time response study from 1 minute to 2 hours demonstrates a clearly time dependent activity. The most active component is sage extract which has a PFU of 85% for HSV-1 and 95% for HSV-2. Moreover, the activity of HSV-1ACV^{res} is

[14] ACV^{res} is a strain of HSV-1 which does not respond to acyclovir and therefore is ineffective.

reduced for about 85% by peppermint and for up to 97% by sage. This study shows that the extracts interacts with the virus before entering the host cell, which indicates an effect on the virion envelope. In comparison to essential oils, which only inhibits extracellular virus, aqueos extracts also interfere with the adsorption of the virus to host cells. Another interesting point of this study is the significant inhibtion of more than 90% for HSV-1-ACV^{res}. This indicates that the extracts have a different mechanismus of action with HSV compared to acyclovir.

2.5. Mentha piperita

Mentha piperita from the *Lamiaceae* family is a perennial plant native to Europe. The plant grows up to 50-100 centimeters. It is a hybrid between *Mentha aquatica* and *Mentha spicata*. Peppermint oil, the essential oil of *Mentha piperita*, is mainly produced in the U.S. and it is widely used for flavouring and in fragrance properties. The leaves are also popular in the food industry and for cosmetic treatments (McKay and Blumberg, 2006). The leaves contain 1,2 -3,9% (v/w) essential oil. The major components of peppermint oil are of menthol, menthon, isomenthon, methylacetate, cineole, limonene and carvon. Cineole and limonene are in a ratio of 3 to 1. An oil meeting this demand is considered to be a native peppermint oil. In table 3., *Mentha piperita* essential oils from different locations are compared.

	mentho l	mentho n	isometho n	methylacetat e	cineo l	limonen e	carvo n
Germany, Schuhmache r et al. (2003)	42,8	14,6	5,9	4,4	3,8	1,2	0,6
Italy, Clark and Menary (1981)	38.70	18,10	-	4,8	8,9	6,8	-

Table 3. *M. piperita L*. essential oil components (% v/v)

The study of Schuhmacher et al. (2003) examines the virucidal effect of peppermint oil on Herpes simplex viurs type 1 and type 2 in vitro. To measure the cytotoxicity, peppermint oil is dissolved in ethanol, added to the medium at a concentration of 1%. RC-37 (African green monkey kidney cells) are used in 0,001 - 0,01% drug-containing medium and is incubated for 4 days. Low concentrations up to 0,01 % show no visibly changes in the cell, whereas at the concentrations of 0,03% cell death is monitored. The TC_{50} of peppermint oil for RC-37 is determined at 0,0014%. To evaluate the virucidal action of peppermint oil against herpes virus, concentration from 0,0001-0,06% are exposed in suspension assays. The IC_{50} is determined at 0,002% and 0,0008% for HSV-1 and HSV-2, respectively. Peppermint oil clearly shows a dose-dependent effect against HSV-1 and HSV- 2. At a non cytotoxic concentration of 0,01% the virucidal activity is reduced significantly by 82% for HSV-1 and 92% for HSV-2, respectively, even with increasing concentrations of peppermint oil, HSV-1 and HSV-2 are reduced to 94% and 92%, respectively. The study also demonstrates a clearly time-dependent virucidal activity of the increasing concentrations in the period time

from 1min to 4 hours. After 2h peppermint oil has an activity of 98% after 3h incubation more than 99%. To investigate the antiviral action of mode, the essential oil is added at different steps of the viral replication. Pretreatment cells do not show any significant alteration in the virus replication, but pretreated HSV-1 and HSV-2 show a reduction of 82% and 92%, respectively. No significant effect is observed when the essential oil is added during the absorption period. In comparison, acyclovir has a reduced plaque formation by 79,8% against (HSV-1) and 72,9% (HSV-2) when viruses are pretreated prior infection or when added during adsorption. In this study the antiviral activity of an acyclovir resistant HSV-1 strain is also analyzed. As a result the essential oil with the concentration of 0,01% reduces the activity by 99%.

Basically, the result shows the antiviral activity of peppermint oil against herpes simplex virus in vitro. The effect happens to be before adsorption or during adsorption of the virus. This also indicates that peppermint oil possibly interacts with the viral envelope and the glycoproteins. Moreover, a significant reduction of acyclovir resistant HSV-1 virus is observed. Indeed, there is a different mechanism of peppermint oil unlike acyclovir, which antiviral effect is due to the interference with the DNA polymerase inside the cell. This outcome can be of particular importance for patients who frequently suffer from HSV, especially those who are immunocompromised. Since the exact mechanism of inhibition by the essential oil is not yet evaluated, further investigations have to be done in this particular aspect.

2.6. Myrtaceae essential oils

2.6.1. Leptospermum scoparium

Leptospermum scoparium is a member of the *Myrtaceae* family and is native to the south region of Australia and New Zealand. It is a shrub or a small tree, which can reach the height of 8 meters (Lis-Balchin and Hart, 1998). The essential oil obtained from *Leptospermum scoparium* is also called manuka oil. The main components of manuka oil are terpinene-4-ol (1,76% v/v), isoleptospermone (3,94% v/v), viridiflorene (4,40% v/v), flavesone (4,50% v/v), δ-cadinene (6,02% v/v), leptospermone (14,36% v/v), and calamene (16,93% v/v) (Reichling et al. 2005).

In the study of Rechling et al.in 2005 the virucidal activity of the essential oil of Leptospermum scoparium against HSV-1 and HSV-2 is evaluated. For the cytotoxic evaluation manuka oil, leptospermone and flavasone are dissolved in ethanol with the final concentration of 1% when adding to the medium. Concentrations from 0,0001% - 0,1% (v/v) of the components are incubated on RC-37 cells for 4 days. Manuka oil has a non cytotoxic concentration up to 0,003% (v/v), leptospermone and flavesone show no morphologic changes. The TC_{50} for manuka oil, leptospermone and flavasone are 0,004%, 0,01% and 0,1%, respectively. In order to analyze the antiviral effect against HSV, viruses are added to serial dilution of manuka oil in ethanol in suspension assays. Ethanol with the concentration of 1% has no effect on the virus titers. The plaque formation is inhibited in a dose-dependent manner and the IC_{50} of manuka oil values 0,0001% (v/v) and 0,00006% (v/v) for HSV-1 and HSV-2, respectively. When the cells are pretreated with manuka oil no virus reduction is observed, indeed a significant

infectivity reduction is discovered on the pretreated virus (99,5% for HSV-1, 89,9% for HSV-2). A moderate reduction (40,5% for HSV-1, 54,2% for HSV-2) happens when manuka oil is added during adsorption, but no significant changes are observed when manuka oil is added after penetration of HSV-2, on the contrary HSV-1 is reduced by about 41%.

The work of Schnitzler et al. (2008) deals with the cytotoxicity of different *Myrtaceae* essential oils i.e. cajuput oil (*Melaleuca cajuputi*), clove oil (*Syzygium aromaticum*), kanuka oil (*Kunzea ericoides*) and manuka oil (*Leptospermum ericoides*). The essential oils are analyzed by gas chromatography. According to Schnitzler et al. (2008) the major compounds of cajuput oil are 1,8-cineole (61,16 % v/v), α-pinene (9,05% v/v), limonene (5,99%), β-pinene (3,82% v/v), α-terpineol (3,50% v/v), δ-terpinene (3,10% v/v), p-cymene (2,0% v/v), β-caryophyllene (1,46% v/v) and terpinolene (1,25% v/v). Clove oil consists mainly of eugenol (74,98% v/v), β-caryophyllene (14,95% v/v), α-humulene (1,79% v/v) and caryophyllenoxide (0,34% v/v). The main component for kanuka oil is α-pinene (70,63% v/v) and the one of manuka oil is leptospermone (14,36% v/v). For the experiments Vero cells and RC-37 cells are used. The cytotoxicity is performed in a standard neutral red assay. After 4 days of incubation, with increasing concentrations of ethanol the medium is removed. The cells are incubated for another 3h with neutral red. Firstly, the cytotoxicity of ethanol is analysed and the non-toxic concentration is observed at 1,5% for Vero and RC-37 cells, and the TC_{50} 3,2% for Vero cells and 2,6% for RC-37. Then various concentrations of the essential oil from 1% to 0,0001% are incubated in cell medium. TC_{50} for manuka oil on RC-37 cells is determined at 0,0042%, on Vero cells a similiar percentage is observed. In the study of Schnitzler et al. (2008) the antiviral activity of manuka oil

is tested against herpes simplex virus type 1 and HSV-1 acyclovir resistant isolates. With the maximum noncytotoxic concentration of 0,001%, manuka oil was incubated in susupension assays for 1h. In the plaque reduction assay, the infectivity of HSV-1 and acyclovir resistant HSV-1 isolates is reduced by 99%.

Generally these studies demonstrate the antiviral activity of the essential oil of *Leptospermum scoparium.* Compared to its components flavesone and leptospermone the plaque formation of HSV-1 is significantly reduced by the essential oil after the virus penetration (Reichling et al. 2005). The essential oil is able to inhibit the HSV-1 replication in the host cell, which needs further investigations in this aspect. Another outcome of a study (Schnitzler et al. 2008) is the high acitivity (>99%) against acyclovir restistant HSV-1 virus. This interesting finding show that manuka oil has a different mechanism as acyclovir, which is still unclear. Nevertheless, these results have significant inputs for further developement of antiviral targets.

2.6.2. Eucalyptus globulus

Eucalyptus globulus is a plant from the Myrtaceae family and it is native to Australia and subtropic regions. The tree can grow up to 70 metres high and the essential oil is obtained from leaves and branches. Eucalyptus oil is used for the treatment of respiratory tract disorders and infections. The main components are eucalyptol (=1,8-cineol), α-pinene, borneol and myrtenol (Hänsel, Hölzl 1996, p. 151-152).

	1,8-cineole	β-pinene	tricyclene	myrcene	terpinen-4-ol	α-terpineol
Rocha Vilela et al. (2009)	89,95	1,64	2,95	0,49	0,72	0,62
Derwich et al. (2009)	22,35	5,2	-	1,15	3,1	1,0

Table 4. *Eucalyptus globulus* essential oil components % Area

The study of Cermelli et al. (2007) deals with the antiviral activity of Eucalyptus oil against adenovirus (ADV) and mumps virus (MV). The cytotoxicity test results a concentration of 0,25µl/ml, which is the hightest nontoxic concentration. In the antiviral test eucalyptus oil exibits a high activity against MV compared to ADV. Since the ADV and MV are enveloped viruses, one explanation can be the direct effect on the virus particles.

The study of Schnitzler et al. (2001) examines the antiviral activity of EUO against Herpes simplex virus. EUO exibits an IC_{50} at 0,009% and 0,008% for HSV-1 and HSV-2, respectively. EUO causes a reduction of infectivity of 57,9% (HSV-1) and 93,9% (HSV-2). In another experiment EUO indicates an effect before or during adsorption, which also suggests a binding to virion envelope so that an entry to the cell hosts are inhibited.

Eucalyptus oil posses antiviral activity against HSV-1 and HSV-2 in vitro. Further investigations have to be carried out to detect the antiviral agents.

2.6.3. Melaleuca alternifolia

Melaleuca alternifolia is a member of the Myrtaceae family and is a native plant in Australia. The plant grows in a restricted area of northern New South Wales. *Melaleuca* essential oil is one of the popular oils that is known for its antiseptic effects. It is also called tea tree oil and has been traditionally used as topical formulations to cure cutaneous infections. Tea tree oil (TTO) is extracted from leaves and branches, there are about 1,8% essential oil (Blaschek et al., 2007). The essential oil has a high amount of terpinen-4-ol and γ-terpinene, and moderate amounts of 1,8-cineole, p-cymene, α-terpinene, terpinolone and α-terpineol. In table 5 the main compounds of the essential oil are listed.

	terpinen-4-ol	δ-terpinene	1,8-cineole	p-cymene	α-terpinene	terpinolone	α-terpineol
Shellie (2003)	36,71	22,20	2,49	2,52	10,10	3,53	2,74
Brophy and Davies (1989)	40,10	23,0	5,1	2,9	10,4	3,1	2,4

Table 5. *Melaleuca* essential essential oil components (% w/w)

In the study of Garazzo et al. (2009) the antiviral activity of *Melaleuca alternifolia* essential oil in vitro is investigated. The essential oil and its compounds, terpinen-4-ol, α-terpinene, δ-terpinene, p-cymene, terpinolene and α-terpineol, are tested against influenza A/PR8 virus subtype H_1N_1 in Madin-Darby Canine Kidney

(MDCK) cells, HSV-1 and HSV-2 in vero cells, Echovirus 9 (Hill strain) in LLC-MK2 cells, Poliovirus 1 (Sabin strain), Coxsackievirus B1 and Adenovirus 2 in HEp2 cells. A 10% dillution of the components in DMSO are added to the medium with different concentrations from 0,1% (v/v) to 0,001% (v/v). The cytotoxicity is measuered by light microscopy where cell morphology is evaluated, the cell growth is observed by the MTT method after 24, 48 and 72h. In this study terpinolene, α-terpinene and δ-terpinene value 0,012 % (v/v) CD_{50}[15] demonstrate the hightest cytotxic ability, wheras terpinen-4-ol, p-cymene and α-terpineol have a CD_{50} of 0,05 % (v/v), TTO a CD_{50} of 0,025% (v/v).

In the screening of the antiviral activity TTO, terpinen-4-ol, terpinolene and α-terpineol show an inhibitory effect on influenza virus A/PR8 replication below the CD_{50}. For the experiment 0,5ml of virus suspension and different concentrations of the compounds are incubated for 1h at 37°C. The ID_{50} of the essential oil and its components are 0,0006% (v/v), 0,0025% (v/v) for terpinen-4-ol, 0,0012% (v/v) for terpinolene and 0,025% (v/v) for α-terpineol. The other compounds are found to be ineffective against influenza virus and none of the compounds show any activity against polio 1, adano 2, ECHO 9, Coxsackie B1 and influenza virus. TTO (0,125% v/v) shows a slight reduction against HSV-1 and HSV-2.

The study of Garazzo et al. (2011) deals with the activity of *Melaleuca alternifolia* oil and its components on influenza virus A/PR/8. This study is based on the results of the previous study of Garazzo et al. in 2009, where the inhibititory effect of TTO on influenza is demonstrated on the concentration below the cytotoxic dose. TTO and its components terpinen-4-ol, terpinolene and α-terpineol are diluted in DMSO to a concentration of 5% (v/v), oseltamivir and ribavirin are used

[15] CD_{50} (median curative dose) a concentration that causes symptoms in 50% of the cell medium.

as reference compounds, macrolide antibiotic bafilomycin A1 (Sigma) is used as reference compound blocking the vacolar H+-ATPase proton pump. For cell cultures, Madin-Darby canine kidney (MDCK) cells are used, for working stock solution RPMI (Roswell Park memorial institute medium) are prepeared. The infectivity of virus stock and virus yield is determined by the MTT method. In the neuroaminidase inhibition assay the virus stock solution is used and the virus yield is evaluated by measuring by haemagglutinin units (HAU).

The influenza virus replication is analyzed in MDCK cell after infecting with HAU/ml and in CPE_{50}[16] assays. The two methods show a concordant result. For the evaluation of the effect of TTO and its compounds on different steps of replication of influenza, the compounds are added at different times-period after infections. TTO, terpinen-4-ol, terpinolene and α-terpineol interfere with an early step of the replication, especially when the compounds were added within 1 h after adsorbation, while no reduction is determined if added more than 2 hrs. At 120min a slightly reduction is observed. The early interaction with the viral replication indicates the virucidal effect or the protection action for the MDCK cells. But as a result no virucidal effect as well as protective action for the cells are demonstrated. In other experiments the influence of the test compounds on the viral adsorption are tested. Therefore, infective center assays and haemoagglutionation inhibition assay are used. In the infective center assay MDCK cells are incubated with the virus compound mixtures for 120 min at 4°C, at temperatur where the viruses are inhibited to enter the cells. Afterwards the unobsorbed virus and free compounds are washed away with RPMI. As a result none of the compounds interfere with the

[16] CPE_{50} (cytopathic effect), structural changes in a host cell resulting from viral infection. CPE occurs when the infecting virus causes lysis (dissolution) of the host cell or when the cell dies without lysis because of its inability to reproduce.

cellular attachment of the virus. The haemoagglutination inhibition assay does not show any interaction with the viral adsorbtion.

In the neuroaminidase inhibition assay, the inhibitory effect on the viral neuroaminidase in comparison with oseltamivir is evaluated. TTO and its components do not show any inhibitory effect, only a slight effect from TTO (about 35%) is observed.

In this study the effect of TTO on acidification of cellular lysosomes is studied by vital staining with acridine orange (AO). This experiment is carried out to examine if TTO and its components exert an inhibitory effect on the acidification of intracellular compartments like endosomes and lysosomes inhibiting the influenza virus growth in MDCK-cells. Acridine orange (AO) is a weak base, which is absorbed by the living cells and protonated in endosomes/lysosomes. MDCK-cells are incubated with or without the TTO compound in different cencentrations in RPMI and at the end of the incubation the cells are treated with AO. The experiment is carried out in the dark and is examined through flourescence light microscopy. Untreated MDCK stained with AO cell components such as nuclei, nucleoli and the cytoplamsma show green flourescensce. The granular pattern in the cytoplasma shows an orange flourescense, which is caused of the acidification of the lysosomes. At a concentration of 0,01% (v/v) of TTO, when treated with acridine orange 4h before, a complete disappearance of the orange flourescense occures, whereas the green flourescense remaines. When treated with 100 and 10nM of bafilomycinA1 the same results are observed. On the same and lower concentration (0,0025% v/v) the cytoplasmic orange flourescense is still the same. Terpinen-4-ol demonstrates a cleary acridine orange accumulation. When the cells are treated with 0,01% (v/v) TTO for 4 h, afterwards washed and then incubated

again for another 2h without the compound, the lysosomes in the MDCK-cells show acidification. This impliments the reacidification after treatment with TTO and terpinen-4-ol and moreover, the cell morphology is not influenced by the treatment at all. To sum up, this study showed the inhibitory effect of TTO against influenza virus in MDCK-cells through interferance with acidification of lysosomes, which could initiate viral uncoating.

The study of Schnitzler et al. (2001) examines the antiviral activity of Australian tea tree oil and eucalyptus oil (EUO) against herpes simplex virus in cell culture. The cytotoxicity of the essential oils on RC-37 cells is determined with the neutral red assay. The TC_{50} of value of TTO is determined at 0,006% and of EUO 0,03%. The virucidal activity of the TTO and EUO in different concentration from 0,00001-0,01% is tested against HSV-1 and HSV-2. The compounds are diluted in ethanol and 1% ethanol is used as control. The IC_{50} values are evaluated at 0,0009% (HSV-1) and 0,0008% (HSV-2) for TTO, 0,009% (HSV-1) and 0,008% (HSV-2) for EUO. Eucalyptus oil has a lower virucidal effect. In the plaque formation both essential oils, eucalyptus and tea tree oil, show a dose-dependent effect. TTO reduces the cytotoxicity of HSV-1 and HSV-2 by 98,2% and 93,0% at a noncytotoxic concentration of 0,003%, wheras eucalytpus oil shows a reduction of 57,9% and 75,4% for HSV-1 and HSV-2, respectively. In addtion, the antiviral activities at different periods of time are determined. When pretreating the cells with tea tree and eucalyptus essential oil no virucidal reduction is observed. On the contrary, pretreatment virus prior infection shows a significant reduction of plaques, TTO values a reduction of 98,2% and 93,0% for HSV-1 and HSV-2. EUO has a reduction value of 57,9% for HSV-1 and 75,4% for HSV-2. No reduction is observed during the absorbtion period, TTO has a reduction of 39,0% for HSV-1

and 37,7% for HSV-2 and by about 40% reduced with EUO for HSV-2. Besides no significant effect occures when the essential oil is added after the adsorption period. This study demonstrates that TTO has an antiviral effect on the free virus, and that TTO may interact with the viral envelope and gylcoproteins. This has already been analyzed in another study, where the antiviral activity of the essential oil of *Melaleuca alternifolia* against tobacco mosaic virus is studied (Bishop, 1995). The essential oil is tested for antiviral activity against tobacco mosaic virus. In this experiment a pre-inoculum spray of the essential oil is applied to plants of *Nicotiana glutinosa*. The solutions contain 100ppm, 250ppm and 500ppm of oil in distilled water. To increase in solubility, a suitable amount of oil is mixed with 0,1 mL of Tween 80, which contains 0,05 mL ethanol and then is filled up to 200 mL with distilled water. Spray solutions of water/ethanol and water/ethanol/Tween80 are used as control. The plants are sprayed with the solution and are left to dry for 15 minutes. Afterwards the virus inoculum is sprayed on the same plant. The experiment has an overall 10-day period length, and every other day local lesions of the treatments are examined. After 10 days the leaves are removed and are analyzed. Each leave is measured and the lesion number per cm^2 leaf area is calculated. As a result, the experiment demonstrates a significant decrease in the lesion number, when the essential oil is sprayed on first before the virus inoculum.

Many authors report the antiviral activities of *Melaleuca alternifolia* essential oil. The study of Garrazo et al. (2009) demonstrates the antiviral effect against influenza A virus on MDCK cells, which is based on the main component terpinen-4-ol. Wheras on the replication period of HSV-1 and HSV-2 viruses the essential oil is not effective, which contradicts the results of the study of Schnitzler et al. (2001), where the antiviral effect on the free virus is demonstrated. Additionally,

Bishop et al. (1995) reported an antiviral effect on the nonenveloped plant virus (tabacco mosaic virus) as well. In the second study of Garrazo et al. (2011) the antiherpetic effect is proven. The viral replication is significantly inhibited when the essential oil is added during infection. This indicates the interferance of acidification of the lysosomes that could initiate viral uncoating. TTO possess antiviral activity and terpinen-4-ol is one of the main compounds, which is very effective.

2.7. Oreganum vulgare, Syzygium aromaticum

Oreganum vulgare is a member of the Lamiaceae family. It grows naturally in warm regions in western, southern and central Asia and in mediterranean regions, too. *Oreganum* is a prennianal herb and grows up from 20-80 centimeters. The flowers are purple with a length of 3-4 millimeters. The main compounds of the essential oils are carvacrol, thymol, terpinene, pinene, ocimene and caryophyllene (Table 6.).

	carvacrol	thymol	γ-terpinene	β-pinene	ocimene	caryophyllene
Mediterranean	19.54	2,0	11,57	1.05	16,75	2,19
Mexican	29.80	12.30	0,40	0,40	19,40	4.80

Table 6. *Oreganum vulgare L*. essential oil components % Area, Karimi et al. (2010)

Syzygium aromaticum (cloves) is a member of the Myrtaceae family. Cloves are native to Indonesia and are widely used as a spice. It is an evergreen plant and grows up to 12 meters. The main compound of the essential oil is eugenol. Other compounds are eugenyl acetate, caryophyllene oxide. A comparison of the compounds from 2 authors is listed below (Table 7.).

	eugenol	eugenyl acetate	caryophyllene oxide
Nasar et al. (2007)	71,56	8,99	1,67
Chaieb et al. (2007)	88,58	5,62	-

Table 7. *Syzygium aromaticum* essential oil components % Area

In the work of Siddiqui et al. (1996) the antiviral activity of oregano and clove oils on HSV-1 and Newcastle disease virus is tested. For the experiments Lily Laboratories Culture Collection monkey kidney (LLCMK2), human lyrngaleal carcinoma (Hep-2), African green monkey kidney (Vero) and human embryonic lung (WI-38) cells are used. Various dilutions of the oils in organic solvents including dimethyn sulfoxide are prepared, the oil emulsion contained 0,1% agar. This emulsion with oil-coated agar has an initial dilution of 1:50. The rest is diluted in PBS (phosphate buffered saline) with 2% foetal bovine serum.

For cytotoxicty screening, double dilution of oregano and clove oils are added to the cell cultures. In the first 12h, the 0,002% solution destroyed the vero and Hep-2 cells. The experiment shows a linearly dependent toxicity and clove oil has a

higher toxicity than oregano oil. Due to the high cytotoxicity of the oils, further experiments either as desinfectant or local medicament for superficial virus lesions are examined. The experimental result demonstrates that the initial dilutions (0,02%; 0,01%; 0,004%) of oregano and clove oil destroyed the cells. As a consequence the viruses are not able to replicate. Higher dilutions up to 0,00025% show a dose-dependent response on destroying enveloped viruses. In the electron micrographs both oregano and clove oils show distraction of the enveloped viruses, whereas non-enveloped viruses such as ADV-3 (Adenovirus serotype 3) and PV-S (Potatovirus type S) are not affected.

These experiments demonstrate the active lipide lesion of RNA and DNA viruses, but on non-enveloped viruses both essential oils are not effective. Oregano and clove oils have the abilitiy for lysis by affecting the viral envelope of RNA and DNA viruses.

2.8. Oenanthe crocata, Ridolfia segetum

Oenanthe crocata is a perennial plant which belongs to the Apiaceae family. The plant is widely spread in the Mediterranean region. It grows wild along rivers and contains a toxic compound oenanthotoxin, which causes neuromuscular spasm of mimic. *Ridolfia segetum*, an annual plant also belonging to the Apiaceae family can be found in cornfields and is mostly used in the folkmedicine, especially for the treatment of digestive disorder (Bicchi et al. 2009).

In the study of Bicchi et al. (2009) the antiviral activities of the essential oils of *Ridolfia segetum* and *Oenanthe crocata* against HIV-1 virus are investigated. For the experimental analysis the two plant material is collected in San Basilio (*O. crocata*) and Monastir (*R. segetum*) in Sardinia. Aerial parts of the plants are

steam destillated for 5 hours and the yields of the essential oils are 0,4% v/w for *R. segetum* and 0,004% v/w for *O. crocata*. Components of *Onanthe crocata* and *Ridolfa segetum* essential oil are listed below (table 8. and table 9.).

	heptanal	β-pinene	terpinen-4-ol	1,8-cineole	sabinene
Bicchi et al. (2009)	0,49	10,81	1,84	-	25,67
Bonsignore et al. (2004)	8,1	2,2	1,1	21,7	-

Table 8. *Onanthe crocata* essential oil components % Area

	α-thujene	α-pinene	α-phellandrene	β-phellandrene	α-terpinolene
Bicchi et al. (2009)	0,41	3,23	53,34	9,11	20,48
Pala-Paul et al. (2002)	0,5	1,4	62,0	2,9	8,4

Table 9. *Ridolfa segetum* essential oil essential oil % Area

For the acitivity screening, two enzyme associated activities of the HIV-1 reverse transcriptase (RT), RNA-dependent DNA polymerase (RDDP) activity and ribonuclease H (RNase H) activity are evaluated. *Ridolfa segetum* essential oil inhibits the HIV-1 RT RDDP-associated activity in a dose dependent matter (IC_{50}=

0,095mg/ml), but does not show any activity on the HIV-1 RT RNase. *Onanthe crocata* essential oil is less active (IC_{50}=0,36mg/ml) against HIV-1 RT RDDP and the HIV-1 RT associated RNase-H activity is not inhibited. In the cxtotoxicty assay, the ability of the essential oils on K_{562}[17] are tested. Both essential oils have a dose dependent K_{562} cytotoxcity.

In the study of Bonsignore et al. (2004) the antiviral activity of *Oenanthe crocata* against HSV-2 (Herpes simplex virus type 2), 1S (Sabin type polio virus) and WSN (type A polio virus) is evaluated. The assays do not show any significant antiviral activity for this essential oil.

The study of Bicchi et al. (2009) demonstrates the antiviral activity of *Ridolfa segetum* against HIV-1 RT RDDP activity, which hypothesizes that components of the essential oil have the ability to inhibit the virus. On the other hand *Oenanthe crocata* essential oil is not effective against the many viruses tested in both studies. *Ridolfa segetum* seems to be a promising target, but still further evaluations need to be done regarding the antiviral components.

2.9. Salvia species

The genius *Salvia* is a member of the Lamiaceae family, which contains over 900 species. The most popular plants are *Salvia officinalis* (sage) and *Salvia fructicosa* (*S. triloba*). *Salvia officinalis* is native to Southern Europe as well in Central Asia and the U.S. *Salvia* has been applied in folkmedicine and it is known for numerous effects. It is used for treatments of colds, bronchitis and menstrual disorders to name but a few. Especially in Europe it is used orally as a mouthwash or gurgle

[17] K_{562} is a chronic myeloid leukemia cell line.

against oropharyngeal inflamations, (Topcu, 2006). In table 10. compounds of the *Salvia fructicosa* essential oil are listed.

	1,8-cineole	α-thujone	β-thujone	camphor	β-caryophyllene
Sivropoulou et al. (1997)	47,48	4,32	7,61	9,04	1,69
Pitarokili et al. (2003)	35,5	1,1	0,8	8,7	-

Table 10. *Salvia fructicosa* essential oil components % (v/v)

2.9.1. Salvia fructicosa

In the study of Sivropoulou et al. (1997) the antiviral activity of *Salvia fructicosa* essential oil is evaluated. For the experiments vero cells derived from the kidney of African Green Monkey in DMEM (Dulbecco's Modified Eagle Medium) are used, with 10% fetal calf serum (FCS). Dilutions of 1:500, 1:1000 and 1:2000 (v/v) of the compounds in ethanol are applied for various incubation periods (24, 48 or 72h). After incubation DMEM is removed and the cells are calculated with the trypan blue-exclusion method. The dilution of 1:500 shows complete cell death within 24h, while on the 1:1000 dilution an almost complete mortality is observed. The 1:2000 dilution has a smaller decrease compared to the other dilutions. Thujone shows the hightest cytotoxicity with 95% reduction at 1:1000 dilution. For the evaluation of the antiviral action against HSV-1 virus, various concentrations of the essential oil are incubated for 30 min. At the concentration of 0,2%, an 80% virus inactivation results. When the concentration is doubled, the effect speeds up to

almost $4\log_{10}$. Among the compound tested, thujone shows the most active virucidal effect. With the dilution of 0,1% virus particles were inactivated by 95%, while 1,8-cineole and camphor has a 35% and 0% virus inactivation, respectively. Even higher concentration of thujon (0,2%), the virucidal action was accelerated by 5 $\log_{10}$. This study also demonstrated the antiviral effect of some other compounds such as borneol, bornyl acetate and isoboreneol.

2.9.2. Salvia limbata C.A.Mey., Salvia sclarea

The next study also deals with the antiviral activity of the essential oils of *Salvia limbata* C.A. Mey. and *Salvia sclarea* L (Ögütcü et al., 2008). The main components of *Salvia limbata* essential oil are spathulenol (29,3 %), β-eudesmol (7,4%), 1,8-cineole (3,2 %), bicyclogermacrene (2,3%) and β-pinene (2,2%) based on area peaks. *Salvia sclarea* consists of germacrene (24,72%), β-caryophyllene (16,24%), bicyclogermacrene (9,63%), linallyl acetate (5,52%) and α-copaene (3,78%). For the experiments MDCK (Madin-darby canine kidney) and MDBK (Madin-Darby bovine kidney) in Dulbecco's Eagle medium with fetal calf serum (FCS) and antibiotics are used. 4-fold dilutions of the samples in growth medium are incubated with the cell monolayers and are microscopically observed. The cytopathic effect (CPE) is evaluated under a score schema, and the amount is calculated after 48-72h. *Salvia sclarea* essential oil has a TC_{50} of 0,003 mg/ml both for MDCK and MDBK cells. The antiviral effect against influenza virus and against HSV-1 and HSV-2 is lower than the cytotoxic effect. This study demonstrates no significant antiviral effect for the essential oils from *Salvia limbata* and *Salvia sclarea*.

2.9.3. Salvia cedronella

The study of Alim et al. (2009) examines the antiviral activity of the essential oil of *Salvia cedronella* Boiss aganist influenza virus in MDCK cells and 2 herpes viruses in MDBK cells. 92 components are detected by means of GC-EIMS (gas chromatography-electron impact mass spectrometry). The major components of the essential oil are 1,8-cineole (13,3%), α-pinene (10,1%), caryophyllene oxide (9,8%) and sabinene (7,3%). As a result the methanolic extract shows an EC_{50} of 0,30 mg/ml against influenza virus (A/Weybridge) and for influenza (A/Aichi) the EC_{50} values of 0,60 mg/ml. The methanolic extract also shows an antiherpetic effect (TC_{50}= 0,60 mg/ml for HSV-1, TC_{50}= 0,50 mg/ml for HSV-2).

In these studies the antiviral effect of selected *Salvia* species are evaluated. *Salvia fructicosa*, shows a high antiviral activity among these popular species. Especially thujone has found to be the most active compound (Topcu, 2006). In addition, *Salvia cedronella* posses antiviral activity, which is demostrated by Alim et al. (2009). In contrary, *Salvia limbata* and *Salvia sclarea*, analyzed by Ögütcü et al. (2008), demonstrate a poor antiviral activity. This can be possibly due to the geographical variance and the variaties of the compounds of *Salvia* species, which probably have an influence on the viral activities.

2.10. Santalum album L.

Santalum album L. (Santalaceae) known as sandalwood, is a plant native to the south of India and the Malayan Archepelago. The essential oil is usually found in the roots and woods and it has been used as an antimicrobial in Asia. It is one of the oldest and high priced oils used in the parfumery, cosmetic and aromatheraphy industries. The main compounds are α-santalol (50-60%) and β-santalol (20-25%) (Krotz and Helmchen, 1994). In table 11. the compounds of *Santolina insularis* essential oil are listed.

	α-santalene	cis-α-santalol	(Z)-trans-α-bergamotol	cis-β-santalol	cis-lanceol
Braun et al. (2003)	0,7	41,1	6,4	19,8	1,4
Minh Tu (2007)	0,81	42,9	5,16	18,69	6,6

Table 11. *Santolinum insularis* essential oil components % Area

In the work of Benencia and Courreges (1999) the antiviral activity of sandalwood oil against herpes simplex virus is evaluated. For the experiment Monkey kidney Vero cells, which were grown in minimum essential medium (MEM) with 5% heat inactivates calf serum and 50μg/ml gentamicine are used. The cytotoxicity assay shows no effect below the hightest concentration tested (60μg/ml). No morphologic changes are observed in this experiment, and the trypan blue exclusion method demonstrates no significant changes, too. For antiviral

experiments vero cells were infected with 80 PFU and immediately incubated for 1h at 37°C. Different concentrations of sandalwood from 7,5-60μg/ml are added on infected cells and after 2 days of incubation the virus plaques are counted according to the MTT colorrimetric assay. As a result sandalwood shows a dose-dependent inhibition of HSV-1 and HSV-2 replication, which is affirmed of the reduced number of PFU in the treated cultures. HSV-1 has an EC_{50} of 25, which is more significant compared to HSV-2. When the essential oil (60μg/ml) is mixed with 10^6 PFU of either HSV-1 or HSV-2 no virucidal effectivity is observed.

This study demonstrates the antiviral effect of sandalwood against HSV-1 and HSV-2. The experiments indicate the influence of the essential oil on the replication of the virus in treated cells. Since the detailed mechanism is yet unclear, further investigations have to be carried out.

2.11. Santolina insularis

Santolina insularis is a genius of the Asteraceae family, which is native to mediterranean regions. The aerial parts amount about 1,6% essential oil. The main compounds of the essential oil are, α-pinene (2.11 %), camphene (8,47%), cineole (9,01%), 3,3,6-trymethyl-1,5-heptadien-4-one (21,18%), bornyl acetate (6,35%), camphor (1,68%), borneol (4,23%), aromadendrene (0,765), 10-H-cyclopropyl-1,1,7-trimethyl-methylen-decahydro azulene (12,7%) and muurolene (0,94%), (Poli et al. 1997).

In the study of De Logu et al. (2000) the effect of *Santolina insularis* essential oil against HSV-1 and HSV-2 is investigated. Two methods are used to test the cellular toxicity of *Santolina insularis.* At first, for cell growth examination the trypan

blue dye exclusion test is used. *Santolina insularis* essential oil is incubated in African green monkey kidney cells (Vero) for 72h at 37°C. As second method the MTT test is used. Here, monolayers of vero cells are incubated with the essential oil for 48h. Afterwards the medium is replaced with a solution of tratzolium bromide (MTT, Sigma). In the trypan blue exclusion test the CC_{50} values are 105μg/ml against vero cells, in the MTT the results are similar (CC_{50} = 112μg/ml).

In the plaque reduction assay the antiviral activity was tested against HSV-1 and HSV-2. Monolayers cultures of Vero cells grown in RPMI (Roswell park memorial institue cell medium) were infected with 200-250 PFU of the virus, some were also performed by incubating about 200-250 PFU of the virus with the essential oil at a concentration of 30-0,03μg/ml at 37 or 4°C for different time periods up to 2h. In other experiments vero cells were pre-incubated with the essential oil for 1h at 37°C before infection with the virus. The plaque reduction assay shows both before and during adsorption a concentration-dependent inhibition of plaque formation by *Santolina insularis*. At a concentration of 0,88 μg/ml a 50% inhibition is observed, an 80% inhibition at 1,87 μg/ml for HSV-1. For HSV-2 the 50 and 80% inhibition are at the concentrations of 0,7 and 1,25 μg/ml, respectively. When HSV-1 and HSV-2 are pre-incubated for 2h at 37°C a higher inhibition is observed (50% inhibition at 0,31 and 0,26 μg/ml; 80% inhibition at 0,78 μg/m and 0,89 μg/m for HSV-1 and HSV-2, respectively). The inactivation is clearly dependent on the length of exposure. When viruses are pre-incubated for 15 min before adsorption, HSV-1 incubation (50% inhibition at 6,39 μg/ml, 80% at 12,1 μg/ml) are higher than HSV-2 (50% inhibition at 7,66 μg/ml, 80% at 21,29 μg/ml). Compared to the pretreatment with essential oil no changes are observed, also no inhibition is observed when the pre-incubated cells with essential oil are infected afterwards

with the virus. Moreover, no antiviral activity is shown when the virus is incubated before virus adsorption.

In the yield reduction assay vero cells were infected by HSV-1 for 1h at 37°C. After absorption *Santolina insularis* essential oil with concentrations from 40 to 2,5 μg/ml is added. The assay shows a dose-dependent activity against HSV-1. 80% inhibition is observed at 40 μg/ml of essential oil, 42,7 % at 10 μg/ml. In another experiment, when the vero cells were pre-incubated with essential oil for 1h at 37°C, the inhibition is higher (84,2 and 55,7 % at 40 and 10 μg/ml, respectively).

In another assay the antiviral activity is evaluated during the viral adsorption, in which the infected cells are incubated with the essential oil. As a result a concentration-dependent reduction is observed for both HSV-1 and HSV-2. In additon, HSV-2 plaque developement is completely abolished by *Santolina insularis* at a concentration of 40 μg/ml after 24h post-infection. At 20 μg/ml (after 24h) and 40μg/ml (after 48h) concentration a significant reduction is observed. Also against HSV-1 a significant plaque reduction (>50%) is observed at the concentrations of 40 and 20μg/ml after 72 and 90h, respectively.

In the attachment assay a 50% inhibition is observed at a concentration higher than 30μg/ml for HSV-1 and HSV-2 compared to the controls. This result indicates a direct effect on the virus. In addition, vero cells are infected with HSV-1 and HSV-2 at 4°C in order to inhibit penetration, afterwards the temperatur is increased to 37°C while adding the essential oil. This experiment demonstrates no inhibition effect. Since the experiment is carried out above room temperature (37°C), one explaination for the result is, that the essential oil is already evaporated. Equally no inhibition effect is detected by the post-attachment virus neutralization assay.

To sum up, this study shows the antiviral activity of *Santolina insularis* essential oil

against HSV-1 and HSV-2 in vitro. HSV-1 and HSV-2 have a CC_{50}/IC_{50} [18] ratio of 127 and 160, respectively.

Santolina insularis essential oil inactivates the virus effectively. The experiments show that the essential oil directly inactivates the virus particles by preventing the adsorption to the host cells. The attachment of the virus is not inhibited and virus penetration is not prevented when adding the essential oil after adsorption. The essential oil also shows a reduction in the plaque development assay, where the essential oil spread the cell-to-cell virus in virus-infected cells. The virus inhibition is slightly observed when the cells were pretreated with *Santolina insularis* essential oil before virus absorption (yield reduction). This indicates an intracellular effect.

2.12. Teucrium species

Teucrium arduini belongs to the Lamiaceae family and is widely spread in Mediterranean regions from the western Balkan, along the Adriatic coast from the Istra Peninsula in Croatia in the north to Albania in the south. It has been used for many thousand years and has many effects like antiinflamatory, antimicrobial and antiulcer. The main compounds of the essential oil are β-caryophyllene (19,9%), caryophyllene oxide (14,6%), β-farnesane (5,6%) and limonene (4,7%) based on the total peak area (Dunkie et al. 2011).

In the study of Dunkie et al. (2011) *Teucrium arduini* essential oil is tested against tabacco mosaic virus (TMV) and cucumber mosaic virus (CMV). Spray solutions of the essential oil, β-caryophyllene and caryophyllene oxide are applied to the infected plants of *Chenopodium amaranticolor* and *Ch. Quinoa*. The essential oil

[18] CC_{50}/IC_{50} is the selectivity index (SI) which calculated from the cytotoxic and inhibition concentration.

exibits an inhibition of 25,7% for TMV and 21,9% for CMV infection. In this study the main compounds are also applied individually and show an inhibition of 30,8% and 36,9% for β-caryophyllene and caryophyllene oxide against CMV, respectively. Wheras for the inhibition of TMV a low effect is demonstrated. One explanation can be the synergistic effect of the essential oil against TMV compared to the individual compound. All in all, β-caryophyllene and caryophyllene oxide posses higher antiviral activities against CMV than TMV.

In the study of Bezic et al. (2011) the antiphytoviral activity of Croation *Teucrium* Species such as *Teucrium polium*, *Teucrium flavum*, *Teucrium montanum* and *Teucrium chamaedrys* against cucumber mosaic virus is analyzed. The major compounds of the essential oils are are β-caryophyllene and /or geracrene D (Table 12.). Spray solutions of each *Teucrium* species is applied to *Chenopodium quinoa*, which are infected with cucumber moisaic virus (CMV). The experiments show a significant reduction of CMV infections by the mean of number of lesions. The essential oil from *T. montanum* (44,3%) demonstrates the highest reduction, followed by *T. polium* (41,4%), *T. chamaedrys* (25,7%) and *T. flavum* (22,9%).

	Bezic et al. (2011)	Bezic et al. (2011)	Bezic et al. (2011)	Bezic et al. (2011)	Dunkic et al. (2011)
	T. polium	*T. flavum*	*T. montanum*	*T. chamaedrys*	*T. aruduini*
β-caryophyllene	52,0	23,1	7,1	47,6	19,9
germacrene D	8,7	15,3	17,2	29,0	-
α-pinene	-	10,5	-	-	-
β-pinene	-	-	12,3	-	-
caryophyllene oxide	-	-	-	-	14,6

Table 12. *Teucrium species* essential oil components % Area

These studies confirm the antiviral activities of selected *Teucrium* species. β-caryophyllene the main compound of the essential oils is responsible for the effective antiviral activity against CMV. Also other compounds may contribute to this effect as well. To analyze the mechanism for the antiviral activity further evaluations are needed.

2.13. Thymus transcaspicus

Thymus transcaspicus (Khorasan thyme) belongs to the Lamiaceae family, which is native to Iran and Turkmenistan. *Thymus* sp. has been used in folk medicine for its antiseptic properties. It is still used for several treatments, such as respiratory

diseases, colds and coughs (Teuscher et al. 2004). In table 13 the main compounds are listed.

	thymol	γ-terpinene	carvacrol	ρ-cymene
Behravan et al. (2011)	56,4	7,7	7,6	6,3
Tabrizi et al. (2010)	54,3	4,2	8,4	-

Table 13. *Thymus transcaspicus* essential oil components % Area

In the study of Behravan at al. (2011) the antiviral activity of *Thymus transcaspicus* essential oil is investigated. For the experiments *Bacillus* phage CP51 (bacterial virus) and a plaque reduction assay are used. Different dilutions of the essential oil are pre-incubated with phage CP51. As a result dilutions of 10^{-2}, 10^{-3} and 10^{-4} (v/v) showed a significant reduction of plaque reduction units (>50%). Similar results are obtained when *Bacillus* phage CP51 and dilutions of the essential oil are incubated together. As a result a significant reduction (>50%) is observed at the dilutions of 10^{-2}, 10^{-3} and 10^{-4} (v/v). This study demonstrates an antiviral effect of *Thymus transcaspicus* essential oil. The absorption of the virus is inhibited and the transfection process of the virus is influenced, which is demonstrated by the significant growth reduction observed in this study.

2.14. Spices

In the study of Romeilah et al. (2010) the antiviral activities of 7 essential oils from *Allium cepa* l., *Allium sativum*, *Cuminum cyminum* (seeds), *Corriandrum sativum* (herb and seeds), *Petroselinum sativum* (herb) and *Ocimum basilicum* (herb) against herpes simplex virus type 1 are investigated. The main compounds of these essential oils are linalool (coriander herb, seeds and basil oils), cuminaldehyde (cumin oil), diisoporpyl trisulfide (onion oil), di-2-propenyl disulfide, methyl-2-propenyl trisulfid and di-2-propenyl trisulfide (garlic oil), myristicin and apiol (parseley) (Table14. -19.).

	diisopropyl trisulfide	isopropyldithioisopropane	2-tridecanone	dipropyl disulfide	methyl propyl trisulfide
Allium cepa (bulbs)	20,96	18,10	10,45	8,83	8,10

Table 14. *Allium cepa* essential oil components % Area

	di-2-propenyl disulfide	methyl-2-propenyl trisulfide	di-2-propenyl trisulfide	disulfide	diallyl tetrasulphide
Allium sativum (bulbs)	25,18	23,80	21,05	4,6	3.56

Table 15. *Allium sativum* essential oil components % Area

	cuminaldehyde	caryophyllene ocide	β-pinene	geranyl acetate
Cuminum cyminum (seeds)	60,01	6,12	4,89	4,11

Table 16. *Cuminum cyminum* essential oil components % Area

	linalool	γ-terpinene	limonene	camphor
coriander herb oil	68,36	3,11	2,47	2,41
coriander seeds oil	73,79	4,31	3,59	4,43

Table 17. *Corriandum sativum* essential oil components % Area

	myristicin	apiol	α-pinene	β-pinene	elemicin
Petroselinum sativum (herb)	25,20	18,23	16,16	11,16	4,30

Table 18. *Petroselinum sativum* essential oil components % Area

	linalool	1,8-cineole	β-farnesene	α-guaiene
Ocimum basilicum (herb)	55,55	11,67	7,10	6,14

Table 19. *Ocimum basilicum* essential oil components % Area

The evaluated essential oils are cultivated in Egypt and are assayed on infected vero cell lines. 3 different concentrations are incubated. The antiviral activity shows a dose dependent manner. Basically, the highest concentration (1000µg/ml) shows a herpetic effect; among them are onion, garic, cumin, coriander seeds, basil and parsley essential oil. Garlic oil has the highest EC_{50} (320µg/ml). The lowest EC_{50} is evaluated for coriander herb oil (2045µg/ml).

This study demonstrates the antiviral activities of the essential oils of *Allium cepa* L. (bulbs), *Allium sativum*, *Cuminum cyminum* (seeds), *Corriandrum sativum* (herb and seeds), *Petroselinum sativum* (herb) and *Ocimum basilicum* (herb) against herpes simplex virus 1. These findings can be resulted of the various compounds of the essential oils, which have imparted the different antiviral activities. The essential oils have potential as natural antiviral agents, but still further studies are needed to evaluate the antiviral mechanism.

In the study of Loizzo et al. (2008) antiviral activities of the essential oils of seven Lebanon spices are analyzed. Essential oils of *Laurus nobilis*, *Juniperus oxycedrus* ssp. *Oxycedrus*, *Thuja orientalis*, *Cupressus sempervirens* ssp. *pyramidalis*, *Pistacia palaestina*, *Salvia officinalis* and *Satureja thymbra* are tested against SARS-CoV (severe acute respiratory syndrome-corona virus) and HSV-1

(herpes simplex virus type 1) virus replication in vitro. In table 20. (page 56) the main compounds of the essential oils are listed. As a result *L. nobilis* oil demonstrates the highest antiviral activity against SARS-CoV (IC_{50}=120µg/ml), followed by *T. orientalis* (IC_{50}=130µg/ml) and *J. oxycredrus* ssp. *oxycedrus* (IC_{50}=220µg/ml). Additionally, *L. nobilis* also achieved the most effective activity against HSV-1 virus (IC_{50}=60µg/ml). In terms of safety level, which describes the SI (selectivity index) based on SARS-CoV, *L. nobilis*, *T. orientalis* and *J. oxycredus* ssp. *oxycredus* exibit a higher SI compared to glycyrrhizin (SI=1,2).

Among the spices analyzed in this study *L. nobilis* essential oil shows a high antiviral activity against SARS and HSV-1 virus. One explanation for the results could be the composition of the essential oil, which contains a high amount of β-ocimeme (21,83%), 1,8-cineole (9,43%) and compounds like eremanthin (3,65%) and dehydrocostuslactone (7,52%). Among the compounds, only 1,8-cineole has been analyzed for its antiviral effect (Astani et al. 2010), wheras β-ocimeme, eremanthin and dehydrocostuslactone need further investigations. *J. oxycedrus* ssp. *oxycedrus* and *S. thymbra* also exibit an antiherpetic effect. These essential oils are potential agents for treatment of viral infections.

	Ln	Joo	To	Csp	Pp	St	So
α-pinene	3,67	27,40	35,72	53,56	6,18	10,15	4,72
sabinene	-	-	-	-	17,07	8.64	6,79
β-pinene	2,14	-	-	-	6,48	-	3,01
β-myrcene	-	18,90	-	-	-	-	-
α-phellandrene	-	7,10	-	-	-	-	-
γ-3-carene	-	-	9,48	-	-	-	-
α-terpinene	-	-	-	18,9	-	-	-
p-cymene	-	-	-	-	-	10,76	-
limonene	-	6,7	-	-	8,56	-	-
1,8-cineole	9,43	-	-	-	-	-	43,62
β-ocimene	21,83	-	-	-	-	-	-
γ-terpinene	-	-	-	-	-	7,56	-
α-thujone	-	-	-	-	-	-	12,9
camphor	-	-	-	-	-	-	5,71
thymol	-	-	-	3,84	-	9,92	-
carvacrol	-	-	-	-	-	4,98	-
α-cedrol	-	-	9,55	-	-	-	-
eremanthin	3,65	-	-	-	-	-	-
dehydrocostuslactone	7,52	-	-	-	-	-	-

Table 20. Components (% Area) of essential oil from *L. nobilis* (Ln), *J. oxycedrus ssp. oxycedrus* (Joo), *T. orientalis* (To), *C. sempervirens ssp. pyramidalis* (Csp), *P. palaestina* (Pp), *S. officinalis* (So)

2.15. Selected Essential Oils

In the study of Minami et al. (2003) the antiherpetic effect of 12 essential oils in vitro is examined. The test compounds are *Cupressus sempervirens* (cypress), *Juniperus communis* (juniper), *Melaleuca alternifolia* (tea tree), *Ocimum basilicum album* (tropical basil), *Mentha piperita* (peppermint), *Origanum majoranum* (majoram), *Eucalyptus globulus* (eucalyptus), *Ravensara aromatica* (ravansara), *Lavendula latifolia* (lavender), *Citrus limonum* (lemon), *Rosmarinus officinalis* (rosemary), and *Cymbopogon citratus* (lemongrass). At a concentration of 1% most essential oils reduce the HSV-1 growth with the exception of cypress, juniper and tropical basil. Lemongrass has the highest antiviral activity, at 0,1 % concentration the growth is completely reduced. In the determination of mode of anviral activity no effect resulted.

In conclusion, lemongrass demonstrates the strongest antiviral activity in a concentration dependent manner. This study suggets the direct inactivation of the viral particles. Lemongrass oil is a promising agent for treating HSV-1 infections, but further studies on animal models have to be carried out.

In the study of Koch et al. (2008) the antiviral activity of essential oils against HSV-2 are investigated. The test compounds are essential oils from anis (*Illicium verum*), hyssop (*Hyssopus officinalis*), thyme (*Thymus vulgaris*), ginger (*Zingiber officinale*), camomile (*Matricaria recutita*) and sandalwood (*Santalum album*). In the cytotoxicity screening sandalwood is the most cytotoxic essential oil, the lowest is anis oil. The IC_{50} are listed below (table 1.). For the evalution of the antiviral activity a significant reduction is observed when HSV-1 are pretreated with the test compounds prior infection ranging from 65% for camomile oil up to

>90% for hyssop oil, thyme and ginger oil. This indicades that the virus is affected before adsorption to the host cells. This may be due to the direct interaction with the viral envelope and gylcoproteins.

	anise oil	hyssop oil	thyme oil	ginger oil	camomile oil	sandalwood oil
IC_{50}(%)	0,03	0,006	0,007	0,001	0,00015	0,005

Table 21. IC_{50} of selected essential oils (Koch et al., 2008)

All in all, essential oils posses antiviral activity and may be possible agents for topical treatments of herpes simplex type 2, especially camomille oil with a high selectivity index.

In the study of Schnitzler et al. (2007) essential oils of ginger (*Zingiber officinale*), thyme (*Thymus vulgaris*), hyssop (*Hyossopus officinalis*) and sandalwood (*Santalum album*) are analyzed. Their antiviral activity against acyclovir-sensitiv strain KOS[19] and acyclovir-resistant HSV-1 is tested. Hyssop oil has the highest SI (CC_{50}/EC_{50}) of 75, followed by ginger (20), the lowest SI is calculated for thyme and sandalwood (both 7). In the experiment, when the virus are pretreated with the essential oils prior infection, a significant reduction is observed for the acyclovir sensitiv and drug resistant HSV-1 strains (95,9% to 99,9%). This result shows that the essential oils affect the virus before adsorption and have a different mechanism than acyclovir.

In general, these studies proves the antiviral effects of essential oils against HSV-1 and HSV-2. Among them, lemongrass shows a high effect against HSV-1 (Minami et al. 2003). In the screening against HSV-2, camomile oil inhibits the viral

[19] KOS is a genome strain of a HSV-1 virus.

activity significantly (Koch et al. 2008). All the experiments demonstrate the antiviral effects of the essential oils before the cell is infected with the virus. Interestingly, the essential oils also posses viral activity against acyclovir resistant HSV, which emphazises the different mechanism of the essential oils compared to acyclovir. They are in particular of great interest for topical treatment for recurrent HSV infections.

2.16. Selected Monosubstances

2.16.1. Isoborneol

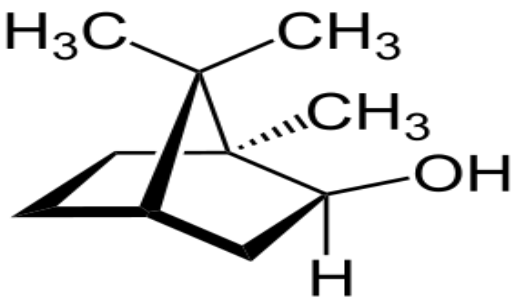

Figure 2. (+) - Isoborneol, CAS 16725-71-6

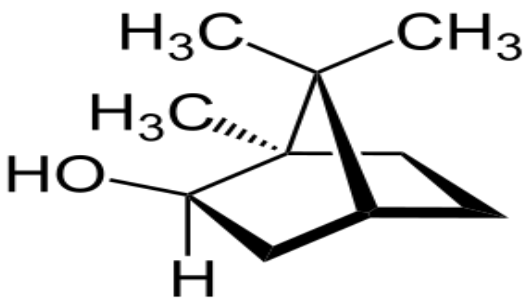

Figure 3. (-) - Isoborneol, CAS 10334-13-1

Isoborneol is a monoterpene alkohol and is the isomer of borneol. It can be found as a single enantiomer and also as the recemate. Borneol is present in many essential oils especially from the Pinaceae family, in citronella oil, in camphor oil, in rosemary, lavender and olibanum oils. Borneol is responsible for its characterisitic camphoraceous odor and burning taste. Borneol can be oxidized to camphor and forms a colourless crystall. It is used for cosmetic products and in the parfumery industry. (Bauer et al. 1990, p 49; Blaschek et al. 2007, 135-136; Falbe and Regitz, 1997, p 1995).

In the study of Armaka et al. (1999) the antiviral effect of isoborneol against Herpes simplex 1 is investigated. Isoborneol is a monoterpene and can be found in several plants like *Salvia fructicosa*. In the study of Sivropoulou et al. (1997) the essential oil of *Salvia fructicosa* is investigated and isoborneol is suggested to be one of the compounds which contributes to the antiherpetic effect.

The experimental study of Armaka et al. (1999) shows that isoborneol significantly reduces the activity of HSV-1 (86%) at a concentration of 0,1%, even at higher concentration up to 1% the effect is accelerated. In a further experiment the effect of isoborneol on viral replication is evaluated. At a concentration of 0,06% the viral growth is completely inhibited, a 96% reduction is observed at a concentration of 0,03%. Moreover, isoborneol inhibits the glycosylation of the virus protein without affecting the cellular host cell. Comparing to borneol, no inhibition is observed. Isoborneol has a specific binding on the glycosylation, which is independent of the viral thymidine kinase. One explanation can be that the infected cells are more sensitive and isoborneol can easily penetrate the cell affecting the glycosylation. This study shows that isoborneol inhibits the HSV-1 gylcosylation specificly. Further studies are needed relating to this results, claryfing if the penetration is dependent on the infection.

2.16.2. Eugenol

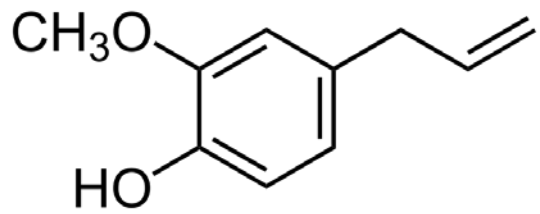

Figure 4. Eugenol, CAS 97530

Eugenol is a phenylpropane and a liquid with a light yellow colour. It can be found in clove oil (*Syzygium aromaticum)* and in the leaves of cinnamon (*Cinnamomum verum*) with an amount >90%, and small amounts in basil (*Ocimum basilicum*) and in bay leaves (*Laurus nobilis*). Eugenol has an antiseptic and anesthetic effect. In the dentistry it is applied as a paste during a root canal treatment. Mouthwash also contains eugenol with a concentration of 1-5%. Besides it is used in the parfumery and cosmetic industry, for manufacturing of vanillin through oxidation and in the agriculture to lure insects. Eugenol irritates the skin and also can have side effects in the gastrointestinal system. (Bauer et al., 1990, p. 101; Blaschek et al. 2007, p. 78-80; Teuscher et al. 2004, p. 420-421).

In the study of Benecia and Courreges (2000) the activity of Eugenol on Herpes simplex virus is investigated. In short, eugenol reduces the viral activity of HSV-1 and HSV-2 significantly. The antiviral effect is dose dependent and the inhibition of HSV-2 is slightly higher than HSV-1. IC_{50} of eugenol are 25,6µg/ml (HSV-1) and 16,2 µg/ml (HSV-2). Additonally, eugenol has no cytotoxic effect at the tested concentration, which is from 15-250µg/ml. In another experiment eugenol is combined with acyclovir on HSV-1 and HSV-2. The IC_{50} of HSV-1 for eugenol is

25,6µg/ml, 0,30µg/ml for acyclovir and for HSV-2 16,2µg/ml and 0,27µg/ml, respectively. The maximum effect is observed at lower concentrations of eugenol and acyclovir. For eugenol the antiviral effect is higher on HSV-2 with up to 40% when applied alone, wheras on HSV-1 the effect is under 10%. When using the maximum concentrations of acyclovir (1,2µg/ml) and eugenol (120µg/ml) together, the lowest effect is observed. There is no explaination given by the authors for this result. In this study the antiviral effect is also tested in vivo. Mice with corneal infection received different dilutions of eugenol, resulting in a delay of the disease symptoms.

The study demonstrate the antiviral activity in vitro and in vivo. Experiments in vitro show a higer effect on HSV-1. Other findings are the synergistic effects of acyclovir and eugenol, which suggest that both compounds inhibit the viral replication differently. In the mice model eugenol is effective against HSV-1, the herpetic keratitis in the cornea is delayed. Eugenol has a great effect for topical treatments, especially in combination with acyclovir.

2.16.3. Monoterpenes

The study of Astani et al. (2010) examines the antiviral activity of selected monoterpenic compounds: α-terpinene, γ-terpinene, terpinen-4-ol, α-pinene, p-cymene, α-terpineol, citral, 1,8-cineole and thymol. Among the essential oils eucalyptus oil, tea tree oil and thyme oil are determined against herpes simplex virus type 1 in vitro. All tested essential oils and monoterpenes at a maximum nontoxic concentration between 20µg/mL (citral) and 1250µg/mL (1,8-cineole) inhibit virial activity by >96 % and >80%, only 1,8-cineole exibits a lower activity. The highest selectivity index is observed for tea tree oil (SI=60), followed

by α-terpineol (18,2) and α-pinene (17,8). When pretreating the hostcells with the compounds prior to infection, eucalyptus oil, tea tree oil and α-pinene demonstrate minor effects. But in the pretreatment of HSV-1 with the compounds a significant reduction is observed (>96%) for all essential oils and for α-terpinene, γ-terpinene and α-pinene. When adding after penetration no sigificant results are observed, only α-terpinene and 1,8-cineole show a moderate reduction.

The results show that the tested compounds inactivate the herpes virus directly, probably via interference with the virion envelope. HSV-1 is inhibited before adsoption. By now, it is unclear, if the compounds bind the virus proteins, which is necessary for its adsorption and penetration into the host cells.

2.16.4. Phenylpropanoids and sesquiterpenes

In the study of Astani et al. (2011) the antiviral activities of phenylpropanoids and sesquiterpenes are screened. Trans-anethole, eugenol, β-eudesmol, fernesol, β-caryophyllene and β-caryophyllene oxide are examined against HSV-1 virus. The noncytotoxic concentration is 9µg/ml for β-caryophyllene oxide and β-eudesmol and 100µg/ml for star anise oil from *Pimpinella anisium*. The TC_{50} are 18µg/ml for β-caryophyllene oxide and 160µg/ml for star anise oil. For antiviral activity the IC_{50} for HSV-1 ranges 0,25µg/ml for β-caryophyllene and 35µg/ml for eugenol. Star anis oil is the most effective test compound with a concentration dependent effect, and has the highest SI (selectivity index) of 160 followed by β-caryophyllene (SI=140). Star anis oil inhibits viral replication by >99%, trans-anethole, β-caryophyllene and farnesol only >90% at the maximum nontoxic concentration. For the mode of antiviral action, the test compounds show no significant effects

when the host cells are pretreated. Wheras on pretreated HSV-1 with star anis oil, phenylpropanoid or sesquiterpenes the infectivity is reduced by 99% by star anis oils followed by β-caryophyllene (98%). A plaque reduction is observed on all other compounds with a percentage about 60 and 90%, only eugenol shows no significant effect in this study.

This paper reports a high antiviral effect of star anis oil, also phenypropanoids and sesquiterpenes show an significant effect. These compounds are often found in essential oils and obviousely contribute to the antiviral effect. Another aspect is the selectivity index, compounds with high SI like star anis oil and β-caryophyllene are very promising agents and could be applied to topical treatments.

3. Discussion

Essential oils have been used as different properties for many thousand years. They were popular in the culinary industry as spices, in the perfumery industry and also for medicinal treatments. There are many reports about their various effects like antimicrobial, antirepelent, antidesinfectant and also antiviral. Especially the antiviral properties are an interesting target for new agents. Since viral infections are a worldwide health problem, there is a need for new antiviral agents. One of the problems is also the development of resistancy. Most affected are patients with surpressed immunology, specifically HIV patients or patients who are suffering from recurrent infections like herpes. By now there are only a few antiviral agents available, which are effective. Most of the antiviral drugs have their activity intracellularly, like acyclovir, when the cells are already infected.

Many reports confirmed the antiviral activity of essential oils. A lot of the discussed reports are tested on viruses like herpes simplex virus type 1 and type 2 and influenza virus. They are the most widely spread viruses. Herpes is most widely spread infection disease on the skin. Only a few reports deal with the antiviral effect against HIV virus.

One of the most popular essential oil is from the Myrtaceae family. There are many reports that prove the antiviral activity of the essential oils from this family. Most of them are used in health care products, in the cosmetic industry and as disinfectant. Examples are eucalyptus oil, the essential oil from *Leptospermum scoparum*, tea tree oil and cloves. All of the plants are native to Australia. Eucalyptus oil shows a high antiviral activity against mumps virus (Carmelli et al. 2007) and HSV-1 and HSV-2 (Schnitzler et al. 2007). The antiviral

activity may base on the 1,8-cineole, which is one of the main compounds of the essential oils. The experiments demonstrate that the target of the test compounds is the viral envelope.

Manuka oil, which is obtained from *Leptospermum scoparium,* also shows an antiviral effect. When the virus is preatreated with manuka oil before infecting the host cell, a significant reduction is observed for HSV-1 (99,5%) and HSV-2 (89,9%), (Reichling et al. 2005). Two characteristic compounds are leptospermone and flavesone, which also demonstrate a reduction of 79,9% and 99,1%, respectively. Accordingly, the essential oil as well as leptospermone and flavesone have an antiherpetic effect. Moreover, manuka oil also inhibits acyclovir resistant HSV-1 virus significantly at 99% (Schnitzler et al. 2008). This finding shows the different mechanism in comparison to acyclovir, which is a nucleosid analoga and inhibits the DNA polymerase.

In addition, tee trea oil from *Melaleuca alternifolia* was also proven to have antiviral activity against influenza virus A (Garrazo et al. 2009, Garrazo et al. 2011). The experiments demonstrate that tee trea oil, terpinen-4-ol, terpinolene and α-terpineol are effective during the viral replication against influenza virus. The inhibition is demonstrated when the essential oil is added during infection, which also indicates the interference of acidification of the lysosomes. Lysomes contain acid hydrolase enzymes, which prevent the virus from uncoating. Terpinen-4-ol, a main compound of the essential tee trea oil shows an effective inhibition of the influenza virus. A significant antiherpetic (HSV-1) effect is demonstrated from the essential oil of *Melaleuca armillaris* (>99%) followed by *Melaleuca leucadendrom* (92%) and *Melaleuca ericifolia* (91,5%), (Farag et al., 2004). The different effect is due to the various compounds of these essential oils. *M. armillaris* contains 33,3%

of 1,8-cineole and 18,79% terpinen-4-ol. *M. ericifolia* has a high amount of methyl eugenol (96,84%) and *M. leucadendrom* of 1,8-cineole (64,3%). Tea trea oil has also an antiviral effect on noneveloped viruses such as tabacco masaic virus. In the plant system a reduction of lesion caused by the tabacco mosaic virus is demonstrated (Bishop et al. 1995).

There are many reports from the Lamiaceae family as well. Essential oils (lemon balm) from *Mellissa officinalis* are effective against HSV-2 virus (Allahverdiyev et al. 2004). The main components among are caryophyllen, citral a, citral b and citronellal, which can be responsible for the antiherpetic effect. In the study of Schnitzer et al. 2008) the antiherpetic effect is proven. When the virus is pretreated with balm oil prior infection a significant reduction is determined. Both viruses are influenced by the essential oil before adsorption, which works differently compared to acyclovir. Another study from Nolkemper et al. 2006 analyzed the antiviral activity of several aquoes extract from lamiacae species, and as a result the extracts interacts with the virus before entering the host cell. *Salvia officinalis* is the most active component. Besides *Salvia officinalis* and *Mentha piperita* demonstrate an antiviral activity against acyclovir resistant HSV-1 virus. The aquoes extracts interact with the virus before entering the host cell, which is another, prove on different mechanism compared with acyclovir.

Another example of a Lamiaceae species, which also interacts with both HSV virus types before entering the host cell, is the essential oil from *Mentha piperita* (Schuhmacher et al. 2003). The experiments show the antiviral activity of the essential oil. It is still unclear if the antiviral effect acts before or during adsorption. In addition *Mentha piperita* has a stronger antiviral effect on HSV-1 virus. The main compounds of *Mentha piperita* are menthol, menthon and methylacetate. It

might be that the main compounds that contribute to the antiviral effect or the essential oil itself. Menthol has a hyperemic effect and cooling effect and has also a weak local anaestetic effect. It is ensured that the essential oil acts different compared to acyclovir. The individual compounds may have a stronger effect when applied but still further researches of the compounds have to be carried out. Nevertheless is a promising drug for the topical treatment of recurrent herpes virus.

Another species from Lamiacae is *Oreganum vulgare*. Oreganum is well known as a spice in the Medditeranean regions. In the experiments of Siddiqui et al. (1996) the antiviral effect of oregano against HSV-1 and New Castle virus are proven. The essential oil interacts with the lipide lesion of the viruses by destroying it through lysis. Oregano has a high amount of carvacrol and ocime, which can be responsible for the antiviral effect. Furthermore, spices like *Allium cepa*, *Allium sativum*, *Corriandrum sativum*, *Petroselinum sativum* and *Ocimum basilicum* show an antiherpetic effect (Romeilah et al., 2010). The antiviral effect is a result of the different compounds of the essential oils. The main compounds of this essential oil are linalool, cuminaldehyde, diioporpyl trisulfide, di-2-propenyl disulphide, methyl-2-propenyl trisulfid and di-2-propenyl trisulfide, myristicin and apiol. Another spice that shows a high antiviral activity against HSV-1 virus is the essential oil from *Laurus nobilis*, also against SARS (severe acute respiratory syndrome) a high antiviral effect is observed (Loizzo et al. 2008). The main compounds of this essential oil are alpha-ocimeme (21,83%), 1,8-cineole (9,43%) and also small amounts of eremanthin (3,65%) and dehydrocostuslactone (7,52%).

All of these compounds in particular spices are potential antiviral agents and they have to be analyzed how they exactly affect the virus. On the other hand essential

oils have the ability to penetrate the host cell and therefore interact with the virion. Besides, they can also affect the virus before entering the host cell. In the time assay the antiviral effect can be evaluated, where the different stages of virus replication is happening. It is of interest if the main compounds of the species, which have been evaluated, demonstrate the same antiviral effect.

The next famous species is *Salvia* from the lamiacae family, which have been reported by some athors. *Salvia fructicosa* demonstrates a high antiviral activity; in particular thujone has the most active effect (Topcu, 2006). Furthermore the ethanolic extract of *Salvia cedronella* also posseses an antiviral effect against HSV-1, HSV-2 and influenza virus (Alim et al. 2009). Other species like *Salvia limbata* and *Salvia sclarea* the antiviral activity is low (Ögütcü et al. 2008). One explaination can be the geographical location and the variaties of compounds, since Salvia has more than 900 different species. *Salvia fructicosa* has a high amount of thujone compared to *Salvia limbata* and *Salvia sclarea*, this can be also a reason for the poor antiviral activity, since thujone mainly contributes to the antiviral effect. Besides compounds like borneol, bornyl acetate and isoborneol also inhibits the virus effectively.

Isoborneol has been evaluated in the work of Armaka et al. (1999). The effect on viral replication is analyzed. Isoborneol inhibits the HSV-1 gylcosylation and the host cell stays spared, it penetrates the infected cells easily and affects the glycosylation through a specific binding. Compared to the isomer borneol no antiviral effect is observed.

Teucrium species, also from the Lamiaceae family, are analyzed by Dunkie et al. (2011). The essential oil of *Teucrium arduini* inhibiton of the tabacco mosaic virus is 25,7% and of Cytomegalie virus (CMV) 21,9%. The main compounds

caryophyllene and caryophyllene oxide show higher inhibition against CMV compared to the essential oil (30,8 and 36,9%). Even the results are not significant; the experiments demonstrate a different antiviral effect of the essential oil and its components. In the essential oil the individual components may act differently and work synergistically against the virus. There is an indication that other compounds are involved in the antiviral effect. On the contrary the main components caryophyllene and caryophyllene oxide demonstrate a higher antiviral effect when applying individually. There is a possibility that the components itself directly inactivates the virus particles, which justify the higher effect than the essential oil. In the study of Bezic et al. (2011) various Teucrium species are analyzed. The essential oil of *Teucrium montanum* demonstrates a reduction of 44,3% for CMV, which is the highest reduction, compared to other Teucrium species. *T. montanum* essential oil only consists 7,1% (Area) of α-caryophyllene compared to T. arduini (19,9%). The highest amount of a compound, which is found in *T. montanum* is germacrene D (17,2% area). So germacrene D can also be the source of the antiviral effect. There can also be a synergistic effect of the various compounds within the teucrium species. It is still unclear if germacrene D can inhibit the CMV or how the virus can be influenced if applied individually.

α-Caryophyllene has been evaluated in the study of Astani et al. (2011). α-Caryophyllene demonstrates a significant effect against HSV-1 virus, in particular when the virus is pretreated with α-caryophyllene (98%). Besides the selectivity index (SI) of α-caryophyllene is 140, which is very high. A compound with high SI is considered to be a safe agent and can therefore be used for topical treatments.

From the Asteracae family *Artemisia arborescens* and *Santolina insularis* have been evaluated. The essential oil of *Artemisia arborescens* inhibits the virus

diffusion from cell-to-cell in infected HSV-1 cells (Saddi et al., 2005). The virus is not able to multiplicate and the essential oil may directly interact with the lipide membrane, either by destroying the membrane or penetrating the virus envelope. It is still unclear if it has the same mechanism like acyclovir. Another example is *Santolina insularis*, also a member of the Asteraceae family. In the study of De Logu et al. (2000) the antiviral effect of the essential oil of *Santolina insularis* against HSV-1 and HSV-2 has been proven. The attachment of the virus to the host cell is prevented. Here, the essential oil also inactivates the virus particle directly. Besides when the cells are pretreated before virus attachment a slight virus inhibition is observed. This outcome indicates an intracellular effect. Acyclovir only has an effect if the host cell is already infected. *Sanotolina insularis* essential oil can possibly prevent the virus spreading, and also unifected cells can be "saved" from new virus attachment. But there is a need for further evaluation to analyse how the essential oil exactly works. To consider the components of the essential oil, they have to be investigated if the monosubstances have the same antiviral effect when it is applied individually. *Santolina insularis* is a promising target because among other aspects a high selectivity index is calculated.

In the screening of an antiviral drug against influenza virus, the volatile oil from *Cynanchum stautonii* is investigated in the study of Wang et al. 2004. Both in in vitro and in vivo experiments the antiviral activity against the virus is proven. The analysed compounds are mostly unknown, like (E,E)-2,4-Decandienal, 3-Ethyl-4-methylpentanol, 5-pentyl-3H-furan-2-one, (E,Z)-2,4-decadienal and 2(3H)-Furanone, dihydro-5-pentyl. The viral agent has to be investigated to clarify how the individual components contribute to the antiviral effect. *Cynanchum stautonii* has been traditionally used in china and used to be a safe agent. For the treatment

of influenza neuroaminidase inhibitiors like amantadine, romantadine for influenza A and for influenza A & B zanamivir and oseltamivir are used. *Cynanchum stautonii* is a promising agent and further studies are needed to evaluate if there is a similarity to neuroaminidase inhibitors or if the anitiviral mechanism is totally different from the current antiviral drugs.

In the study of Bicchi et al. 2009 the antiviral activities of *Ridolfia segetum* and *Oenanthe crocata*, both from the Apiaceae family against HIV-1 virus are investigated. *Oenanthe crocata* did not show any effect compared to *Ridolifia segetum*. The main components of *Ridolifia segetum* are α-thujene, α-pinene, α-phellandrene, β-phellandrene and α-terpinolene. These components contribute to the antiviral effect. HIV-1, which causes the acquired immunodeficiency syndrome (AIDS), is a deadly worldwide disease. Most of the drugs are toxic and have a lot of side effects, besides resistancy can develop*. Ridolifia segetum* can contribute to new findings of antiretroviral drugs.

In the study of Minami et al. (2003) essential oil from *Cymbopogon citratus* (lemongrass) demonstrate a high antiviral activity against HSV-1 compared to the other essential oils, which are also tested. The experiments demonstrate that lemongrass is more effective than tea trea, which is has also been demonstrated for its high antiviral effect. Again a direct inactivation of the virus particle is suggested. When the HSV-2 virus is pretreated with essential oils like camomile oil, hyssop oil, thyme and ginger oil a significant reduction is observed. This indicates an interaction with the viral envelope and glycoproteins (Koch et al., 2008). Essential oils can also be effective against aycyclovir resistant HSV-1 virus (Schnitzler et al., 2007). These are *Zingiber officinale* (ginger), *Thymus vulgare* (thyme), *Hyossopus officinalis* (hyssop) and *Santalum insularis* (sandalwood).

When the acyclovir resistant virus is pretreated, a significant reduction is observed (95,9% to 99,9%). There is a different mechanism of the essential oil compared to acyclovir.

There are also studies about monosubstances like isoborneol and eugenol (Armaka et al. 1999; Benecia and Courreges 200) available. Isoborneol inhibits the HSV-1 glycosylation speficially and is a promising agent for the antiviral therapy and therefore studies on animal models have to be attempted. Eugenol, a phenylpropane demonstrates the antiviral effect against HSV-1 and HSV-2, whereas a higher effect on HSV-1 is demonstrated. In addition a synergistic effect is observed, when both acyclovir and eugenol are tested. This also suggests that the effect of eugenol is differently compared to acyclovir. This is very promising for combination therapy when for example resistancy is developed. Eugenol is suitable for topical treatments. Moreover, in the mice model, eugenol is able to delay the herpetic keratitis. There have to be more studies concerning the dose of eugenol, because the highest concentration tends to be not effective.

Monoterpenes like α-terpinene, γ-terpinene and α-pinene show an antiviral effect against HSV-1 when the virus is pretreated with the test compounds before infecting the host cells (Astani et al. 2010). This proves that these compounds interact with virion envelope, but the exact mechanism is still unclear. The monoterpenes reduce the viral infection by >80% compared to eucalyptus oil, tea tree oil and thyme oil with a reduction of >96%. The complexe mixture of the essential oil has a higher antiviral activity than the selected monoterpenes. Tea tree oil has a high amount of terpinen-4-ol (36, 71% w/w), a moderate amount of γ-terpinene (22, 2% w/w) and only a low amount of α-terpinene (2,74% w/w), (Shellie 2003). Regardless of the low amounts of γ-terpinene and α-terpinene,

they both demonstrate a significant effect compared to the main component terpinen-4-ol. Regarding the selectivity index, tea tree oil revealed the highest amount (60) followed by α-pinene (17,8), α-terpinene (6,5) and γ-terpinene (5,4). The essential oil seems to be a very promising agent with a high selectivity index. The monoterpenes with low SI are not suitable for topical application.

4. Conclusion

There are many reports that prove that essential oils and their components do have antiviral properties. In the folk medicine the essential oils have been used and applied for particular diseases for centuries and a lot of studies prove the scientific evidence.

At present there are different mechanisms of antiviral activity of different essential oils. One of the important aspects is the composition, which has a big influence on the antiviral effect of the essential oil. The exact composition varies, which is mainly due to the geographical location as well as the growth condition and plantation. It is a fact that also other components, which are present in small amounts, contribute to the antiviral effect or reveal to be the main antiviral agent compared to the essential oil. Therefore isolated monosubstances also demonstrate an antiviral effect, which is often reported by some authors. But still further screening is needed to evaluate the exact antiviral mechanism. There are many proves that essential oils or their single components have a different mechanism compared to antiviral drugs like acyclovir. Many of the studys are in vitro experiments and only a few have been carried out in animal models.

In conclusion, essential oils have a potential as an antiviral agent and there are many promising components, which can contribute to the development of new drugs.

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7. Lebenslauf

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