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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 (250×4.6 mm i.d., 5 μm particle size; Waters, Wexford, Ireland) fitted with a security guard C_{18} ODS (4×3.0 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.