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# Antiviral activity of *Rhus aromatica* (fragrant sumac) extract against two types of herpes simplex viruses in cell culture

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We report on the antiviral potency of an aqueous extract of root/stem bark of *Rhus aromatica* (fragrant sumac extract) against herpes simplex virus type 1 and type 2 in cell culture (RC-37 cells) using a plaque reduction assay. The extract exhibited a high level of anti-HSV activity with $IC_{50}$-values of 0.0005 % for HSV-1 and 0.0043% for HSV-2 as well as high selectivity indices (SI) of 5400 for HSV-1 and 628 for HSV-2. In order to determine the mode of antiviral action, the fragrant sumac extract was added at different times to the cells or viruses during the viral infection cycle. At maximum non-cytotoxic concentration (0.25%), plaque formation was significantly reduced by more than 99% when *herpes simplex* viruses were pretreated with the plant extract for 1 h prior to cell infection. When the host cells were pretreated with the fragrant sumac extract for 1 h prior to virus infection, the infectivity of viruses was reduced by 50% for HSV-1 but only moderately for HSV-2. No antiviral effect was seen when the plant extract was added to already infected host cells. Based on these findings the plant extract seems to interact not only with the viral envelope but also with the surface of the host cells impairing the ability of herpes simplex viruses to adsorb to and penetrate into the host cells. In conclusion, the aqueous fragrant sumac extract revealed a strong antiviral activity against HSV-1 and HSV-2 *in vitro*.

## 1. Introduction

The genus *Rhus* (sumac) comprises more than 250 species and is well characterized by phenolics and triterpenes. *Rhus* species grow in temperate and tropical regions worldwide and have been used as spice or medicinal herbs for hundreds of years (Rayne and Mazza 2007).

*Rhus aromatica* Aiton (Anacardiaceae), the fragrant sumac, is an aromatic, deciduous, small bushy shrub with yellowish catkin-like flowers preceding dark-red berries. The stem is growing 6 to 12 feet high, leaves alternate and trifoliate. The shrub is native in the rocky regions of Eastern United States. Aqueous extracts of plant root or stem bark exhibited anti-inflammatory and anti-microbial effects. Also inhibition of muscarinic receptor-mediated contraction of human bladder was demonstrated. These properties support the traditional use of *Rhus aromatica* for the treatment of urinary incontinence, overactive bladder, cystitis, functional bladder problems and certain types of uterine hemorrhages (Effenberger and Schilcher 1990; Brantner and Chakraborty 2001; Borchert et al. 2004).

Herpes simplex virus type 1 (HSV-1) causes orofacial herpetic infections. More than 90% of the population is latently infected by this virus for the whole life, some people are affected by recrudescence more or less frequently, which manifests clinically as labial herpes (Whitley and Roizman 2001). Genital herpes is an important sexually transmitted disease that is usually caused by herpes simplex virus type 2 (HSV-2) (Leung and Sacks 2000). A vaccine against HSV-1 and HSV-2 is not available, but there are several synthetic drugs for treatment, e.g. acyclovir, that interferes with viral metabolism and interrupts DNA synthesis of herpes progeny (Leung and Sacks 2000; Whitley and Roizman 2001). While few examples for plant-derived products in topical application already exist (Reichling 1999), there is still a great demand for new and more effective anti-herpesvirus agents. Aqueous and alcoholic extracts of *Rhus* species are well known for their antimicrobial activities, although limited information is available on their antiviral properties (Lin et al. 1999; Kurokawa et al. 1999; Rayne and Mazza 2007; Gu et al. 2007; Wang et al. 2008). For *Rhus javonica* and *Rhus succedanea* antiviral activity against herpes simplex viruses was shown (Lin et al. 1999; Kurokawa et al. 1993, 1999). In a former screening experiment, May and Willuhn (1978) could demonstrate an antiviral effect of fragrant sumac extract against HSV-1.

In the present study, the antiviral potency and mode of action of an aqueous extract of root/stem bark of *Rhus aromatica* against HSV-1 and HSV-2 in cell culture was assessed using a plaque reduction assays.

## 2. Investigations and results

### *2.1. Chemical characterization of the aqueous fragrant sumac extract by HPLC analysis*

Former chemical investigation of root/stem bark of *Rhus aromatica* revealed about 8% tannins, gallic acid, flavonoids like quercetin and quercitrin, the phenolic compound orcin-ß-D-glucoside, triterpenes like oleanon aldehyde, oleanol aldehyde, ß-sitosterin and stigmast-7-en-3-ß-ol as well as 0.01 to 0.07% essential oil with the major components geranyl acetone, α-ambrinol, dihydro-γ-ionone, farnesyl acetone and dinorlabdenons (Effenberger and Schilcher 1990).

While a dry aqueous stem/root bark extract of fragrant sumac (fragrant sumac extract) was in the focus of this study, we identified gallic acid as its leading compound. Using HPLC with gallic acid as authentic reference substance, gallic acid could be identified in the fragrant sumac extract. Quantitative analyses revealed a content of about 150 mg gallic acid per gram extract.

### *2.2. Cytotoxicity*

For evaluation of cytotoxic properties of the fragrant sumac extract, host cells (RC-37) were seeded into 96-well plates and serial dilutions of the drug were added onto sub-confluent cells. After 3 days of incubation at 37 °C, cytotoxicity of the plant extract was determined with a standard neutral red assay. $CC_{50}$ value and maximum non-cytotoxic concentration were determined from dose-response curves. A clear concentration-dependent effect was demonstrated. The maximum non-cytotoxic concentration of the fragrant sumac extract was determined at 0.25%, the $CC_{50}$ values was fixed at 2.7%. Experiments to assess the cytotoxic property of biological compounds on eukaryotic cells (Halle and Göres 1987) indicated a very moderate toxicity of the fragrant sumac extract.

### *2.3. Mode of antiviral action*

HSV replication is characterized by a complex sequence of different steps at which antiviral agents might interfere. In order to investigate the inhibitory effects on herpes simplex viruses in detail, fragrant sumac extract was added at the maximum non-cytotoxic concentration at different stages of the viral infection cycle. The percent reduction was calculated relative to the amount of viruses produced in the absence of the extract.

Pretreatment of host cells with the fragrant sumac extract displayed a reduction of plaque formation of about 50% for HSV-1 and only a moderate reduction for HSV-2 (Fig. 1). On the other hand, pretreated HSV-1 and HSV-2 particles with fragrant sumac extract 1 h prior to host cell infection caused a significant decline in the amount of plaques. At maximum non-cytotoxic concentration, infectivity was reduced by more than 99% for both HSV-1 and HSV-2. In contrast, when the fragrant sumac extract was added to the overlay medium after penetration of the viruses into host cells, plaque formation was not reduced. Acyclovir showed the highest antiviral activity when added during the replication period with inhibition of the viral replication of 98.6% for HSV-1 and HSV-2. Acyclovir inhibits specifically the viral DNA polymerase activity during the replication cycle when new viral DNA is synthesized. However, no effect on viral replication was detected when cells or viruses were pretreated with acyclovir (data not shown).

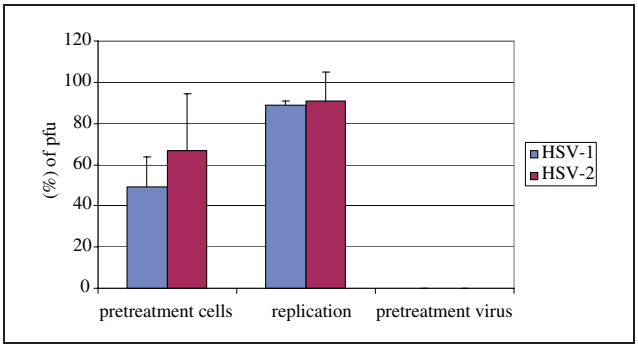


Fig. 1: Antiviral effect of fragrant sumac extract against HSV-1 and HSV-2 by incubation at different periods of time during infection. The extract was added at the maximum noncytotoxic concentration of 0.25%. Cells were pretreated with the extract prior to virus infection (pretreatment cells), viruses were pretreated prior to cell infection (pretreatment virus), fragrant sumac extract was added to the cells after penetration of the viruses into cells (replication). Experiments were repeated independently and data presented are the mean of three experiments

### *2.4. Antiviral dose-response assay*

The effect of fragrant sumac extract on HSV-1 and HSV-2 multiplication *in vitro* was determined using a plaque reduction assay. Serial dilutions of this drug ranging from 0.0001% to 0.25% in nutrient medium were incubated with herpes simplex viruses for 1 h at 37 °C and subsequently adsorbed to host cells. The concentration of fragrant sumac extract, which inhibited plaque reduction by 50% ($IC_{50}$), was determined from dose-response curves (Table).

The 50% inhibitory concentrations ($IC_{50}$) for HSV-1 and HSV-2 of fragrant sumac extract were 0.0005% and 0.0043%, respectively. The results are presented as percentage of control and represent the average of three independent experiments. In plaque reduction assay, the fragrant sumac extract revealed a concentration-dependent antiviral effect, when HSV-1 and HSV-2 were incubated with the drug 1 h prior to inoculation of host cells. Concerning the $IC_{50}$ values, HSV-1 displayed a 10 times higher susceptibility to the fragrant sumac extract than HSV-2.

Selectivity indices for the fragrant sumac extract were calculated as the ratio $CC_{50}/IC_{50}$. For both types of viruses

**Table: Determination of the 50% inhibitory concentration ($IC_{50}$) of fragrant sumac extract against HSV-1 and HSV-2**

| Concentration of the fragrant sumac extract | HSV-1 | HSV-2 |
|---|---|---|
| (in %) | pfu (%) | pfu (%) |
| 0.00005 | 96.0 ± 4.2 | 117.0 ± 26.9 |
| 0.0001 | 87.5 ± 3.5 | 125.0 ± 43.8 |
| 0.00025 | 68.5 ± 20.5 | 117.0 ± 38.2 |
| 0.0005 | 50.0 ± 8.5 | 111.0 ± 42.4 |
| 0.00075 | 46.0 ± 4.2 | 89.0 ± 17.0 |
| 0.001 | 39.3 ± 14.8 | 93.5 ± 29.0 |
| 0.005 | 11.0 ± 7.8 | 45.0 ± 5.7 |
| 0.01 | 2.5 ± 4.0 | 12.5 ± 4.9 |
| 0.05 | 0.0 | 0.0 |
| 0.10 | 0.0 | 0.0 |
| 0.25 | 0.0 | 0.0 |

Viruses were incubated for 1 h at room temperature with increasing concentrations of the plant extract and immediately tested in a plaque reduction assay. Pfu = plaque forming unit. Experiments were repeated independently and data presented are the mean of three experiments

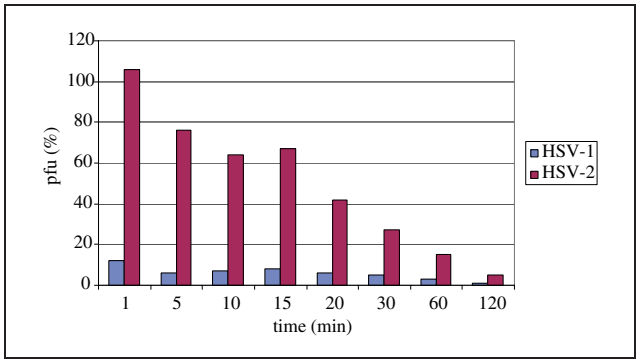


Fig. 2: Illustration of the time-dependent antiviral effect of fragrant sumac extract. Viruses were pretreated with the plant extract in the noncytotoxic concentration of 0.0025% for different amounts of time as described in experimental

very high selectivity indices could be determined, 5400 for HSV-1 and 628 for HSV-2. These SI-values demonstrate a great distance between cytotoxicity and antiherpes activity of the fragrant sumac extract.

### *2.5. Antiviral time-response assay*

In order to examine a possible time-dependence of the antiviral effect, HSV-1 and HSV-2 were incubated with fragrant sumac extract in the noncytotoxic concentration of 0.0025%, a 100 fold lower concentration than the maximum noncytotoxic concentration, for different amounts of time ranging from 1 min to 120 min as described below.
It is worth mentioning that inactivation of HSV-1 and HSV-2 by the fragrant sumac extract was significantly different. Already 1 min after incubation with the extract tested, a plaque reduction of about 90% for HSV-1 has been observed, after 120 min of incubation the infectivity (plaque reduction) of HSV-1 was even reduced by about 99%. In contrast, HSV-2 revealed less susceptibility against the fragrant sumac extract expressed in a more gradually descending of plaque reduction over time (see Fig. 2).

## 3. Discussion

HSV infections continue to increase (Cassady and Whitley 1997) and resistance to all major anti-herpetic drugs has become a current problem especially in immunocompromised patients (Fields 1989; Safrin et al. 1994; Stranska et al. 2005). Therefore, new anti-HSV drugs continue to be developed (Waugh et al. 2002). Various phytochemicals have been shown to possess *in vitro* antiviral activity and may be a source of new antiviral agents (Reichling 1999). Within the genus *Rhus*, the species *Rhus chinensis* and *Rhus javanica* have been shown to exhibit antiviral properties. A petroleum ether fraction as well as an ethanolic fraction of *Rhus chinensis* revealed potent anti-HIV-1 activities in vitro. The antiviral effects may be based on compounds with a benzofuranone-type skeleton (Gu et al. 2007). Furthermore, a hot-water extract of *Rhus javanica* displayed oral therapeutic properties against HSV-1 in mice (Kurokawa et al. 1993). The biological activity could be addressed to moronic acid (Kurokawa et al. 1999).
Aqueous extract of stem/root bark of *Rhus aromatica* has been reported to exhibit anti-inflammatory and anti-microbial effects. Also inhibition of muscarinic receptor-mediated contraction of human bladder was demonstrated. *Rhus aromatica* has been used for more than 100 years for the treatment of urinary incontinence, overactive bladder, cystitis, functional bladder problems and certain types of uterine hemorrhages (Effenberger and Schilcher 1990).
In the present study, the inhibitory effect and mode of antiviral action of an aqueous fragrant sumac extract against HSV-1 and HSV-2 infection *in vitro* was explored using plaque reduction assays. The cytotoxic concentration of the fragrant sumac extract which reduced viable cell number by 50% ($CC_{50}$) and the inhibitory concentration of the extract which inhibited plaque numbers by 50% ($IC_{50}$) were determined from dose-response curves.
The fragrant sumac extract demonstrated a very low cytotoxic activity against RC-37 cells in vitro. On the other hand, the same extract exhibited a high level of anti-HSV-1 and anti-HSV-2 activity with $IC_{50}$ values of 0.0005% and 0.0043% as well as selectivity indices of 5400 and 628, respectively. In order to determine the mode of antiviral action, fragrant sumac extract was added to cells or viruses at different times during the infection cycle. At maximum noncytotoxic concentration, plaque formation was significantly reduced by more than 99% when herpesviruses were pretreated with the fragrant sumac extract. When the host cells were treated with the extract for 1 h prior to virus infection, the plaque formation was reduced by 50% for HSV-1 and only moderately for HSV-2.
Apparently, pretreated herpesviruses are very sensitive to the fragrant sumac extract tested. This finding suggests that fragrant sumac extract interferes with virion envelope structures or is masking viral compounds which are necessary for adsorption or entry into host cells. Furthermore, the inhibition of HSV-1 and HSV-2 replication appears to occur before entering the cells but not after penetration of the viruses into the cells. It remains to be determined whether the inhibitory effect is due to binding of some constituents of the extract to viral proteins involved in host cell adsorption and penetration or is due to damage viral envelope. In addition to that, the fragrant sumac extract revealed for HSV-1 a dual mode of action. Some compounds of the fragrant sumac extract may interact not only with the viral envelope but also with the surface of host cells impairing the ability of herpesviruses to adsorb to and penetrate into the host cells. In contrast to the fragrant sumac extract, acyclovir demonstrated antiviral activity only during intracellular replication of the herpesviruses.
According to our findings, a therapeutic application of a fragrant sumac extract containing cream, or oral dosage form as antiviral agents in recurrent herpesvirus infection or as protecting agent appears to be promising.

## 4. Experimental

### *4.1. Rhus aromatica (fragrant sumac) extract*

Preparation of the dry fragrant sumac extract: sumac bark (cortex arbores et radicis) was extracted with water. After evaporation of the aqueous solvent, the ratio of herbal drug to drug preparation (= dry extract) was 5–7 : 1. For further details see Effenberger (1990).
Preparation of test-solution (fragrant sumac extract): Test-solution was prepared by adding 2 g dry sumac extract to 100 ml water, followed by sterile filtration. Serial dilutions ranging from 0.0001% to 10% and 0.0001% to 0.25% in nutrient medium were analysed for cytotoxicity and antiviral effects, respectively.

### *4.2. Chemical characterization of fragrant sumac extract by HPLC*

The identity of the fragrant sumac extract was determined by HPLC using gallic acid as the leader substance. Chromatographic parameters: Varian HPLC system consisting of a pump, autosampler, UV-detector; HPLC column: Hypersil ODS, 125 mm × 4 mm, 5 µm; volume injected: 10 µl, flow: 1.0 ml/min.; mobile solvents: methanol 50 ml, water 950 ml, $H_3PO_4$ 1.0 ml; gradient: isocratic; detection: UV 254 nm; authentic substance: gallic acid. The gallic acid content was calculated using an external calibration method.

Extract preparation: 50 mg of dry sumac extract was dissolved in water for chromatography and shaken in an ultrasonic bath for 15 min. After cooling the sample was filtered in vials through membrane filter and injected into the HPLC. For further details see Effenberger (1990).

### *4.3. Acyclovir*

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

### *4.4. Cell culture and viruses*

RC-37 cells (African green monkey kidney cells) were grown in monolayer culture with Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin as described previously (Schuhmacher et al., 2003). Cells were seeded into 96-well and 6-well culture plates for cytotoxicity and antiviral assays, respectively. HSV-1 strain KOS and HSV-2 strain HG52 were cultivated as confluent cell monolayers.

### *4.5. Cytotoxicity assay*

For cytotoxicity assays, cells were seeded into 96-well plates and incubated for 24 h at 37 °C as described previously (Schnitzler et al. 2001). Briefly, the medium was removed and fresh DMEM containing appropriate dilutions of fragrant sumac extract was added onto subconfluent cells in eight replicates for each concentration. Wells containing nutrient medium but no drug were also included on each plate as controls. After 3 days of incubation, the cytotoxicity of the extract was determined with a standard neutral red assay. The cytotoxic concentration of the drug which reduced viable cell number by 50% ($CC_{50}$) was determined from dose-response curves. Additionally the maximum non-cytotoxic concentration of the fragrant sumac extract was determined.

### *4.6. Mode of antiviral action*

In order to determine the mode of antiviral action for fragrant sumac extract, cells were pre-treated with the plant extract before viral infection, viruses were incubated with this drug before cell infection or cells were incubated with the plant extract after the penetration period of the virus into the host cells as described previously (Reichling et al. 2005). After 3 days of incubation the monolayers were fixed with 10% formalin, stained with 1% crystal violet and plaques were counted. The aqueous fragrant sumac extract was used at the maximum non-cytotoxic concentration. Each assay was run in five replicates, the number of plaques of drug-treated cells and viruses were compared to untreated controls to calculate the extent of plaque reduction (in % of control). Acyclovir was used as positive antiviral control.

### *4.7. Plaque reduction assay*

Inhibition of virus replication was examined by a plaque reduction assay. Serial dilutions of fragrant sumac extract were incubated with herpesvirus ($2 \times 10^3$ plaque forming unites) for 1 h at room temperature and subsequently added to cell monolayers at 37 °C for another 1 h. The remaining inoculum was removed and the infected cells were overlaid with medium containing 0.5% methylcellulose. Each assay was performed in 5 replicates. After 3 days of incubation, monolayers were fixed with 10% formalin, stained with 1% crystal violet and subsequently plaques were counted. By reference to the number of plaques observed in virus-infected untreated controls, the concentration of fragrant sumac extract which inhibited the plaque number by 50% ($IC_{50}$) was determined from dose-response curves (Reichling et al. 2008).

### *4.8. Antiviral time-response assay*

HSV-1 and HSV-2 were incubated at 37 °C with fragrant sumac extract in the noncytotoxic concentration of 0.0025 % for different amounts of time ranging from 1 min to 2 h. After 1, 5, 10, 15, 20, 30, 60 and 120 min of incubation, an aliquot was removed and assayed for remaining infectivity on confluent monolayer of RC-37 cells in 6 well plates by plaque reduction assay. Each assay was performed in 5 replicates.

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