Antiviral Research 89 (2011) 9–18

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Oligomeric proanthocyanidins from *Rumex acetosa* L. inhibit the attachment of herpes simplex virus type-1

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ARTICLE INFO

Article history:
Received 2 August 2010
Received in revised form 6 October 2010
Accepted 28 October 2010

Keywords:
Rumex acetosa
Proanthocyanidins
Epicatechin-3-O-gallate
Galloylation
Herpes simplex virus type-1
Attachment
Penetration
Organotypic skin model
Antiviral

ABSTRACT

The polyphenole-enriched acetone–water extract R2 from the aerial parts of *Rumex acetosa* L. containing high amounts of oligomeric and polymeric proanthocyanidins and flavonoids was tested for antiviral activity. R2 exhibited strong antiviral activity against herpes simplex virus type-1 (HSV-1) while the replication of adenovirus 3 was not affected. By plaque reduction test and MTT assay on Vero cells, the HSV-1-specific inhibitory concentration (IC_{50}) and cytotoxic concentration (CC_{50}) were determined. R2 exibited an IC_{50} of 0.8 μg/mL and a selectivity index (SI) (ratio of IC_{50} to CC_{50}) of approximately 100 when added to the virus inoculum for 1 h at 37 °C prior to infection. The antiviral activity was due to the presence of flavan-3-ols and oligomeric proanthocyanidins in the extract. Structure–activity analyses indicated that flavan-3-ols and proanthocyanidins with galloylation at position O-3 are highly potent compounds (SI > 40), while ungalloylated compounds did not exhibit antiviral effects (SI < 1).

R2 and a major proanthocyanidin from R2, epicatechin-3-O-gallate-(4β → 8)-epicatechin-3-O-gallate abolished virus entry into the host cell by blocking attachment to the cell surface. When added after attachment at a concentration of ≥12.5 μg/mL, R2 inhibited also penetration of HSV-1 into the host cell. R2 and epicatechin-3-O-gallate-(4β → 8)-epicatechin-3-O-gallate were shown to directly interact with viral particles leading to the oligomerisation of envelope proteins as demonstrated for the essential viral glycoprotein gD.

Using raft cultures with three-dimensional organotypic human skin equivalents it was shown that treatment of cultures with R2 after infection with HSV-1 resulted in a reduced viral spread.

1. Introduction

Recurrent infections with herpes simplex virus type-1 (HSV-1) are a frequent cause of orofacial and ocular mucocutaneous lesions. After primary infection, HSV-1 establishes life-long latency in sensory neurons of the peripheral nervous system. Sporadic reactivation of latent virus triggered by environmental, emotional or physical stress can lead to virus replication followed by recrudescent infection. Although clinical manifestations of primary and recurrent HSV-1 infection vary significantly, beyond the neonatal period most HSV-1 infections of the immunocompetent host are self-limiting. Severe, generalized HSV-1 infections are observed in newborns or immunocompromised individuals. Furthermore, HSV-1 infrequently causes sporadic encephalitis, a devastating disease in the absence of early and sufficient antiviral treatment. For details concerning the pathogenesis of HSV-1 infection, see Koelle and Corey (2008).

The first critical step in primary HSV-1 infection is viral entry into orolabial epithelial cells. Virus entry is mediated by the viral glycoproteins gB, gC, gD, gH and gL. For review on the molecular mechanisms of viral entry see (Akhtar and Shukla, 2009; Akhtar et al., 2008; Spear, 2004; Campadelli-Fiume et al., 2007).

After successful entry into the host cell, viral gene expression and replication ensue, followed by rapid cell-to-cell spread within the epithelium. In addition, the virus rapidly reaches trigeminal ganglia by infecting sensory neurites innervating the infected epithelium, and establishes life-long latency within infected sensory neurons (Akhtar and Shukla, 2009). Despite the fact that most HSV-1 infections can be treated efficiently by DNA polymerase inhibitors such as aciclovir and related compounds, drug-resistant HSV-1 strains may develop especially during long-term treatment of immunocompromised individuals. Resistance is mostly due to mutations abolishing or strongly diminishing the expression of a functional thymidine kinase protein. Point mutations in the viral DNA polymerase are less frequently responsible for resistance (Whitley et al., 1998).

Numerous reports are dealing with natural products as antiviral compounds against HSV-1. Reviews comprising data from such

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doi:10.1016/j.antiviral.2010.10.007

surveys (Khan et al., 2005; Namba et al., 1998; McLaughlin, 2008) indicate that in many cases plant-derived polyphenols exhibit anti-HSV activity mostly by influencing the early phases of virus infection. Polyphenols are a large and highly heterogenous group of natural products, with the general characteristics of multiple hydroxylation of complex aromatic systems. Besides chalcones, flavonoids with different degrees of oxidation, anthranoids, hypericins, lignans, depsides and the various groups of tannins, especially the class of non-hydrolyzable tannins, in particular proanthocyanidins seem to influence HSV entry into host cell (Cheng et al., 2002; Shahat et al., 2002). Similar antiviral effects of proanthocyanidins are described also for other virus families, e.g., such as influenza A virus (Droebner et al., 2007; Ehrhardt et al., 2007). Proanthocyanidins are a complex class of oligo- to polymeric compounds with building blocks from flavan-3-ols (e.g., catechin, epicatechin, gallocatechin, epigallocatechin, afzelechin, epiafzelechin, etc.). These different flavan-3-ols can be condensed covalently by a linkage *via* $4 \rightarrow 6$ or $4 \rightarrow 8$ (B-type oligomers) or by an additional ether bridge (A-type oligomers). The degree of polymerization, the stereochemistry of the interflavan linkage, and the stereochemistry of the building blocks can be highly variable. Additionally, many of these proanthocyanidins are secondarily substituted by excessive galloylation, acetylation, etc. Thus, detailed investigations concerning mechanistic activity and structure–activity relations are difficult. Therefore, proanthocyanidin-enriched plant extracts used for antiviral testing are in many cases complex mixtures of many of these derivatives. Only on rare occasions (De Bruyne et al., 1999; Cheng et al., 2002) defined compounds have been used for detailed investigations of antiviral activity.

In this study the potential anti-HSV-1 activity of a proanthocyanidin-enriched extract and a defined dimeric proanthocyanidin from *Rumex acetosa* L. (Polygonaceae) denominated also as "common sorrel" was evaluated. The aerial parts of *R. acetosa* are used traditionally for treatment of skin irritations and diarrhoea. Modern phytotherapeutical preparations with nationally registered drug status in Europe contain extracts from *R. acetosa* for treatment of acute and chronic infections of the upper respiratory system (Guo et al., 2006). Recently, the phytochemical composition of extracts has been extensively investigated, indicating the presence of monomeric flavan-3-ols (catechin, epicatechin, epicatechin-3-O-gallate), A- and B-type procyanidins and propelargonidins (15 dimers, 7 trimers, 2 tetramers) and a phloroglucinol derivative beside different flavonoids in an acetone–water extract of the aerial parts of *R. acetosa* (Bicker et al., 2009).

Our results indicate that the procyanidin-enriched, standardized extract of *R. acetosa* exhibits strong anti-HSV-1 activity by selectively interfering with viral entry.

2. Materials and methods

2.1. Plant material and extraction

Dried plant material of *R. acetosa* L. (Herba Rumicis acetosa concis., Batch No. 43146115) was obtained from Caesar & Loretz GmbH, Hilden, Germany. 100 g of dried plant material was exhaustively extracted (Bicker et al., 2009) with 1 L cold acetone/water (7:3) with a ratio of plant material to extract of 15.5:1. The extract was evaporated in vacuo, filtered to remove the precipitated chlorophyll, defatted with petroleum benzene and freeze-dried to yield the crude extract (R2). Oligomeric proanthocyanidins beside minor amounts of flavanoids were determined by RP-HPLC. Isolation and analytical characterisation of defined proanthocyanidins from R2 was performed as described in detail by Bicker et al. (2009); epicatechin **1**, epicatechin-3-O-gallate **2**, epigallocatechin **3**, epigallocatechin-3-O-gallate **4**, gallocatechin **5**, epicatechin-$(4\beta \rightarrow 8)$-epicatechin (syn. procyanidin B2) **6**, epicatechin-3-O-gallate-$(4\beta \rightarrow 8)$-epicatechin-3-O-gallate (syn. procyanidin B2-3,3′-di-O-gallate) **7**, epicatechin-3-O-gallate-$(4\beta \rightarrow 6)$-epicatechin-3-O-gallate **8** and gallic acid had purity > 95% as determined by HPLC (RP18, λ_{max} 280 nm); ESI-MS and $^{13}C/^{1}H$ NMR did not indicate the presence of relevant impurities.

2.2. Viruses, cells and viral infections

Vero cells (African green monkey kidney cells) were propagated in minimal essential medium (MEM, Biochrom, Berlin, Germany) supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM L-glutamin, non-essential amino acids (1×), 100 μg/mL penicillin/streptomycin.

HSV-1 strain 17 syn+ was grown on Vero cells, and infectious titers were quantified by plaque assay. The antiviral assays were performed using serum-free medium. To obtain a highly concentrated virus suspension, virus-containing cell culture supernatants were harvested and centrifugated at 19,000 × *g* for 3 h. The virus pellet was resuspended in 100 μL PBS and centrifuged through 25% (w/v) saccharose at 150,000 × *g* for 2 h to remove remaining culture medium constituents and cell debris.

Adenovirus type 3 (AdV 3) was grown on MRC5 cells (human lung fibroblasts). Virus was quantified by serial dilution and titration-assay. The $TCID_{50}$ (50% tissue culture infectious dose) was calculated using the formula of Spearman–Kärber

$$TCID_{50} = \log(\text{highest dilution giving 100\% CPE}) + \frac{1}{2} - \frac{\text{total number of test units schowing CPE}}{\text{number of test units per dilution}}$$

Antiviral assay: The antiviral activity was assayed with MTT assay as essentially described for HSV-1. 100 TCID50 AdV 3 per well were applied and cultures were stained after 5 days. Additionally, the antiviral activity of R2 against AdV 3 was determined by serially titrating the incubation mixture on Vero cells.

2.3. Cytotoxicity assay

The cytotoxicity of R2 was assayed using the MTT assay (Mosmann, 1983). 10^4 Vero cells per well were seeded into 96-well culture plates (Greiner Bio-one) and cultivated for 24 h. The medium was removed, cells were washed with PBS and various concentrations of R2 in serum-free medium were added. After 72 h culture medium was removed, cells were rinsed twice with PBS and MTT reagent (2.5 mg/mL 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromid in PBS) was added (50 μL/well). After 4 h of incubation, the MTT-reagent was removed and formazan generated in the cells was dissolved in 50 μL DMSO/well. Absorbances were measured at 492 nm (reference wavelength of 650 nm) in an ELISA microplate reader.

2.4. Antiviral assay

2.4.1. MTT-assay

The antiviral assay was done similar to the cytotoxicity assay (Section 2.3), with HSV-1 (1×10^4 pfu/well) added to the cells additionally. Cells were seeded and cultivated for 24 h before starting the assay. In the basic assay, HSV-1 was preincubated with R2 for 1 h at 37 °C and Vero cells were incubated for 48 h with the R2/HSV-1 mixture. In modified assays, cells were either preincubated with R2 alone prior to infection with HSV-1 or HSV-1 and R2 were added simultaneously to the cells without preincubation, respectively.

The antiviral activity was calculated according to the following formula (Pauwels et al., 1988):

$$\text{antiviral activity}\,(\%) = \frac{(OD_T)_{HSV} - (OD_C)_{HSV}}{(OD_C)_{mock} - (OD_C)_{HSV}} \times 100\%$$

$(OD_T)_{HSV}$ represents the optical density of cells, which were infected by HSV-1 (index: HSV) and treated with R2 (Index: T = treated). $(OD_C)_{HSV}$ corresponds to the optical density measured for the untreated HSV-1-infected cells (index: C = control) and $(OD_C)_{mock}$ is the optical density of untreated, mock-infected cells.

2.4.2. Plaque reduction assay

1.25×10^5 Vero cells per well were seeded into 24-well culture plates for 24 h. HSV-1 (100 pfu/200 μL) was incubated with R2 for 1 h at 37 °C. Medium was removed from the cultures, cells were washed with PBS and the HSV-1/R2-suspension was added. After 1 h of cultivation, the inoculum was discarded, infected cells were washed with PBS and overlaid with medium containing 0.5% carboxymethylcellulose. After 4 days of incubation, cells were fixed with 3.7% formalin, stained with 1% crystal violet, and virus plaques were counted. The antiviral activity was calculated by the following formula:

$$\text{antiviral activity}\,(\%) = 1 - \frac{\text{plaque number (assay)}}{\text{plaque number (control)}} \times 100$$

To determine the influence of R2 on virus replication and the release of the virus, the plaque reduction assay was modified. Cells were infected with 100 pfu HSV-1/well in the absence of substance. After an incubation period of 1 h, the inoculum was removed and R2 dissolved in overlay medium was added.

2.5. Penetration assay

2.5.1. Modified plaque reduction assay

The effect of R2 on viral penetration was determined by a modified plaque reduction assay. In contrast to the basic assay, cells were treated with R2 after virus attachment to the cell surface. Penetration of HSV-1 during the attachment and treatment phase was prevented by strictly performing all steps at 4 °C. Cells were washed once with ice-cold PBS, and an HSV-1 inoculum (200 μL/well) corresponding to 50 pfu/well in the standard plaque reduction assay was allowed to attach to the cells at 4 °C. After 30 min, the inoculum was removed, cells were washed once with ice-cold PBS, medium containing a 2-fold serial dilution of R2 was added and cells were incubated for another 30 min at 4 °C. Following, cells were shifted to 37 °C to initiate viral penetration. After an incubation period of 30 min at 37 °C, supernatants were discarded and cells were treated for 45 s with low pH citrate buffer (135 mM NaCl, 10 mM KCl, 40 mM Na-citrate pH 3.0) to stop penetration and to inactivate attached, non-penetrated virions. Low pH citrate buffer was removed by washing twice with PBS, and overlay medium was added. Further cultivation and quantitation of plaques was performed as described for the basic plaque assay. Mock-treatment of attached virus, and inactivation of attached mock-treated virus by low pH citrate buffer immediately prior to the 37 °C shift served as controls, respectively.

2.5.2. Immunofluorescence assay

Cells were seeded in chamber slides (8-Well, Nalge Nunc, Naperville, IL) and cultivated up to 50% confluence. 1×10^7 pfu HSV-1/chamber, preincubated with R2, were added to cells at 4 °C. After 1 h of incubation, the inoculum was removed, cells were rinsed with PBS and fixed with methanol (−20 °C). After incubation with blocking-solution (2% BSA/PBS), HSV-1 specific antiserum (rabbit anti HSV-1, Dako, Denmark, dilution 1:1000) was added. After 1 h cells were rinsed with PBS and incubated with the second antibody (Biotin-SP-conjugated AffiniPure F(ab')2 Fragment Goat Anti-Rabbit IgG, Dianova, Hamburg, dilution 1:500) After washing the cells again, detection solution was added (CyTM3-conjugated streptavidin, Dianova, Hamburg, dilution 1:500), additionally cells were stained with DAPI (1:2000).

2.6. Immunoblotting

HSV-1 (6.5×10^7 pfu/mL) enriched and purified by ultracentrifugation as described above was incubated with R2 (1 mg/mL) for 0, 0.5, 1, 2, 4, 6, 8, 24 h, as control HSV-1 alone was incubated. The suspension was mixed with sample buffer (NuPAGE® LDS Sample Buffer, Invitrogen, Carlsbad, CA) and incubated for 10 min at room temperature. Proteins were separated electrophoretically on SDS-PAGE (NuPAGE® Novex Bis–Tris Gel, Invitrogen, Carlsbad, CA) and electroblotted onto PVDF membrane. Nonspecific binding of antibodies was blocked by incubation in PBS containing 1% non-fat dry milk powder for 1 h. The membranes were incubated with anti-gD antibody (IV.3.4) diluted 1:500 overnight. After washing the membranes with 2% BSA/PBS, blots were incubated with biotin-conjugated goat anti-mouse antibody (biotin-SP-conjugated AffiniPure F(ab')2 fragment goat anti-mouse IgG, Dianova, Hamburg, dilution 1:1000) for 1 h. Membranes were washed again with 2% BSA/PBS and incubated with peroxidase-conjugated streptavidin diluted 1:1000 for 1 h, washed again and stained with 4-chloro-1-naphthol.

2.7. Full thickness skin model

The skin models (Phenion GmbH, Düsseldorf, Germany) were scratched with a cannula to wound superficially the epidermis and 2×10^4 pfu HSV-1, suspended in 2 μL MEM, were carefully pipetted into the scratches (addition of the virus suspension onto the intact epidermis did not lead to an infection). After 10 min R2 was added into the cut to simulate a therapy during the first symptoms of HSV-1 infection (single dose). Additionally, infected skin models were treated three times a day (repeated dose). As control, infected models were treated with PBS. After 48 h the skin models were fixed in 4% formaldehyde/PBS and histological slides from paraffin-embedded material were prepared. Deparaffination was done by incubating them twice with xylol (10 min) and rehydration with descending concentrations of ethanol (96–50%). Sections were digested for 5 min using trypsin/EDTA at 37 °C. Enzyme activity was stopped by washing the sections with cold PBS (4 °C). To block nonspecific antibody binding, the sections were treated with 2% BSA/PBS. The immunostaining was done as described for the adsorption assay.

2.8. Real-time PCR

HSV-1 DNA was extracted with AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) according to Manufacturer's instruction. To isolate DNA, defined pieces of the raft cultures were homogenized in RLT lysis buffer using the TissueRuptor (Qiagen, Hilden, Germany).

Quantification of HSV-1 genomes by real-time PCR was done using ICP27-specific (UL54) primers (0.4 μM) and a dual-labeled TaqMan probe (0.2 μM) (Biomers, Ulm, Germany) as reported by Cohrs et al. (2000). As housekeeping gene ß-globin was quantified using the following primers at 0.5 μM (Biomers, Ulm, Germany):

β-Globin-fw: 5′ CCC TGC TAC GTT TAT CTG ATT GAG 3′.
β-Globin-bw: 5′ CCC ACA GGA TAC TTG GCT ATG G 3′.

Reactions were performed using the LightCycler® (Roche) system, Light Cycler® Fast Start DNA Master SYBR Green I Kit and LightCycler® TaqMan® Master-Kit.

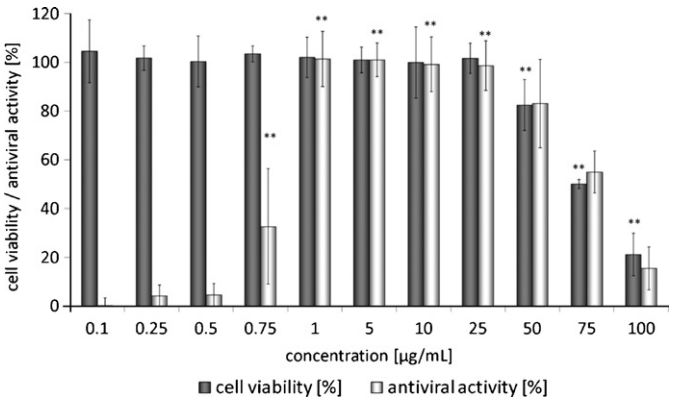

Fig. 1. Antiviral and cytotoxic activity of R2 on Vero cells. 1×10^4 pfu HSV-1/well in serum-free medium (antiviral activity) or serum-free medium (cytotoxic activity) were incubated with R2 at the concentrations indicated for 1 h at 37 °C. 48 h after adding the reaction mixtures to 96-well plates, the antiviral activity and cell vitality were determined by MTT assay as described in Section 2. Values represent ± SD of 6 independent experiments. $^{**}P < 0.01\%$ (one-way ANOVA).

For the determination of viral genome levels, Ct values of viral target sequences were normalized to threshold cycles of ß-globin ($Ct_{HSV\text{-}1} - Ct_{ß\text{-}globin} = \Delta Ct_{sample}$). ΔCt values of mock-treated samples served as control and a relative quantification (RQ) was performed using the ΔΔCt method ($\Delta Ct_{treated\ sample} - \Delta Ct_{mock\text{-}treated\ sample} = \Delta\Delta Ct$, $RQ = 2^{-\Delta\Delta Ct}$).

2.9. HPLC

R2 (1 mg/mL in PBS) was incubated with three different virus concentrations (2×10^5, 2×10^6, 2×10^7 pfu) at 37 °C for 1 h. The virus was removed by ultracentrifugation (Airfuge®, 66.000 × *g*, 40 min). Supernatant was heated at 57 °C for 40 min to inactivate remaining virus particles. To remove the aequeous solvent, samples were concentrated in a SpeedVac® concentrater (Thermo Scientific) at 37 °C. The pellet was dissolved in 140 μL methanol. HPLC was performed on an Alliance 2690 system (Waters, USA), stationary phase Silica Diol, pore size 6 μm, 120 Å, 250 mm × 4.6 mm (Interchim, Montlucon Cedex, France). The binary mobile phase consisted of (A) CH_3CN:HOAc (98:2, v/v) and (B) CH_3OH:H_2O:HOAc (95:3:2, v/v/v). Separations were effected by a linear gradient. Eluent was monitored by UV-detection at 280 nm and by fluorescence detection with excitation at 280 nm and emission at 316 nm.

3. Results

3.1. Rumex extract R2 exhibits concentration-dependent antiviral effects against HSV-1 and does not negatively interfere with cellular metabolism at effective concentrations

The proanthocyanidin-enriched extract R2 from the aerial parts from *R. acetosa* was prepared as described in Section 2.

For evaluation of the cytotoxic effects of *R2* on treated Vero cells the MTT assay (Mosmann, 1983) was used. A significant reduction of the cell vitality occurred at concentrations > 25 μg/mL, corresponding to a CC_{50} of 78.6 ± 12.7 μg/mL (Fig. 1). The antiviral activity of R2 against HSV-1 was also monitored by MTT assay. R2 showed 100% of antiviral activity at extract concentrations > 1 μg/mL corresponding to an IC_{50} of 0.8 ± 0.04 μg/mL (Fig. 1). From these data the selectivity index (SI = ratio of CC_{50}/IC_{50}) was calculated to be 100.7.

3.2. R2 has no activity against adenovirus

To investigate whether R2 exhibits antiviral activity against other structurally non-related viral pathogens infecting the oropharynx experiments were repeated with adenovirus 3. Activity was assessed by determination of 50% tissue culture infection doses $TCID_{50}$ (Hierholzer and Killington, 1996). Even at a concentration of 100 μg/mL R2 showed no antiviral activity against adenovirus 3 indicating a comparative specifity of R2 against herpes simplex virus (data not shown).

3.3. Galloylated polyphenols from R2 are more active than non-galloylated compounds

For structure–activity investigations monomeric flavan-3-ols and dimeric proanthocyanidins which are present in high concentrations in R2 were isolated by preparative fractionation of R2 and subsequent analytical characterisation by MS and NMR studies (Bicker et al., 2009) and comparatively tested on antiviral activity (Fig. 2 and Table 1). Interestingly, epicatechin (Fig. 2, compound **1**) had only moderate antiviral activity at high concentrations (200 μM) without influencing cell physiology to a higher extend. From this an SI < 1 was estimated. In contrast, the respective galloylated epicatechin (Fig. 2, compound **2**) exhibited high anti-HSV-1 activity at concentrations about 20 μM, while the 200 μM dose was clearly cytotoxic (estimated SI = 19).

While epicatechin is a di-hydroxylated flavan-3-ol in the B-ring system, ortho-trihydroxyl groups in the B-ring should increase the activity (De Bruyne et al., 1999). Again we investigated the respective compounds epigallocatechin (Fig. 2, compound **3**), its isomer gallocatechin (Fig. 2, **5**) and epigallocatechin-3-O-gallate (Fig. 2, compound **4**). While epigallocatechin and gallocatechin were only active at high concentrations (200 μM) (SI < 1 for epigallcatechin, SI = 1 for gallocatechin) the galloylated compound epigallocatechin-3-O-gallate **4** exhibited strong antiviral activity in the absence of cytotoxicity at concentrations of about 2 μM (SI > 50). This clearly shows that a tri-hydroxylation in the B-ring is not a prerequisite for anti-HSV-1 activity, since the antiviral profiles of the di- and tri-hydroxylated compounds epicatechin and epigallocatechin are similar. In contrast, galloylation especially of the tri-hydroxylated compound epigallocatechin increases antiviral activity about 100-fold.

To further corroborate this finding, we isolated galloylated and non-galloylated dimeric procyanidins, namely procyanidin B2 (Fig. 2, compound **6**) and procyanidin B2-di-gallate (Fig. 2, compound **7**). Again we found only moderate antiviral activity for the ungalloylated compound at 200 μM (SI = 1), while the di-galloylated procyanidin exhibited strong activity in the low-micromolar range (SI > 40). This indicates that galloylation is a prerequisite for high anti-HSV-1 activity.

The two building blocks of procyanidin B2 are linked by a 4 → 8 interflavan bridge. Additonally, a highly similar compound (Fig. 2, compound **8**) derived from R2 was investigated containing a 4 → 6 interflavan linkage. No significant differences between both compounds were detected, indicating that changes in the planarity of the molecules have no influence on the antiviral activity.

Because galloylation seems to be a prerequisite for antiviral activity also the antiviral activity of free gallic acid (Fig. 2, compound **9**) was tested (Table 1). In the range of 2–200 μM no activity was detected, indicating that solely the coupling of procyanidins with gallic acid forms compounds with antiviral activity.

3.4. Rumex extract R2 affects viral adsorption

To identify which step in the virus life cycle was affected by R2, HSV-1 and extract were preincubated for 1 h, then added to

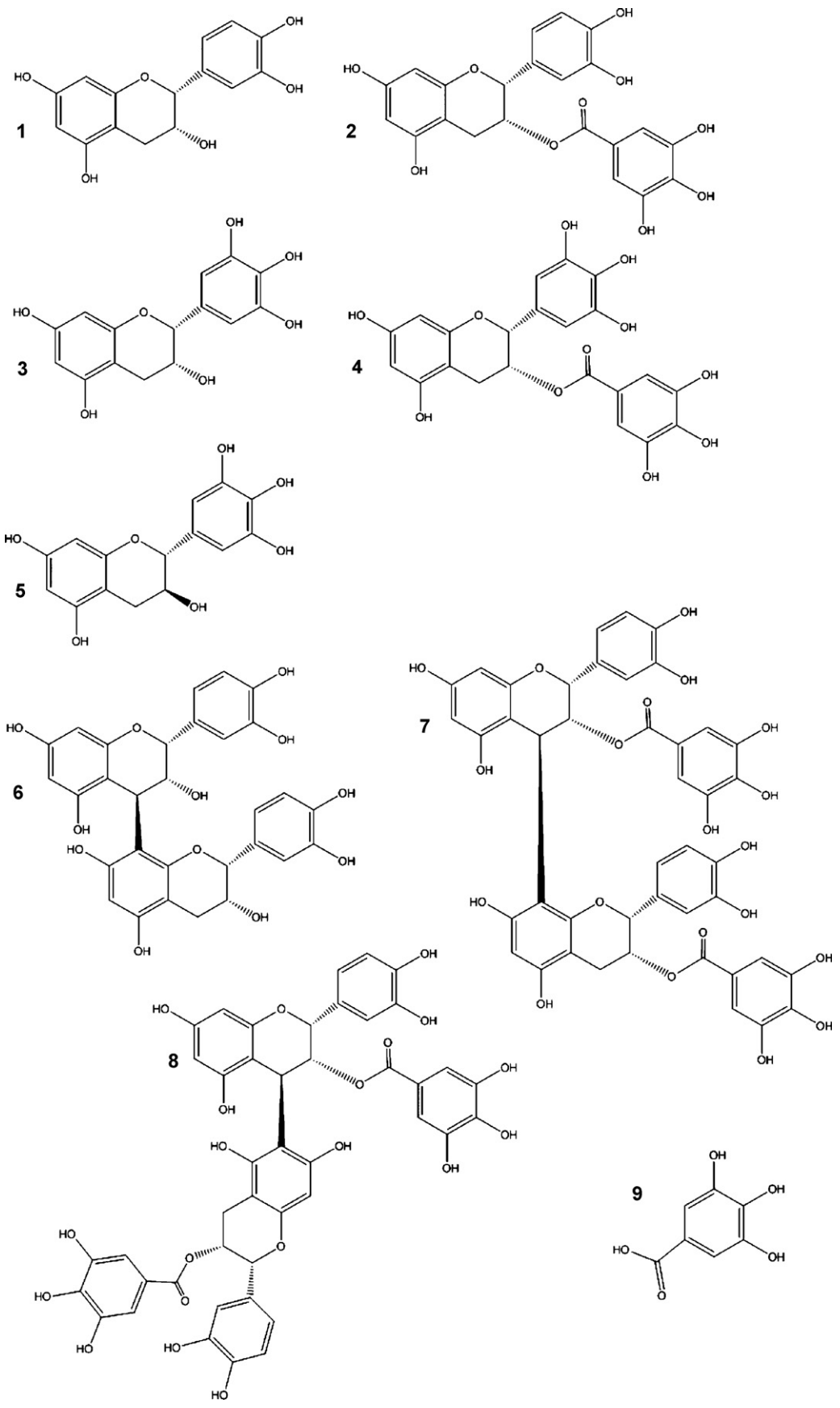

Fig. 2. Structural features of flavan-3-ols, dimeric proanthoycanidins and gallic acid as main compounds from *Rumex acetosa* extract R2.

Table 1
Influence of flavan-3-ols, procyanidins and gallic acid from *Rumex acetosa* extract R2 on cell vitality (MTT test on Vero cells) and respective anti-HSV-1 activity. Values ± SD relate to the respective untreated controls (= 100%).

Compound	Compound No.		Concentration (μM)		
			2	20	200
Epicatechin	**1**	Cell vitality (%)	99 ± 1	102 ± 1	92 ± 5
		Anti-HSV (%)	16 ± 2	12 ± 2	57 ± 1
Epicatechin-3-O-gallate	**2**	Cell vitality (%)	102 ± 2	96 ± 6	22 ± 6
		Anti-HSV (%)	36 ± 0	81 ± 2	10 ± 1
Epigallocatechin	**3**	Cell vitality (%)	101 ± 9	107 ± 8	72 ± 10
		Anti-HSV (%)	4 ± 0	9 ± 0	59 ± 4
Epigallocatechin-3-O-gallate	**4**	Cell vitality (%)	94 ± 2	80 ± 7	13 ± 0
		Anti-HSV (%)	98 ± 4	71 ± 14	−3 ± 1
Gallocatechin	**5**	Cell vitality (%)	99 ± 5	104 ± 1	54 ± 10
		Anti-HSV (%)	3 ± 1	10 ± 9	26 ± 22
Epicatechin-(4β → 8)-epicatechin (Procyanidin B2)	**6**	Cell vitality (%)	97 ± 10	98 ± 8	90 ± 12
		Anti-HSV (%)	1 ± 3	1 ± 1	32 ± 4
Epicatechin-3-O-gallate-(4β → 8)-epicatechin-3-O-gallate (procyanidin B2-3,3′-di-O-gallate)	**7**	Cell vitality (%)	99 ± 2	71 ± 4	13 ± 2
		Anti-HSV (%)	104 ± 7	78 ± 7	2 ± 5
Epicatechin-3-O-gallate-(4β → 6)-epicatechin-3-O-gallate	**8**	Cell vitality (%)	97 ± 7	57 ± 8	18 ± 3
		Anti-HSV (%)	101 ± 17	47 ± 2	3 ± 16
Gallic acid	**9**	Cell vitality (%)	93 ± 2	36 ± 21	2 ± 6
		Anti-HSV (%)	9 ± 3	−9 ± 7	−8 ± 7

the cells, and the inoculum was removed after 60 min. Again R2 inhibited virus replication completely at >1 μg/mL, indicating that the extract acts within the early phase of viral infection. In contrast when HSV-1 was incubated for 1 h with Vero cells and R2 was added later no antiviral effect was measured, indicating that the extract does not act on the post-entry phase.

In order to investigate if R2 interacts with target molecules of the host cells or of the virus, respectively, Vero cells were preincubated with the extract for 1 h, and then infected with HSV-1after removal of the extract. Preincubation of the cells did not evoke any antiviral effect, which clearly indicates that the activity of R2 is caused by direct interaction with the virus.

For confirmation of the results obtained by MTT assay, the effect of R2 on different stages of the HSV-1 replication cycle was monitored by plaque reduction assay. In case of coincubation of the extract with HSV-1 potent antiviral effects were observed. Almost complete inhibtion was still observed at a concentration of 0.01 μg/mL R2 (Table 2, experiment 1). No antiviral effect was observed when R2 was added after completion of the attachment/penetration phase.

In order to clarify, whether R2 was able to inhibit viral penetration, R2 was added after attachment of HSV-1 to the cell surface at 4 °C. After an incubation of 30 min, penetration was initiated by temperature shift to 37 °C. These experiments showed that R2 also inhibits viral penetration when added after attachment of HSV-1 to the cell surface. As compared to pre-incubation with HSV-1 prior to entry, however, significantly higher concentrations of R2, e.g., >6.25 μg/mL, were needed to completely block viral penetration (Table 2, experiment2). Thus, similar to the entry blocker heparin (Lycke et al., 1991), R2 seems to affect virus entry primarily by inhibiting viral attachment.

To visualize the inhibitory effect of R2 on viral attachment, adsorption of treated and nontreated virus particles to Vero cells was analyzed by immunofluorescence (details in Section 2). As shown in Fig. 3 untreated HSV-1 effectively bound to the cell surface at 4 °C, while pretreatment of HSV-1 with R2 abolished virus adsorption to the cell surface. These results further corroborate the antiadhesive activity of R2 on HSV-1.

3.5. Proanthocyanidins from R2 interact with virus surface proteins

R2 comprises a polyphenol enriched extract, consisting mainly of proanthocyanidins and flavonoids (Bicker et al., 2009). For unambiguous identification which compounds interact with HSV-1 analytical investigations were performed by using a selective HPLC system on diol stationary phases, suitable for clustering proanthocyanidins and other polyphenols (Zumdick et al., 2009). By using the UV-spectra of the eluting peaks, the fluorescent behaviour and specific reference compounds the nature of all main peak clusters can be determined. Because R2 as a very complex extract did show overlay of some peaks, the extract was divided by extraction with ethylacetate into a fraction R4 containing flavonoids and low molecular proanthocyanidins (dimers to tetramers) and a fraction R3 with high molecular weight proanthocyanidins. R4 was well suited to be analyzed by HPLC Testing of R4 for antiviral activity indicated no significant qualitative and quantitative differences to those data obtained with R2 (data not shown). Proanthocyanidins from R4 clustered in 3 different peaks (monomeric, dimeric and trimeric proanthocyanidins, named 1–3). The peak areas were taken as 100%. Incubation of a solution of R4 (1 mg/mL) with HSV-1 at 37 °C, 1 h at different virus titers (2×10^5, 2×10^6 and 2×10^7 pfu), followed by removal of the virus particles by ultracentrifugation, HPLC analysis of the supernatant and quantification of the three relevant proanthocyanidin clusters 1–3 indicated a significant reduction of proanthocyanidins concentrations of about 40–50% (Fig. 4A). A similar experiment investigated the influence of HSV-1 on the amounts of flavonoids in the R4 test solution, eluting during HPLC in 4 different flavonoid peaks, named a to d: also in this case HSV-1 provoked a slight reduction of the flavonoid content for about 20%, indicating that also these polyphenols are capable to interact to some extent with the virus particles (Fig. 4B).

Table 2
Antiviral effect of R2 as determined by plaque reduction assay. Data are given as % of plaque reduction in relation to R2 mock-treated controls (*$P < 0.05$, **$P < 0.005$). *Experiment 1*: HSV-1 and R2 were coincubated for 1 h at RT prior to the addition to cells, heparin served as a positive control. *Experiment 2*: R2 was added after attachment of HSV-1 to cells at 4 °C for 30 min, penetration was initiated by temperature shift to 37° and stopped after 30 min by washing the cells with citrate buffer pH 3.0. The efficiency of low pH treatment was controlled by washing nontreated cultures immediately after the attachment period with citrate buffer at 4 °C.

Experiment 1: pre-incubation with HSV-1 for 1 h at RT prior to the addition to cells					
R2					Heparin
10 μg/mL	1 μg/mL	0.1 μg/mL	0.01 μg/mL	0.001 μg/mL	25 IU/mL
100 ± 0**	100 ± 0**	100 ± 0**	98 ± 4**	13 ± 3**	100 ± 0**

Experiment 2: addition to HSV-1 attached to the cell surface at 4 °C					
R2					None
50 μg/mL	25 μg/mL	12.5 μg/mL	6.25 μg/mL	3.125 μg/mL	pH 3.0 wash after attachment at 4 °C
100 ± 0**	89 ± 11**	45 ± 18*	15 ± 29	0 ± 14	100 ± 0

Summarizing these data we suggest that mainly proanthocyanidins are responsible for the interaction with the viral surface, but also flavonoids can have a similar tendency.

3.6. Proanthocyanidins from R2 oligomerize the gD protein from HSV-1

To study the direct interaction of proanthocyanidins with viral envelope proteins, the effect of proanthocyanidin-treatment on the electrophorectic mobility of the essential viral glycoprotein gD was investigated in immunoblots. Glykoprotein D of nontreated HSV-1 particles migrated with an apparent molecular weight of 55–60 kDa (Fig. 5). Incubation of HSV-1 (6.5×10^7 pfu/mL) with R2 (data not shown) or with the major dimeric proanthocyanidin epicatechin-3-O-gallate-(4β → 8)-epicatechin-3-O-gallate (1 mg/mL) isolated from R2 (Fig. 2, compound **7**). Led to a reduction of the major gD band and to the oligomerisation of gD towards higher aggregates (Fig. 5). Soon after addition of the respective proanthocyanidins to HSV-1 the formation of gD dimers and trimers was detected. During longer incubation periods prominent bands corresponding to gD dimers, trimers and higher oligomers became visible. In addition, a characteristic broad 60 to >200 kDa "smear" consisting of gD molecules with reduced electrophorectic mobility was observed in all experiments (Fig. 5). Aggregation of gD was resistant to boiling of protein samples in reducing SDS-PAGE sample buffer, or treatment of the mixture in strong chaotropic environment (urea 8 mol/L at 30 °C). Thus, treatment of HSV-1 with R2 most likely results in the formation of covalent linkages between proanthocyanidins and protein moieties and not in aggregation by non-covalent H-H- or van de Waals bonds.

Aggregation of gD caused by R2 could be easily discriminated from time-dependent self aggregation of gD, which occurred to a much lower extend and only after several hours of incubation (Fig. 5). The finding that proanthocyanidins directly interact with viral glycoproteins such as gD protein may well explain the potential antiadhesive effects of extracts from *R. acetosa*.

3.7. R2 inhibits viral infection in skin keratinocytes and reduces viral spread in organotypic skin equivalents

For further evaluation of R2 the antiviral activity of the extract was studied in HSV-1-infected human keratinocytes (HaCaT cell line). R2 showed essentially comparable antiviral activity as already demonstrated for HSV-1-infected Vero cells (data not shown). In a further set of experiments the effect of R2 on HSV-1 spread in complex three-dimensional human organotypic skin equivalents was investigated.

To enable infection of raft cultures approximately 1 mm deep and 5 mm long cuts were created in the keratinized epidermal surface, followed by infection with HSV-1 (2×10^4 pfu/cut). As shown by immunohistochemistry (Fig. 6) 48 h after addition of HSV-1 raft cultures were predominantly infected in the epidermis, only to a minor extent in the dermis.

For testing of the antiviral effect of R2 in the so-called "single dose experiment", 2 μg of the extract was applied into the cuts 5 min after addition of HSV-1.

Additionally, in the so-called "repeated dose experiment" 2 μg of R2 were applied thrice daily during a 2 days incubation period after HSV-1 infection of raft cultures.

As shown in Fig. 6, in the single dose experiment as well as in the repeated dose experiment R2 was unable to block completely HSV-1 infection of the raft cultures when applied after the addition of virus. Viral spread in cultures treated with R2, however, appeared to be reduced. As compared to single treatment with R2, multiple

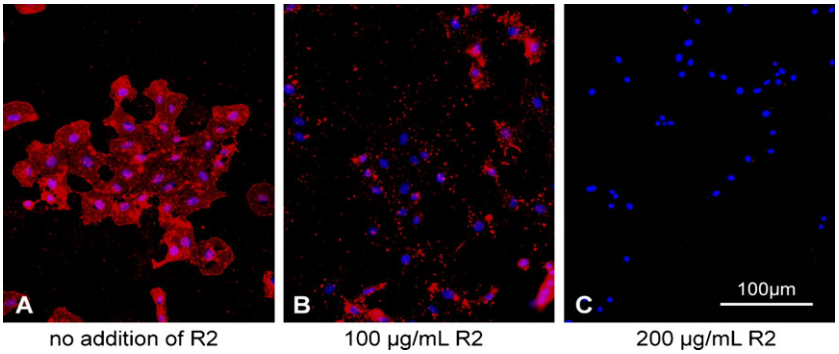

Fig. 3. Effect of R2 on HSV-1 attachment to Vero cells. 1×10^7 pfu HSV-1 were either mock-treated (panel A), pre-incubated with 100 μg/mL R2 (panel B) or 200 μg/mL R2 (panel C), and added for 1 h at 4 °C to Vero cells cultivated on chamber slides. Virus bound to the cell surface was visualized by immunostaining with a polyclonal rabbit serum against HSV-1, biotinylated goat anti-rabbit antibodies, and Cy3-conjuagted streptavidin (red), cell nuclei were stained with DAPI (blue). Scale bar: 100 μm.

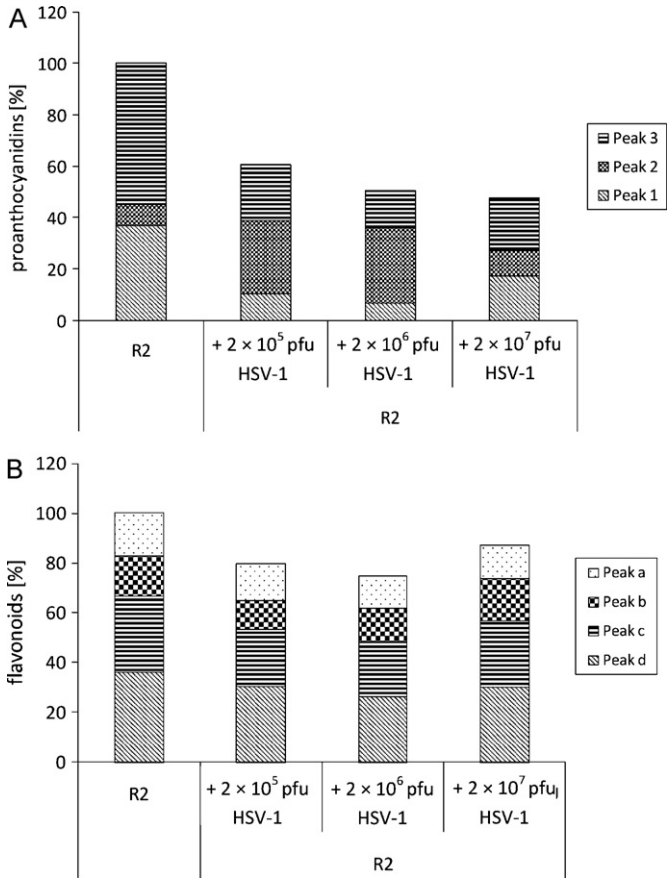

Fig. 4. Relative proanthocyanidin (A) and flavonoid (B) amounts determined by HPLC analysis in R2 after pre-incubation of R4 with different concentrations of HSV-1 (2×10^5, 2×10^6 and 2×10^7 pfu) for 1 h at 37 °C. Proanthocyanidins were analyzed as peaks 1, 2 and 3 while flavonoids eluted as peaks a, b, c and d.

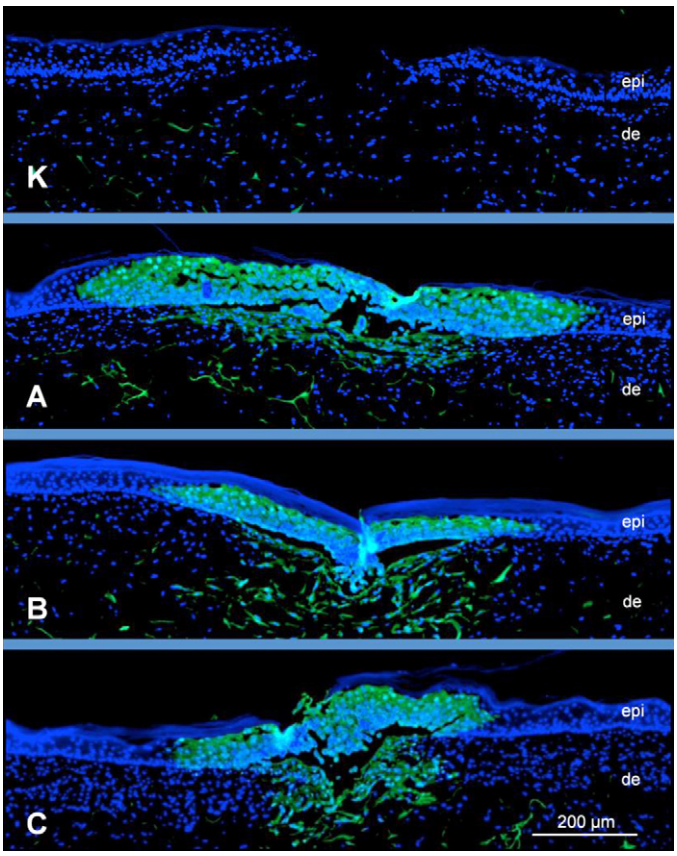

Fig. 6. Effect of R2 on HSV-1 spread in an organotypic human skin model. Histological tissue slides were stained with an HSV-1-specific polyclonal rabbit serum (green) and DAPI (blue) 48 h post infection, scale bar 200 µm: (K) uninfected control tissue, (A) HSV-1 infected tissue, (B) HSV-1 infected tissue, single dose R2 treatment (1 mg/mL, 2 µL), (C) HSV-1 infected tissue, multiple dose R2 treatment (1 mg/mL, 6×2 µL); epi: epidermis, de: dermis.

treatment of raft cultures with R2 seemed to cause additional skin damage (Fig. 6).

Additionally, viral genome replication in the raft cultures was quantified by quantitative real-time PCR. Using the HSV-1 UL54/ICP27 gene as target sequence and the cellular β-globin gene as cellular marker the relative viral load in infected raft cultures was determined as described in Section 2. In two independent experiments a 40–50% reduction of the relative viral load as compared to nontreated cultures was detected. The differences between single and repeated dose treatment were assessed not to be significant (Fig. 7).

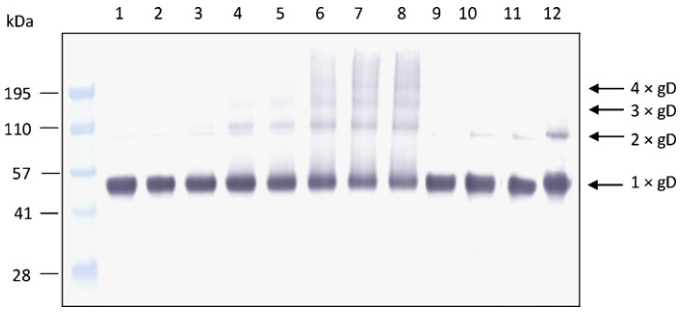

Fig. 5. Epicatechin-3-O-gallate-(4β → 8)-epicatechin-3-O-gallate (compound **7** in Fig. 2) causes oligomerisation of HSV-1 glycoprotein D (gD). Treated and untreated HSV-1 particles were analyzed by SDS-PAGE/Western blot using a gD-specific monoclonal antibody. Lane 1: mock-treated HSV-1 preparation; lanes 2–8: HSV-1 incubated with epicatechin-3-O-gallate-(4β → 8)-epicatechin-3-O-gallate for 30 min, 1, 2, 4, 6, 8, and 24 h; lanes 9–12: HSV-1 incubated with medium only for 1, 4, 8, 24 h. The position of oligomeric forms of gD is indicated.

4. Discussion

The use of plant extracts comprising highly complex mixtures of up to several hundred compounds in antiviral therapy is part of a multi-target therapy: different compounds may act against different molecular targets and inhibit viral infection more effectively than a single compound. In our investigations an acetone–water extract from *R. acetosa* was found to be highly active against HSV-1. Main compounds responsible for the antiviral activity were shown to be the tannin-like flavan-3-ols and oligomeric proanthocyanidins. Additionally, flavonoids present in the extract were shown to interact also with the HSV-1 surface. Interestingly, high activity was found against HSV-1 while adenovirus was not susceptible. This indicates that the tannin-like, adstringent effect of the proanthocyanidins is significantly influenced by their affinity to specific proteins and may be strongly dependent on amino acid composition and hydrophilicity of the proteins (Frazier et al., 2010). It remains to be shown to which extent different virus families are affected by polyphenols: for example other polyphenol-enriched extracts or proanthocyanidins from different plant species were described to be active against herpes simplex virus (for review see Khan et al., 2005), influenza virus (Ehrhardt et al., 2007), enterovirus (Ho et al., 2009) or HIV (Cos et al., 2008; De Bruyne et al., 1999; Feng et al., 2008; Nair et al., 2002). Investigations on defined proanthocyanidins should clarify in future investigations

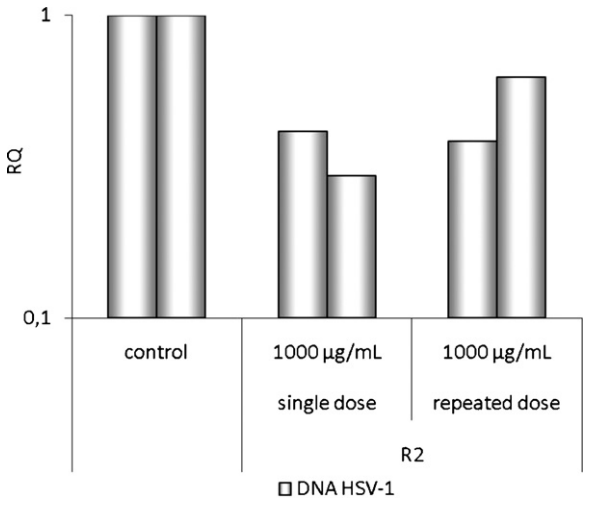

Fig. 7. HSV-1 genome quantification by RT-PCR in infected organotypic skin model treated with R2. The relative viral load (RQ-values) in two independent experiments is shown (A) untreated skin model, negative control; (B) single dose treatment of infected skin with R2 (1000 µg/mL); (C) repeated dose treatment of infected skin with R2 (6 × 1000 µg/mL).

which virus families or species, respectively, can be inhibited by these compounds and if there are any common viral surface features which act as defined molecular targets for polyphenols.

Concerning the physicochemical interactions of the proanthocyanidins with viral surface proteins the presence of galloyl residues, attached to the proanthocyanidins seems to be essential. Ungalloylated proanthocyanidins are only active at higher concentrations, and thus not interesting for any therapeutic use, while galloylation increases activity about 100-fold towards the low micromolar concentration range. On the other side it has to be considered that galloylation also effects cell physiology: galloylated compounds seem to be more cell toxic for eukaryotic cells than the ungalloylated compounds. However, toxic effects are only observed at higher concentrations, while within the effective antiviral doses host cells are not influenced. This correlates also with the high selectivity index (about 100) found for extract R2.

Obviously, dimeric proanthocyanidins with di-galloylation showed a higher selectivity then monomeric flavan-3-ols with one gallic acid ester. An exception was epigallocatechin-3-O-gallate which in contrast to the other tested flavan-3-ols is tri-hydroxylated in the B-ring system and galloylated, e.g., it contains 2 distinct phenolic structures with each 3 ortho-hydroxyl groups. Therefore, these structural properties could be responsible for the observed high activity.

The antiviral effect of R2 was shown to be due mainly to a potent inhibition of viral attachment. At higher concentrations R2 also abolished the penetration of viral particles already attached to the cell surface. Inhibition of the viral adhesion by R2 occurs at lower concentrations than that of the penetration. This may be due to the higher cell and protein load within the respective experimental design, leading to an unspecific binding of proanthocyanidins to cell surfaces and diminishing therefore the amount of R2 available for influencing viral surface proteins. Analyzing the effect of R2 on the structure of viral glycoproteins, we observed aggregation of the essential glycoprotein gD to oligomeric structures. This finding implies that proanthocyanidins may link the protein covalently, and is in good accordance with the model published by Charlton et al. (2002) describing the aggregation of proteins by polyphenols. Most likely, the pronounced antiadhesive effect of proanthocyanidins on HSV-1 is due to direct interactions with the viral envelope, i.e., with the glycoproteins gB or gC mediating viral attachment. Our finding that polyphenols block HSV-1 entry by interacting directly with the HSV-1 surface seems to be interesting in comparison with the well-established effects of proanthocyanidins on HIV (De Bruyne et al., 1999). On one side proanthocyanidins have been reported to down-regulate expression of the HIV-1 entry coreceptors CCR2b, CCR3 and CCR5 on peripheral blood mononuclear cells (Nair et al., 2002). On the other side they inhibit HIV entry most likely by interacting with structures on the viral envelope different from the target of enfurvitide, i.e., the viral fusion protein gp41 (Fink et al., 2009). Therefore, we speculate that polyphenols in general can be assessesd as entry blockers for enveloped viruses.

Beside the basic *in vitro* experiments with monolayer cell cultures the use of organotypic skin equivalents for advanced investigations bears many additional benefits: While investigations with monolayers enable an unambiguous relation between cellular effects and specific cell types, skin equivalents can simulate paracrine interactions between different cell types, e.g., dermal fibroblasts and epidermal keratinocytes. Furthermore skin equivalents are cultivated air-exposed so that the epidermis develops all differentiation states up to the *stratum corneum*. Since the penetration characteristics of skin equivalents are close to the *in vivo* situation, the comparability to human skin is much higher as compared to submerse cultivated monolayer cultures. Data obtained from organotypic skin equivalents indicated that R2 still exerted some antiviral activity even when added immediately after infection of raft cultures with HSV-1.

5. Conclusion

Summarizing these data, *R. acetosa* extract is assessed to be an antiviral system, with a strong influence of viral entry and penetration, which is very economically concerning production and which probably will not lead to the induction of viral resistance (Ehrhardt et al., 2007). Further preclinical and clinical investigations should clarify the clinical potential of such extracts for the therapeutic practice.

Acknowledgments

Thanks to Prof. G. Faller (Karlsruhe) for the preparation of paraffin-embedded tissue sections and to Engelhardt Arzneimittel GmbH, Niederdorfelden, Germany, for financial support.

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