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# Comparative *in vitro* study on the anti-herpetic effect of phytochemically characterized aqueous and ethanolic extracts of *Salvia officinalis* grown at two different locations

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## Abstract

Aqueous and ethanolic extracts of *Salvia officinalis* (Lamiaceae) from two different locations (*Garden* and *Swabian Mountains*) were examined *in vitro* on RC-37 cells for their antiviral activity against herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) using a plaque reduction assay. The 50% inhibitory concentrations ($IC_{50}$) of the extracts for HSV plaque formation were determined in dose–response studies. All extracts tested revealed a high virucidal activity against free HSV-1 and HSV-2. The experimental data exhibited a significant higher sensitivity of HSV against the extracts derived from *Garden* in comparison with those from *Swabian Mountains*. The most active one was the *Garden* 20% ethanol extract with $IC_{50}$ values of 0.18 μg/ml for HSV-1 and 0.04 μg/ml for HSV-2. In order to identify the mode of antiviral action, the extracts were added to the host cells (RC-37) or viruses at different stages of infection. Independently of the location, both types of herpes viruses were considerably inactivated after treatment with the extracts prior to cell infection. Plaque formation was significantly reduced by >90% for HSV-1 and by >99% for HSV-2. Pretreatment of the host cells with both *Garden* and *Swabian Mountains* 20% and 40% ethanolic extracts prior to virus infection revealed a strong reduction of HSV-2 plaque formation by 94% and 70% (*Garden*) and 99% and 45% (*Swabian Mountains*), respectively. In time–activity studies with free HSV-1 over a period of 2 h, a clearly time-dependent activity was demonstrated whereby the ethanolic extracts of both locations revealed a much higher activity than the aqueous ones. The 20% ethanolic extracts of both locations are of special interest and were effective when added to host cells and free virus. A topical application with a dual mode of action would be ideal against recurrent herpes infections.


***Keywords:*** *Salvia officinalis*; Antiviral activity; Herpes simplex virus; Aqueous extracts; Ethanolic extracts

## Introduction

Herpes simplex virus type 1 (HSV-1) and Herpes simplex virus type 2 (HSV-2) are human pathogens of the family of Herpesviridae, which can be distinguished by serological and molecular methods. Both viruses cause recurrent infections of the nervous system located around the lips, in the eyes, in the mucous membrane of the oral cavity and the genitals as well. Only a few drugs are currently available for the treatment of HSV infections such as acyclovir or penciclovir. These substances belong to the synthetic nucleoside analogues

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interfering with viral DNA replication after activation by viral thymidine kinase. More than 90% of mankind are carrier of HSV-1, and 40% suffer from recrudescences. Besides the incidence of infections with HSV-2 (genital herpes) continues to increase (Leung and Sacks, 2000). The development of antiviral drugs with new targets is difficult. Therefore, the interest in alternative antiherpetic agents without severe adverse effects especially from natural origin is increasing.

Many species of the Lamiaceae family are known for their antiviral effect (Saller et al., 2001; Nolkemper et al., 2006). Among them sage (*Salvia officinalis*) is an old remedy that is used in phytomedicine against sore throat and infections of the oral mucous membranes. In the present study plants of *Salvia officinalis* were cultivated at two different locations in South Germany called *Garden* and *Swabian Mountains*. Subsequently, aqueous and various ethanolic extracts were produced from plants of both locations and tested against HSV-1 and HSV-2 *in vitro* using a plaque reduction assay.

## Materials and methods

### Plant material

*Salvia officinalis* L. "Extrakta" was cultivated by WALA Heilmittel GmbH (Bad Boll/ Eckwälden, Germany) at two different locations of the company, in Southern Germany. The first was cultivated at a location with an almost Mediterranean climate (*Garden*; location: Bad Boll; altitude: 449 m; soil: brown Jura; pH: 7.8), while the second was grown at the edge of the woods with a slightly cooler climate (*Swabian Mountains*; location: 7 km off Bad Boll; altitude: 757 m; soil: white Jura; pH: 7.9). The samples of both locations were derived from the harvest of June 2005. Voucher specimens were deposited at the herbarium of the University of Heidelberg.

### Authentic substances

Rosmarinic acid, caffeic acid, apigenin, kaempferol, luteolin, quercetin, apigenin-7-*O*-glucoside, kaempferol-3-*O*-glucoside, luteolin-7-*O*-glucoside, quercetin-3-*O*-glucoside were purchased from Sigma (Taufkirchen, Germany) and Roth (Karlsruhe, Germany). These compounds were used as standards and equally dissolved in 80/20 ($MeOH/H_2O$, v/v) prior to HPLC analyses.

### Preparation of extracts

For preparing the aqueous extracts, 100 ml of boiling water was added to 10 g dried leaves which themselves were obtained by a gentle air-circulation method during 3–4 days at 25–35 °C. The resulting extract was filtered after 15 min and cooled down to room temperature.

Following the instruction for LA-Preparations of the Homeopathic Pharmacopoeia, ethanolic extracts were prepared by WALA Heilmittel GmbH (Bad Boll/ Eckwälden, Germany). Twenty grams of fresh leaves were chopped and subsequently mixed with 80 g aqueous ethanol 20%, 40%, 60% and 80% [v/v], respectively, stirred twice a day and filtrated after 7 days by pressure. The squashed residue was burned and a diminutive portion of the ash was added to the extracts. The resulting extracts were sterile filtrated, serially diluted with distilled water and added to the cell culture medium. To determine their dry weight, all extracts were freeze-dried (Christ Alpha I-6 Heraeus, Hanau, Germany).

### Characterization of aqueous and ethanolic extracts by HPLC and LC–MS analyses

The HPLC-system was a Merck-Hitachi LaChrom Elite (Merck, Darmstadt, Germany) consisting of a pump L-2130, an auto sampler L-2200, a JetStream column oven and a diode array detector L-2450. The Stationary phase was a Sunfire $C_{18}$ column ($250 \times 4.6$ mm i.d., 5 μm particle size; Waters, Wexford, Ireland) fitted with a security guard $C_{18}$ ODS ($4 \times 3.0$ mm i.d., Phenomenex, Rorrance, USA) at a flow rate of 1 ml/min, a constant temperature of 25 °C. Eluent A was 5% aqueous formic acid while B was 100% MeCN. Starting at 100% A, a gradient was followed to 81% A at 30 min, an isocratic step until 55 min and then 0% A at 70 min before re-equilibration to starting conditions.

For quantification of individual phenolic compounds, 1 ml aqueous and ethanolic extracts were dried in vacuum at a pressure of $10^{-3}$ mbar and room temperature (SpeedVac SPD 111 V, Savant, Düsseldorf, Germany) and re-dissolved in 5 ml 80/20 ($MeOH/H_2O$, v/v) prior to injection of 50 μl into the HPLC or LC–MS system. Quantification based on five point calibration ($R^2 > 0.997$) was carried out at 260 nm in duplicate. When no reference substance was available, the particular phenolic compound was quantified based on the calibration data for luteolin-7-*O*-glucoside.

Using the same chromatographic conditions, LC–MS analyses were performed on an Agilent HPLC series 1100 (Agilent, Waldbronn, Germany) equipped with a degasser G1322A, a binary gradient pump G1312A, an auto sampler G1329/1330A, a column oven G1316A, and a diode array detector G1315A connected in series with a Bruker (Bremen, Germany) model Esquire 3000+ ion trap mass spectrometer fitted with an ESI source operating in the positive ionization mode.

### Acyclovir

Acyclovir was purchased from GlaxoSmithKline (Bad Oldesloe, Germany) and dissolved in sterile water.

### Cells and viruses

Monolayer of RC-37 cells (African green monkey kidney cells) was grown with Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS), 100 µg/ml penicillin and 100 µg/ml streptomycin. Cells were seeded out into 96-well and 6-well culture plates for cytotoxicity and antiviral assays, respectively, and incubated at 37 °C in an atmosphere of 5% $CO_2$. *Herpes simplex* virus type 1 (HSV-1) strain KOS and HSV-2 strain HG52 were cultivated on RC-37 cells and used for experiments as described previously (Reichling et al., 2005; Schuhmacher et al., 2003).

### Cytotoxicity assay

For cytotoxicity assays, cells were seeded into 96-well plates at a density of $5 \times 10^4$ cells per well. After incubation for 24 h at 37 °C the medium was removed and 100 µl fresh DMEM containing appropriate dilutions of the extracts were added onto subconfluent RC-37 cells in eight replicates for each concentration of the drug. Cells treated with pure medium and medium supplemented with distilled water or ethanol instead of the extracts tested were used as control. After 72 h of incubation, the cytotoxicity of the extracts was determined by the standard neutral red assay, which quantifies the amount of viable cells after their exposure to toxic substances by measuring the quantity of neutral red dye taken up by the cells (Schuhmacher et al., 2003). The maximum non-cytotoxic concentrations as well as the cytotoxic concentrations of the extracts which reduce cell number by 50% ($CC_{50}$) were ascertained by a dose–response experiment. Presented data are means of three independent replicates.

### Direct plaque assay

Inhibition of HSV replication was examined by plaque reduction assays. Cells were seeded out into 6-well culture plates at a density of $2 \times 10^5$ cells per well 24 h before the experiment. Ten different concentrations of each extract between 0.000097 µg/ml and 224 µg/ml were mixed with $2 \times 10^2$ pfu, respectively, and incubated for 1 h at room temperature. Serial dilutions of the treated virus were adsorbed to the cells for 1 h at 37 °C. After adsorption, the remaining inoculum was removed and the infected cells were overlaid with medium containing 0.5% methylcellulose. Plaque reduction assays were evaluated as described above. The concentrations of the extracts which inhibited the amount of plaques by 50% ($IC_{50}$) were determined from dose–response curves. In all experiments untreated virus infected cells were used as control. The percent reduction was calculated relative to the amount of virus produced in the absence of the compounds. All extracts were dissolved in medium, some of the ethanolic extracts contained ethanol at 1% final concentration and all untreated HSV controls for the ethanolic extracts also contained ethanol at 1% final concentration. Ethanol at 1% final concentration had no influence on viral infectivity.

### Mode of antiviral action

In order to elucidate the mode of antiviral action and to identify the target site, respectively, cells, viruses and extracts were incubated together during adsorption (adsorption), cells were pretreated with extracts before viral infection (pretreatment of cells), viruses were incubated with extracts before cell infection (pretreatment of virus) or after penetration of the virus into the host cells (intracellular replication) as described previously (Nolkemper et al., 2006). Acyclovir was used as control instead of extracts in all experiments mentioned above. After 72 h of incubation at 37 °C the monolayer was fixed with 10% formalin, stained with 1% crystal violet and plaques were counted. All extracts tested were used at their maximum non-cytotoxic concentrations and in all experiments untreated virus infected cells as well as water and ethanol treated viruses were used as control. The number of plaques (pfu; plaque forming unit) of treated cells and viruses were compared to untreated controls to calculate the extent of plaque reduction (in % of control = pfu%), acyclovir was used as positive control.

## Results

### Characterization of plant extracts

Sage leaves contain a range of different phenolic components, especially flavonoids, such as apigenin and luteolin glycosides, 4-hydroxyacetophenone glycoside, phenolic acids such as caffeic acid, carnosic acid and rosmarinic acid, *cis*- and *trans*-p-coumaric acid glycosides as well as oligomeric caffeic acid derivatives such as lithospermic acid, salvianolic acid, salvianolic acid K and sagecoumarin acid (Nolkemper et al., 2006; Lu and Foo, 1999, 2000; Areias et al., 2000; Imanshahidi and Hosseinzadeh, 2006; Lu et al., 1999; Jang et al., 2005). Performing co-injection experiments with authentic reference compounds together with typical UV spectra, relative retention time data and characteristic fragmentation patterns during HPLC–MS analyses, a number of

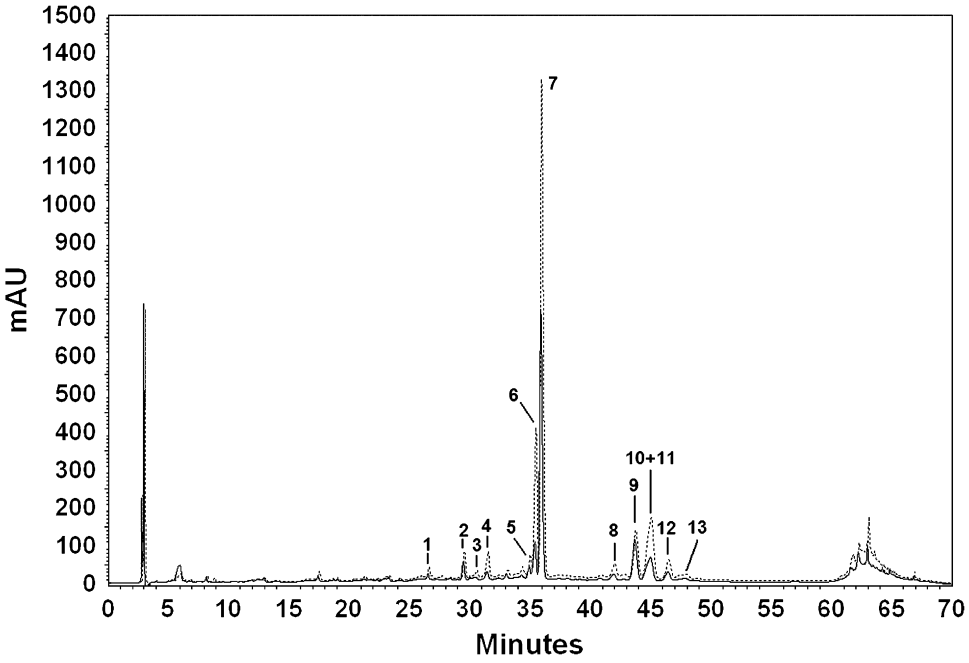


**Fig. 1.** HPLC-DAD-chromatogram of sage extracts from *Garden* (dotted line) and *Swabian Mountains* (solid line) monitored at 260 nm. Peak assignment is given in Table 1.

phenolic compounds could be assigned (Fig. 1, Table 1). Apigenin-7-*O*-glucoside, luteolin-7-*O*-glucoside were unambiguously identified with apigenin- and luteolin-glucuronides as the major components in virtually all extracts. In addition, rosmarinic acid together with other flavonoids especially luteolin derivatives were also detected. Of all extracts tested, the aqueous extracts derived from sage from both locations displayed the largest concentration of phenolics (Table 1, Fig. 1). The aqueous extracts of both locations differed significantly with respect to their quantitative pattern of individual compounds. The *Garden* aqueous extract revealed almost twice the concentrations of apigenin-7-*O*-glucoside, luteolin-7-*O*-glucoside and luteolin-glucuronide compared to the *Swabian Mountains* aqueous extract. This was also true for the total amount of flavonoids and rosmarinic acid determined for both aqueous extracts (*Garden* aqueous extract: 1760.5 mg/l versus *Swabian Mountains* aqueous extract: 854.3 mg/l).

## Cytotoxicity

To discriminate antiviral activities from cytotoxic effects, cytotoxic concentrations which reduce viability of RC-37 cells by 50% ($CC_{50}$) (Table 2) as well as the maximum non-cytotoxic concentrations (Table 3) were determined. The extracts were diluted with distilled water and added to the medium to examine the cytotoxicity of the extracts on RC-37 cells. Cell monolayer was grown in medium containing concentrations between 0.2 and 1497.0 μg/ml of the extracts. After 72 h of incubation, cell viability was determined in the neutral red assay. $CC_{50}$ was determined at 128.8 μg/ml (*Garden* 60% ethanol extract) and up to 733.8 μg/ml (*Garden* 20% ethanol extract). With exception of the *Garden* 20% ethanol extract all other extracts derived from *Garden* were significantly more cytotoxic than extracts derived from *Swabian Mountains* and positively correlated with total phenolic compound content (Table 1). Acyclovir was not cytotoxic at a concentration of 25 mg/ml (data not shown).

## Antiviral activity

For determination of the $IC_{50}$ values of the extracts, HSV-1 and HSV-2 were treated separately for 1 h with various extract concentrations. Afterwards, aliquots of each dilution were added onto the host cells and incubated for 1 h at 37 °C. A clearly dose-dependent activity was demonstrated for both types of viruses. The 50% inhibitory concentrations ($IC_{50}$) of the *Garden* extracts tested were determined to range from 0.03 to 7.5 μg/ml for HSV-1 and 0.02 to 0.75 μg/ml for HSV-2, respectively. In contrast, *Swabian Mountains* extracts tested displayed a significant lower antiviral activity against HSV-1 and HSV-2. The $IC_{50}$ values range between 0.12 and 11.18 μg/ml for HSV-1 and 0.21 and 3.20 μg/ml for HSV-2, respectively (Table 2). Altogether, the ethanolic extracts, independently of their sources, exhibited a significantly higher antiviral activity than the

**Table 1.** Quantitative analysis of major phenolic compounds in sage extracts (mg/l)

| No. | Component | *Garden* aqueous | *Garden* 20% ethanol | *Garden* 40% ethanol | *Garden* 60% ethanol | *Garden* 80% ethanol | *Sw. Mount.* aqueous | *Sw. Mount.* 20% ethanol | *Sw. Mount.* 40% ethanol | *Sw. Mount.* 60% ethanol | *Sw. Mount.* 80% ethanol |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Apigenin-6,8-di-C-glucoside[a] | 10.24±0.04 | 2.04±0.03 | 2.11±0.04 | 2.59±0.08 | 2.13±0.05 | 7.35±0.30 | 2.0±0.01 | 1.87±0.05 | 1.75±0.04 | 1.31±0.00 |
| 2 | Luteolin-di-glucuronide[a] | 41.34±0.57 | 4.06±0.13 | 13.18±0.26 | 13.41±0.07 | 8.07±0.22 | 25.49±1.04 | 4.43±0.08 | 9.45±0.25 | 10.4±0.57 | 6.32±0.01 |
| 3 | Luteolin-glucuronyl-hexoside[a] | 15.63±0.04 | nq | 4.33±0.61 | 5.95±0.48 | 3.66±0.38 | 6.02±0.35 | 1.99±0.07 | 2.56±0.06 | 3.25±0.11 | 1.88±0.02 |
| 4 | Hesperetin-hexoside[a,b]/quercetin-glucuronide[a,b] | 49.48±0.8 | nq | nq | 4.21±0.17 | 3.04±0.06 | 11.91±0.48 | nq | nq | 1.69±0.26 | 1.21±0.11 |
| 5 | Luteolin-glucuronyl-pentoside[a] | 30.14±0.30 | 0.89±0.05 | 9.28±0.14 | 10.48±0.01 | 8.20±0.11 | 17.78±0.15 | 1.67±0.01 | 6.58±0.15 | 8.33±0.1 | 6.41±0.05 |
| 6 | Luteolin-7-*O*-glucoside | 264.93±0.49 | nq | 50.31±0.80 | 99.96±1.73 | 85.54±1.46 | 71.18±0.24 | nq | 16.00±0.28 | 36.12±0.4 | 31.50±0.11 |
| 7 | Luteolin-glucuronide[a] | 863.54±6.23 | 14.26±0.40 | 217.11±4.55 | 248.17±2.86 | 213.97±4.35 | 458.08±3.93 | 22.73±0.17 | 154.10±3.82 | 213.46±5.38 | 162.57±0.11 |
| 8 | Apigenin-7-*O*-glucoside[a] | 33.57±1.04 | nq | 14.09±0.25 | 25.36±0.62 | 22.10±0.39 | 15.38±0.16 | nq | 8.69±0.06 | 14.32±0.11 | 15.48±0.03 |
| 9 | Apigenin-glucuronide[a] | 130.02±0.29 | 28.91±0.44 | 63.25±0.73 | 71.39±1.24 | 63.19±1.18 | 114.06±0.15 | 32.24±0.09 | 92.43±2.03 | 94.82±1.12 | 88.95±0.01 |
| 10+11 | Rosmarinic acid + hispidulin-hexoside[a,c]/luteolin-malonyl-hexoside[a,c] | 242.12±0.47 | 0.69±0.02 | 4.83±0.36 | 15.91±0.46 | 13.05±0.11 | 92.91±0.45 | 0.23±0.02 | 1.25±0.08 | 11.07±0.06 | 8.85±0.01 |
| 12 | Hispidulin-glucuronide[a] | 57.79±0.13 | 12.64±0.30 | 27.50±0.64 | 31.07±0.53 | 27.04±0.57 | 26.53±0.05 | 4.66±0.06 | 15.49±0.54 | 17.76±0.4 | 14.24±0.00 |
| 13 | Luteolin-glucuronyl-coumaroyl-hexoside[a] | 20.73±0.83 | 0.23±0.05 | 1.95±0.07 | 3.37±0.07 | 3.16±0.15 | 7.84±0.09 | 0.48±0.04 | 2.21±0.03 | 3.82±0.07 | 3.08±0.01 |
| | $\sum$ | 1760.5 | 63.72 | 407.95 | 531.87 | 453.15 | 854.53 | 70.43 | 310.63 | 416.79 | 341.80 |

nq—not quantifiable.

[a]Calculated as equivalents of luteolin-7-*O*-glucoside.

[b]Coeluting compounds, assignment not unambiguous.

[c]Minor coeluting compound.

**Table 2.** Determination of the inhibitory concentrations ($IC_{50}$) and selectivity index ($SI = CC_{50}/IC_{50}$) against HSV-1 and HSV-2

| Extract | $IC_{50}$ HSV-1 (μg/ml) | $IC_{50}$ HSV-2 (μg/ml) | $CC_{50}$ (μg/ml) | SI HSV-1 | SI HSV-2 |
|---|---|---|---|---|---|
| *Garden* aqueous | 7.5 | 0.75 | 499.0 | 67 | 665 |
| *Garden* 20% ethanol | 0.18 | 0.04 | 733.8 | 4077 | 18,345 |
| *Garden* 40% ethanol | 0.26 | 0.08 | 240.8 | 926 | 3010 |
| *Garden* 60% ethanol | 0.30 | 0.05 | 128.8 | 429 | 2576 |
| *Garden* 80% ethanol | 0.03 | 0.02 | 163.9 | 5463 | 8195 |
| *Sw. Mount.* Aqueous | 11.18 | 3.20 | 630.5 | 56 | 197 |
| *Swabian Mountains* 20% ethanol | 0.48 | 0.63 | 544.8 | 1135 | 865 |
| *Swabian Mountains* 40% ethanol | 0.75 | 0.21 | 551.4 | 735 | 2626 |
| *Swabian Mountains* 60% ethanol | 0.15 | 0.64 | 554.4 | 3696 | 866 |
| *Swabian Mountains* 80% ethanol | 0.12 | 0.25 | 450.6 | 3755 | 1802 |

**Table 3.** Antiviral effect of the extracts on HSV-1 and HSV-2 by incubation at different stages during infection

| Extract | Virus | MNCC (μg/ml) | Adsorption | Pretreatment cells | Pretreatment virus | Intracellular replication |
|---|---|---|---|---|---|---|
| *Garden* aqueous | HSV-1 | 25.0 | 94±5 | 98±3 | 41±12 | 100±21 |
| | HSV-2 | | 46±8 | 99±5 | 0±1 | 96±11 |
| *Garden* 20% ethanol | HSV-1 | 122.3 | 10±3 | 32±6 | 0±0 | 82±11 |
| | HSV-2 | | 0±0 | 6±3 | 0±0 | 81±6 |
| *Garden* 40% ethanol | HSV-1 | 96.3 | 14±9 | 83±4 | 0±0 | 109±12 |
| | HSV-2 | | 0±1 | 30±5 | 0±0 | 88±9 |
| *Garden* 60% ethanol | HSV-1 | 59.4 | 3±4 | 64±29 | 0±0 | 98±11 |
| | HSV-2 | | 0±1 | 62±7 | 0±0 | 95±6 |
| *Garden* 80% ethanol | HSV-1 | 37.8 | 5±4 | 76±19 | 0±0 | 100±12 |
| | HSV-2 | | 11±4 | 65±22 | 0±0 | 90±11 |
| *Sw. Mount.* Aqueous | HSV-1 | 140.1 | 69±13 | 94±8 | 8±7 | 105±7 |
| | HSV-2 | | 16±16 | 86±3 | 0±0 | 93±4 |
| *Sw. Mount*. 20% ethanol | HSV-1 | 272.3 | 30±6 | 71±18 | 9±5 | 104±3 |
| | HSV-2 | | 1±1 | 1±1 | 0±0 | 70±11 |
| *Sw. Mount*. 40% ethanol | HSV-1 | 91.1 | 39±12 | 98±13 | 0±1 | 109±11 |
| | HSV-2 | | 1±2 | 55±8 | 0±0 | 91±4 |
| *Sw. Mount*. 60% ethanol | HSV-1 | 27.7 | 25±14 | 92±11 | 0±0 | 101±1 |
| | HSV-2 | | 10±5 | 77±9 | 0±0 | 96±1 |
| *Sw. Mount*. 80% ethanol | HSV-1 | 7.5 | 49±23 | 100±8 | 0±0 | 103±5 |
| | HSV-2 | | 19±6 | 84±5 | 0±0 | 94±5 |

Results are given in plaque forming units (pfu) expressed as % of virus control. MNCC = maximum non-cytotoxic concentration.

aqueous extracts. Furthermore, HSV-2 seems to be more sensible against all kinds of extracts than HSV-1. In particular, these differences become highly evident with respect to both *Garden* aqueous extract and *Swabian Mountains* aqueous extract. To evaluate the activity of antiviral agents *in vitro*, the selectivity index ($SI = CC_{50}/IC_{50}$) was determined and is shown in Table 2. The selectivity index describes the ratio between the cytotoxic and the antiviral activity of a substance.

HSV replication is characterized by a complex sequence of different steps which offers the opportunity for antiviral agents to intervene. In order to identify the antiviral target site, all extracts tested were added in maximum non-cytotoxic concentrations at different stages during viral infection (Table 3). Pretreatment of HSV-1 and HSV-2 (pretreatment virus) with the extracts of both locations prior to host cell infection caused a strong and universal inhibition of virus infectivity. The plaque formation was reduced to 9% for HSV-1 (except for *Garden* aqueous extract) and down to 1% for HSV-2, compared to untreated controls where no extracts had been added. On the other hand, pretreatment of host cells (pretreatment cells) with the extracts prior to virus infection revealed different results. While the *Garden* 20% ethanol extract reduced plaque formation to 32% for HSV-1 and down to 6% for HSV-2, the *Swabian Mountains* 20% ethanol extract exhibited a significant plaque reduction to 71% for HSV-1 and down to 1% for HSV-2. In contrast, all other extracts

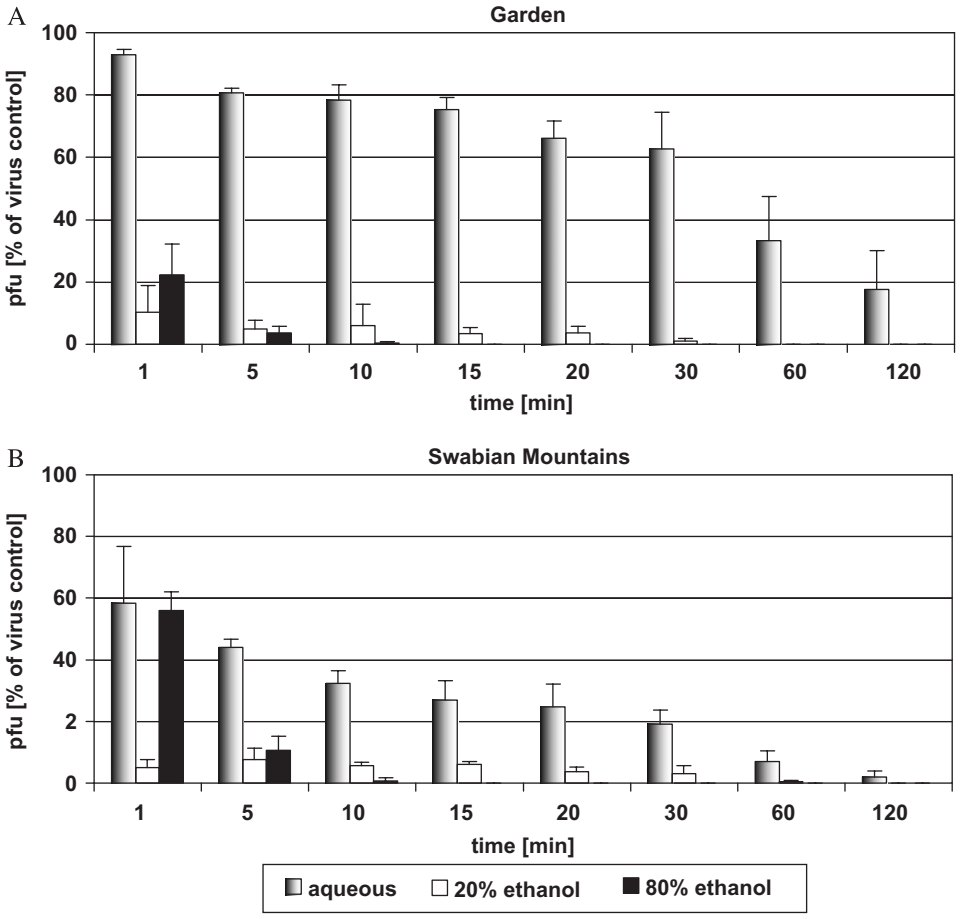


**Fig. 2.** Time-dependent activity of sage extracts from *Garden* (A) and *Swabian Mountains* (B) against HSV-1. Virus was incubated with maximum non-cytotoxic concentrations of the extracts for different periods of time.

showed less antiviral activity. When the extracts were added during the adsorption period, viral amplification was significantly reduced by ethanolic and aqueous extracts for HSV-1 and HSV-2. When the extracts were added to the overlay medium after penetration of the viruses, plaque formation was not significantly reduced. Acyclovir showed the highest antiviral activity when added during the replication period with inhibition of the viral replication of 98.6%. This drug inhibits specifically the viral DNA polymerase during the replication cycle when new viral DNA is synthesized. However, no effect on viral replication was detected when acyclovir cells or viruses were pretreated with acyclovir or when acyclovir was only added during the adsorption phase (data not shown).

In order to examine the time-dependence of the antiviral effect of the sage extracts, HSV-1 was incubated with maximum non-cytotoxic concentrations of selected extracts for different time periods prior to host cell infection, ranging from 1 min to 2 h (Fig. 2). After 1, 5, 10, 20, 30, 60 min and 2 h of incubation, a clearly time-dependent activity could be demonstrated. Even after 10 min of incubation most extracts revealed a significant plaque reduction down to 5% when compared to untreated infected controls. After 2 h of incubation all extracts showed an antiviral activity and decreased viral infectivity down to 2%.

## Discussion

The anti-herpetic effect of sage extracts tested is mainly based on the amount and composition of phenolics in the particular plants. Recently, apigenin and luteolin glucuronides were detected as new sage leaf constituents. In addition, rosmarinic acid, apigenin-7-*O*-glucoside, luteolin-7-*O*-glucoside, hispidulin ( = 6-methoxyapigenin) derivatives are typical phenolics of sage leaves (Lu and Foo, 2000; Areias et al., 2000). Therefore, aqueous and ethanolic extracts of both locations were analyzed for their phenolic composition in the present study. The quantitative pattern of the individual compounds of the *Garden* aqueous extract exceeded significantly those from *Swabian Mountains* aqueous extract indicating a correlation with the growing site of sage. It was previously reported that differences in the

cultivation conditions such as climate and altitude do not only affect the total amount but also the composition of secondary metabolites in plants (Harborne, 1982; Yesil-Celiktas et al., 2007; Spitaler et al., 2006). In order to corroborate these findings further investigations appear to be necessary comprising sage harvests from different locations in consecutive years.

For the topical treatment of recurrent herpes labialis a preparation containing a mixture of sage and rhubarb has been reported. The fixed plant combination was shown to be significantly superior to placebo and equivilant to acyclovir (Saller et al., 2001). In order to identify the antiviral target site of sage extracts, cells and viruses were treated with maximum non-cytotoxic concentrations of the extracts at different stages during viral infection. When HSV-1 and HSV-2 were pretreated with the extracts for 1 h prior to host cell infection, plaque formation was significantly reduced. This result suggests that the extracts interact with structures of the virion envelope which are necessary for adsorption or entry into host cells. When host cells were pretreated with the extracts prior to virus infection, most of the alcoholic extracts displayed a significant diminution of plaque number with highest plaque reduction for the *Garden* 20% ethanol extract. This extract revealed a strong reduction of viral infectivity not only for HSV-1 (68% plaque reduction) but also for HSV-2 (94% plaque reduction). These data indicate that the *Garden* 20% alcohol extract does not only interact with the surface of the host cells but also with the envelope of herpesviruses. Consequently, the *Garden* 20% ethanol extract reveals a double mode of action. To a certain extent, this is also true for some other ethanolic extracts of both locations but not for the corresponding aqueous extracts. These findings are in good agreement with recently published data concerning the anti-herpetic effect of aqueous extracts of sage and related species of the Lamiaceae family (Nolkemper et al., 2006). When the extracts were added during the adsorption period of viruses to host cells, the number of plaque forming units was reduced significantly by all types of extracts tested with exception of *Garden* aqueous extract. In contrast, when the extracts were added to infected cells after the adsorption phase, no or only moderate reduction of plaques was detected. These results suggest that herpesviruses are inactivated before adsorption or during adsorption to cell surface but not after penetration into host cells. This mechanism is different to the mode of action of acyclovir. In contrast, essential oils interfere only with extracellular herpesvirus but not with host cells (Schuhmacher et al., 2003). However, it remains to be clarified whether essential oil compounds (Sivripoulou et al., 1997; Tada et al., 1994) that may partly be co-extracted would contribute to the antiviral capacity of ethanolic sage extracts.

The observed HSV inhibitory effects are considered to be due to some of already known secondary metabolites. Phenolics such as flavonoids, tannins and caffeic acid derivatives are reported to inactivate herpes simplex viruses by blocking ligands or receptors on the surface of viruses and host cells, respectively (Cohen et al., 1964; Kucera and Herrmann, 1967; May and Willuhn, 1978; Reichling, 1999; Kaul et al., 1985; Vanden Berghe et al., 1986; Jassim and Naji, 2003). Furthermore, in aqueous and ethanolic extracts of different *Salvia* species oligomers of caffeic acid derivatives were identified (Lu and Foo, 1999, 2000; Lu et al., 1999) exhibiting antiviral activity (Jang et al., 2005; Thiel et al., 1983; Bailly et al., 2005).

*Garden* extracts generally revealed significant lower $IC_{50}$ values than S*wabian Mountains* extracts. These findings suggest that the $IC_{50}$ values might correlate with the plant location. To corroborate this assumption further investigations are urgently required comprising extracts of sage harvested from consecutive years. In addition, all extracts tested will be qualified for application as adjuvant topical treatment in HSV infections by their SI-values being considered greater than 100. The *Garden* 20% ethanol extract should be favoured due to the dual antiviral effect with respect to HSV-1 and HSV-2.

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