Microbiology

Chemotherapy

Chemotherapy 2012;58:70–77
DOI: 10.1159/000335590

Received: May 6, 2011
Accepted after revision: December 4, 2011
Published online: February 23, 2012

Melissa officinalis Extract Inhibits Attachment of Herpes Simplex Virus in vitro

Akram Astani[a, c] Jürgen Reichling[b] Paul Schnitzler[a]

[a]Department of Infectious Diseases, Virology, and [b]Department of Biology, Institute of Pharmacy and Molecular Biotechnology, University of Heidelberg, Heidelberg, Germany; [c]Shahid Sadoughi University of Medical Science, Yazd, Iran

Key Words
Herpes simplex virus type 1 · Antiviral activity · Melissa extract · Caffeic acid · *p*-Coumaric acid · Rosmarinic acid

Abstract
Background: Extracts and essential oils of medicinal plants are increasingly of interest as novel drugs for antiherpetic agents, since the herpes simplex virus (HSV) might develop resistance to commonly used antiviral drugs. ***Methods:*** An aqueous extract of *Melissa officinalis* as well as phenolic extract compounds, i.e. caffeic acid, *p*-coumaric acid and rosmarinic acid were examined for their antiviral activity against herpes simplex virus type 1 (HSV-1) in vitro. ***Results:*** When drugs were added to HSV-1-infected cells, no antiviral effect was observed as determined by plaque reduction assay and analysis of expression of viral protein ICP0. However, the Melissa extract demonstrated a high virucidal activity against HSV-1, even at very low concentrations of 1.5 μg/ml, whereas similar results for phenolic compounds were only achieved at 100 times higher concentrations. Besides the virucidal activity, the Melissa extract and rosmarinic acid inhibited HSV-1 attachment to host cells in a dose-dependent manner. These results indicate that rosmarinic acid was the main contributor to the antiviral activity of Melissa extract. However, the selectivity index of Melissa extract of 875 against HSV is superior to the selectivity indices of single constituents. ***Conclusion:*** Melissa extract exhibits low toxicity, is virucidal and affects HSV-1 attachment to host cells in vitro.

Introduction

Herpes simplex virus type 1 (HSV-1) is an important pathogen for humans and the discovery of novel effective antiherpetic drugs without adverse effects is of great interest. The primary symptoms of herpes infection include a prodromal flu-like syndrome with fever, headache, malaise, diffuse myalgias, followed by local symptoms consisting of itching and painful papules. Gingivostomatitis and pharyngitis are the most frequent clinical manifestations of the first episodes of HSV-1 infection. After establishing latency, HSV can reactivate, causing frequent recurrent infections in some patients, while most people experience few recurrences [1]. Recurrent herpes labialis is the most frequent clinical manifestation of reactivated HSV-1 infection. The clinical manifestation of the disease exhibits different severity in immunocompetent patients. However, in immunocompromised patients and neonates, herpetic infections can cause serious systemic illnesses [2]. An antiviral treatment for HSV infection is

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0009–3157/12/0581–0070$38.00/0

Accessible online at:
www.karger.com/che

Prof. Dr. Paul Schnitzler
Department of Infectious Diseases, Virology
University of Heidelberg
Im Neuenheimer Feld 324, DE–69120 Heidelberg (Germany)
Tel. +49 6221 56 50 16, E-Mail Paul_Schnitzler@med.uni-heidelberg.de

available since the introduction of acyclovir in the 1970s and is still the most commonly used chemotherapy [3]. This nucleoside analogue functions as DNA chain terminators, ultimately preventing elongation of viral DNA [4]. The development of viral resistance towards antiviral agents enhances the need for new effective compounds against viral infections. Thus, new antiviral agents exhibiting different mechanisms of action are urgently needed.

Medicinal plants produce a variety of chemical constituents with the potential to inhibit viral replication, and compounds from natural sources are of interest as possible sources to control viral infection. These plants have been widely used in traditional medicine to treat a variety of infectious diseases and cancer [5] and represent an abundant source of new bioactive secondary metabolites. *Melissa officinalis* (lemon balm) is a member of the Lamiaceae family and plants of this family are well known in antiviral phytotherapy [6, 7]. Extracts from plants of the Lamiaceae family have been described for antioxidant [8] and antibacterial effects [9, 10] which have been linked to their polyphenolic composition. An antiviral activity of lemon balm aqueous extracts has been described previously [11], but no details on the mode of antiviral action are available. Experimental as well as therapeutic effects of lemon balm extract [12, 13] and rosemary [14] against herpetic infections could be demonstrated in vitro and in vivo. Melissa leaves contain polyphenolic compounds, e.g. caffeic acid derivatives in large proportions such as rosmarinic acid and also some flavonoids [15]. Melissa has been evaluated for its activity against herpes viruses and HIV in some experimental models in vitro [16, 17]. However, Melissa extract and its phenolic components caffeic acid, *p*-coumaric acid and rosmarinic acid have not been analyzed systematically for their antiviral potential. Thus only limited information about Melissa extract and phenolic compounds concerning the inhibition of the viral replication cycle and their mode of antiviral action is presently available.

The goal of the present study is to evaluate the antiviral activity of Melissa extract and selected phenolic components against HSV-1 and their mode of antiviral action.

Materials and Methods

M. officinalis *Extract and Phenolic Components*

M. officinalis extract was supplied by Lomapharm GmbH (Emmerthal, Germany) as dried powder. The drug-extract ratio was 70:1, the dried extract corresponding to 1.43% of the primary raw Melissa leaf material. Usually 10 g raw material, corresponding to 143 mg dried extract, is dissolved in 100 ml of the solvent. A stock solution was prepared by adding 100 ml of boiling water to 143 mg water-soluble Melissa powder followed by sterile filtration. HPLC analysis demonstrated phenolic compounds as constituents, e.g. caffeic acid, *p*-coumaric acid and rosmarinic acid. Besides essential oil, the major nonvolatile constituents in leaves of lemon balm are caffeic acid and its di- and trimeric derivatives, including rosmarinic acid and melitric acids A and B [18–20]. In addition, flavonoids such as luteolin and its glycosides are also found in Melissa leaves [21]. Serial dilutions were analyzed for cytotoxicity on monkey kidney cells and for antiviral effect against HSV. Caffeic acid, *p*-coumaric acid and rosmarinic acid were purchased from Sigma-Aldrich (Taufkirchen, Germany) and Roth (Karlsruhe, Germany) and dissolved in ethanol. Acyclovir was purchased from Mayne Pharma (Munich, Germany).

Cell Culture and HSV-1

RC-37 cells (African green monkey kidney cells) were grown in a monolayer culture with Dulbecco's modified Eagle's medium (DMEM; Gibco, Karlsruhe, Germany) supplemented with 5% fetal calf serum (FCS; Gibco, Karlsruhe, Germany), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Karlsruhe, Germany). Cells were plated out into 96-well and 6-well culture plates for cytotoxicity and antiviral assays, respectively, and propagated at 37°C in an atmosphere of 5% CO_2 [22]. HSV-1 strain KOS was used for all experiments. Viruses were routinely grown on RC-37 cells and virus stock cultures were prepared from supernatants of infected cells and stored at –80°C. Infectivity titers were determined by a standard plaque assay on confluent RC-37 cells.

Cytotoxicity Assay

The effect of Melissa extract and its components on the proliferation of RC-37 cells was determined in 96-well tissue culture plates. A medium containing the appropriate dilution of the extract or compounds was added onto the subconfluent cells in eight replicates for each concentration of the drugs. Wells containing a medium with 1% ethanol but no compounds were also included on each plate as controls. After 3 days of incubation the growth medium was removed and the viability of the drug-treated cells was determined in a standard neutral red assay [23]. The cytotoxic concentration of the drug which reduced the viable cell number by 50% (TC_{50}) was determined from dose-response curves. Additionally, the maximum noncytotoxic concentration of Melissa extract and each compound was determined [16].

Plaque Reduction Assay

The assay was performed as described previously [24]. Briefly, 5×10^5 cells per well were seeded into 6-well culture plates and then incubated until reaching at least 95% confluency. The cell monolayer was then infected with 100 pfu/well of drug-pretreated HSV-1. After incubation for 3 days at 37°C, monolayers were fixed with 10% formalin, stained with 1% crystal violet and subsequently plaques were counted. The minimal concentration of drugs required to reduce plaques by 50% (IC_{50}) was calculated by regression analysis of the dose-response curves [24]. The selectivity index (SI) was determined as the ratio of TC_{50}/IC_{50}.

Detection of ICP0 and gD Expression by Immunofluorescence

A total of 1.5×10^5 RC-37 cells were seeded onto circular glass coverslips (12 mm, Assistent, Hecht, Sondheim, Germany) and

maintained in 24-well plates 1 day before infection. On the following day the cells were infected with HSV-1 (MOI = 1) for 1 h, washed, and cultured in a maintenance media with or without Melissa extract or its components. Cells were washed with PBS, fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 1% bovine serum albumin (BSA) in PBS 5 h and 7 h postinfection (p.i.) for detection of ICP0 and gD, respectively. The coverslips were then incubated for 45 min at room temperature with mouse ICP0 or gD monoclonal antibodies (Virusys, Aachen, Germany). Afterwards, cells were washed and stained with 4,6-diamidino-2-phenylindole (DAPI; Sigma) and FITC-conjugated goat anti-mouse monoclonal antibodies for another 45 min at room temperature. The coverslips were mounted and analyzed using immunoflorescence microscopy [25].

Virucidal Assay and Attachment Assay

To determine the effect of Melissa extract and its components on direct inactivation of virus particles, HSV-1 (2×10^5 pfu/100 μl) was treated with an equal volume of drugs at room temperature. The concentration of Melissa extract was in the range of 1.5–150 μg/ml, caffeic acid 1–100 μg/ml, *p*-coumaric acid 10–1,000 μg/ml, and rosmarinic acid 1–100 μg/ml. The highest concentration indicated was always the maximum noncytotoxic concentration of these drugs. After 1, 2, 4, or 6 h, 1,000-fold dilutions of the mixture were added to RC-37 cell monolayers for 1 h at 37°C. The cell monolayers were overlaid with a media containing 0.5% methylcellulose and 2% FBS to be plaque assayed [26].

The attachment assay described by Cheng et al. [26] was used in this study with a minor modification. Briefly, RC-37 cell monolayers were grown in 24-well culture plates and then prechilled at 4°C for 1 h. The medium was aspirated and the cell monolayer was infected with 100 pfu/well of HSV in the absence or presence of serially diluted drugs up to the maximum noncytotoxic drug concentrations. After further incubating the infected cell monolayer at 4°C for another 3 h, the medium was aspirated to remove the unadsorbed virus. The cell monolayer was then washed with PBS 3 times, overlaid with a medium containing 0.5% methylcellulose, and plaque assayed [26].

Results

Analysis and Cytotoxicity of Melissa Extract and Phenolic Components

HPLC analysis demonstrated the presence of caffeic acid, *p*-coumaric acid and rosmarinic acid in the Melissa extract, at a concentration of 1.6 , 0.1 , and 65.8 mg/g dried extract, respectively. Thus the composition of tested phenolic compounds in the maximum noncytotoxic concentration of Melissa extract (150 μg/ml) is 0.16% (0.24 μg/ml) caffeic acid, 0.01% (0.015 μg/ml) *p*-coumaric acid, and 6.58% (9.75 μg/ml) rosmarinic acid. Aqueous Melissa extract and ethanolic solutions of caffeic acid, *p*-coumaric acid and rosmarinic acid were serially diluted and added to a cell culture medium to examine the effect on the growth and viability of tissue culture cells, always resulting in an ethanol concentration below 1% which had no effect on cells and viruses. The concentration ranges tested for Melissa extract, caffeic acid, *p*-coumaric acid and rosmarinic acid were 1.43–715, 0.01–1,000, 0.01–1,000 and 0.01–1,000 μg/ml, respectively. The maximum noncytotoxic concentrations of these drugs were in the range of 100–1,000 μg/ml, i.e. 100 μg/ml for caffeic acid and rosmarinic acid and 1,000 μg/ml for *p*-coumaric acid, the drug which revealed the lowest cytotoxicity (table 1). The Melissa extract was still noncytotoxic up to a concentration of 150 μg/ml. TC_{50} values were 150 μg/ml for caffeic acid, 350 μg/ml for Melissa extract and >1,000 μg/ml for *p*-coumaric acid.

Table 1. SI of Melissa extract and single compounds against HSV-1

Drug	Max. noncytotoxic concentration, μg/ml	TC_{50} μg/ml	IC_{50} μg/ml	SI
Melissa extract	150 ± 10.0	350 ± 18.2	0.4 ± 0.1	875
Caffeic acid	100 ± 5.1	150 ± 3.7	35 ± 6.3	4.3
p-Coumaric acid	1,000 ± 1.8	>1,000	2.5 ± 3.6	>400
Rosmarinic acid	100 ± 0.1	200 ± 12.0	10 ± 2.1	20

Experiments were repeated independently and data presented were the mean ± SD of three experiments.

Plaque Reduction Assay

Cell monolayers were infected with 100 pfu/well of drug-pretreated HSV-1. The concentration ranges tested for Melissa extract, caffeic acid, *p*-coumaric acid and rosmarinic acid were 1.43–150, 0.01–100, 0.01–1,000 and 0.01–100 μg/ml, respectively. The highest concentration of these drugs was always the maximum noncytotoxic concentration of Melissa extract, caffeic acid, *p*-coumaric acid and rosmarinic acid, i.e. 150, 100, 1,000 and 100 μg/ml, respectively. After incubation for 3 days at 37°C, monolayers were fixed and plaques were counted. IC_{50} was calculated by regression analysis of the dose-response curves. IC_{50} for Melissa extract, caffeic acid, *p*-coumaric acid and rosmarinic acid was determined at 0.4, 35, 2.5 and 10 μg/ml, respectively. The SI was determined as a ratio of TC_{50}/IC_{50}. Melissa extract revealed the highest SI of 875 (table 1).

Expression of Herpes Virus Proteins ICP0 and gD

Expression of early viral protein ICP0 and late viral protein gD in HSV-1-infected cells was analyzed after

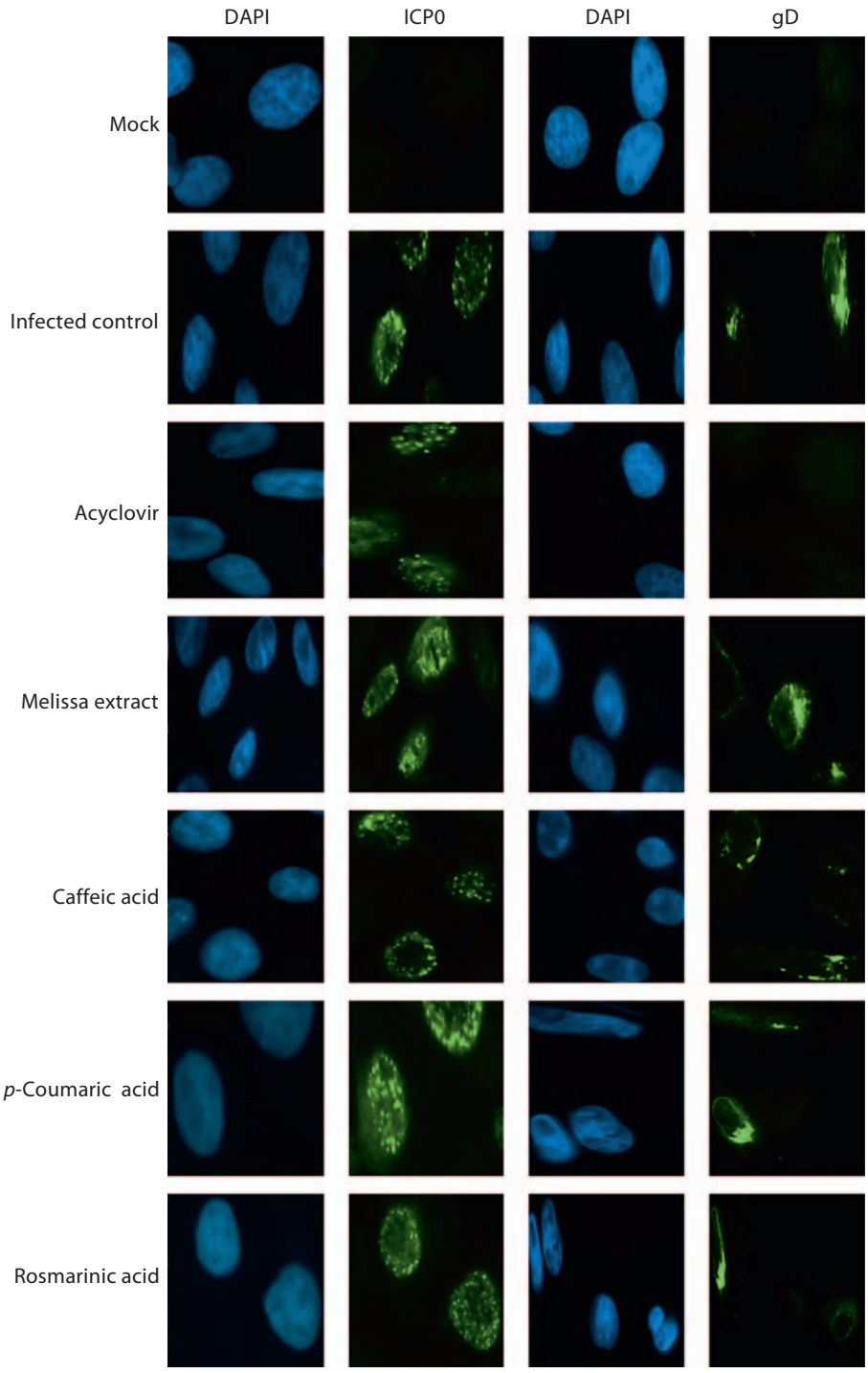

Fig. 1. Effect of Melissa extract and phenolic compounds on HSV-1 protein expression as determined by immunofluorescence microscopy. Infected cells were treated with maximum noncytotoxic concentration of plant extract, compounds and acyclovir 1 h p.i. Cells were fixed and stained at 5 and 7 h p.i. using antibodies against early protein ICP0 and late protein gD. Secondary antibodies were used to label viral proteins with FITC-conjugated goat anti-mouse monoclonal antibodies. Nuclei of cells were stained with DAPI.

mock treatment or drug treatment. All drugs were applied at maximum noncytotoxic concentrations. Infected cells were fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton X-100 5 and 7 h p.i. for ICP0 or gD, respectively, incubated with mouse ICP0 and gD monoclonal antibodies and stained with DAPI and FITC-conjugated goat anti-mouse monoclonal antibodies. Immunofluorescence revealed no effect of tested drugs on the expression of the herpes virus protein ICP0 and some inhibition of gD expression in drug-treated infected cells

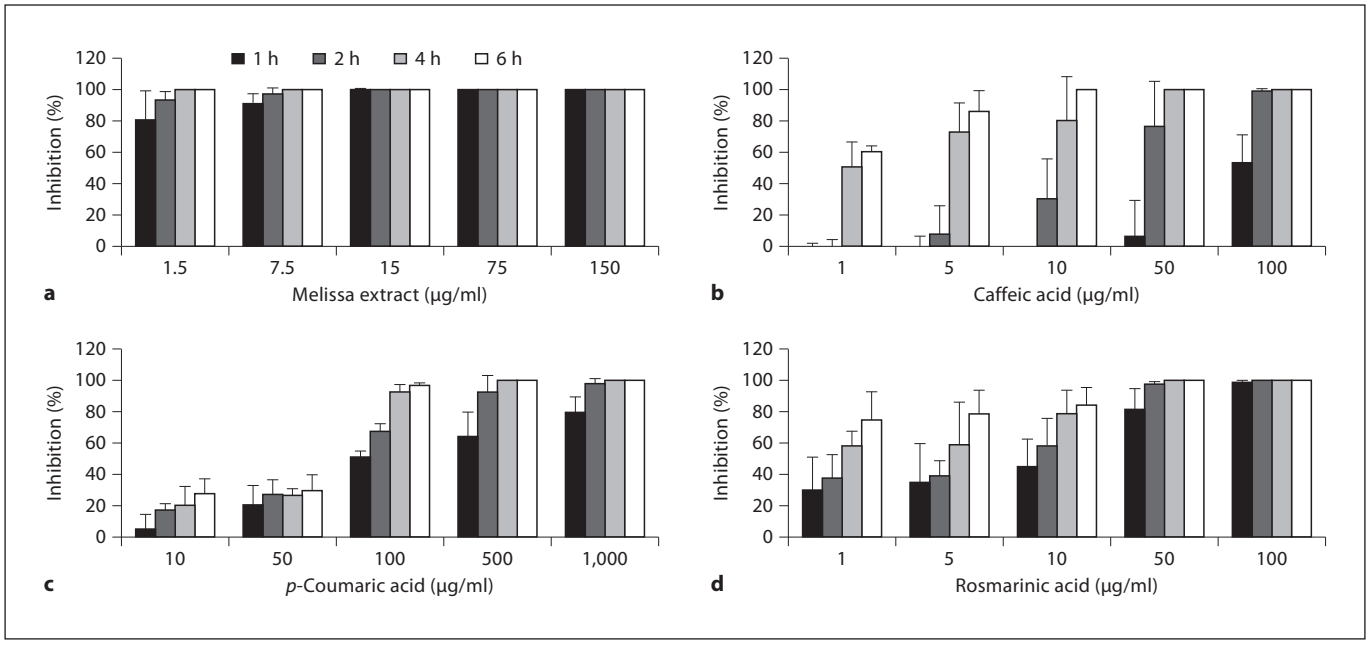

Fig. 2. Virucidal activity of Melissa extract (**a**), caffeic acid (**b**), *p*-coumaric acid (**c**) and rosmarinic acid (**d**). HSV-1 was incubated with drugs at indicated concentrations for 1, 2, 4, and 6 h at room temperature, followed by the dilution of the mixtures prior to their addition to cells. Mean ± SD are representative of three independent experiments.

(fig. 1). Thus some antiviral effect of the tested drugs could be demonstrated in infected cells during the late stage of HSV replication, but a higher potential antiviral effect might be exerted at earlier time points during the viral infection of cells.

Virucidal and Attachment Assay

To determine the virucidal effect of Melissa extract and phenolic components against virus particles, HSV-1 was treated at room temperature with serially diluted drugs, the highest concentration applied being the maximum noncytotoxic drug concentration. After 1, 2, 4, and 6 h, these drug-treated viruses were highly diluted with a medium as typically performed in virucidal assays and added to the RC-37 cell monolayers. Melissa extract and phenolic compounds revealed a dose-dependent virucidal effect against HSV. After incubation of HSV for 1 h with maximum noncytotoxic concentrations of phenolic compounds, viral activity was reduced by 50–99% (fig. 2). In contrast, Melissa extract revealed a >99% virus inactivation at a concentration of 15 μg/ml, a drug concentration tenfold below the maximum noncytotoxic concentration. Thus the plant extract revealed the highest virucidal activity against HSV-1.

In virus attachment assays, again maximum noncytotoxic drug concentrations were applied, i.e. 150, 100, 1,000 and 100 μg/ml for Melissa extract, caffeic acid, *p*-coumaric acid and rosmarinic acid, respectively. Melissa extract and rosmarinic acid showed an inhibition of virus attachment to the host cells. For better comparison of the attachment inhibition of the tested drugs, all maximum noncytotoxic drug concentrations were 1:2, 1:10, 1:20 and 1:100 diluted. Viral attachment was inhibited by 98% with Melissa extract at 7.5 μg/ml, a drug concentration 20 times below the maximum noncytotoxic concentration (fig. 3). However, rosmarinic acid was less effective, even at a maximum noncytotoxic concentration of 100 μg/ml, HSV-1 attachment was only reduced by about 50%.

Discussion

The results of studies on natural compounds are promising since several pure substances of plant origin have been shown to exhibit antiviral activity against HSV [27, 28]. In the present study, the antiviral activity of *M. officinalis* aqueous extract and three phenolic com-

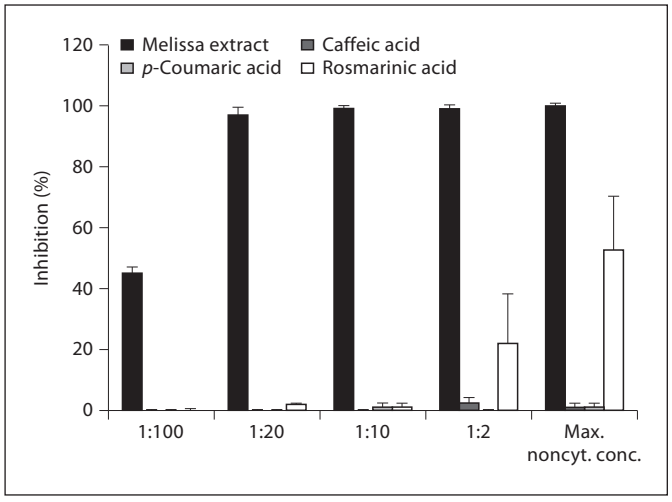

Fig. 3. Effects of Melissa extract, caffeic acid, *p*-coumaric acid and rosmarinic acid on virus attachment. Cell monolayers were prechilled at 4°C for 1 h, then infected with HSV-1 serially diluted drugs and incubated for another 3 h. The percentage of inhibition of the test compound was evaluated by plaque assay. Inhibition of viral attachment was calculated in comparison to untreated controls. Drug concentrations for Melissa extract, caffeic acid, *p*-coumaric acid and rosmarinic acid at the maximum noncytotoxic concentrations (max. noncyt. conc.) are 150, 100, 1,000, 100 μg/ml, respectively; for the 1:2 dilution, drug concentrations are 75, 50, 500, 50 μg/ml, respectively; for the 1:10 dilution, drug concentrations are 15, 10, 100, 10 μg/ml, respectively; for the 1:20 dilution, drug concentrations are 7.5, 5, 50, 5 μg/ml, respectively, and for the 1:100 dilution, drug concentrations are 1.5, 1, 10, 1 μg/ml, respectively.

pounds was analyzed for their antiviral activity against HSV in vitro. Experiments to assess the cytotoxicity of these drugs indicate a moderate toxic behavior in cell cultures according to Halle and Göres [29]. Expression of herpes virus early protein ICP0 was not affected by Melissa extract, though some decrease in expression of the late HSV protein gD was detectable. However, staining of ICP0 was always superior to staining of gD even in untreated HSV-1-infected cells, thus a clear quantification of inhibition of gD expression is not possible. A hydroalcoholic extract of *M. officinalis* had been shown recently to be quite effective against intracellular HSV-2 [30]. However, in our study aqueous Melissa extract as well as phenolic compounds drastically affected the infectivity of HSV-1 only at the early stage of virus replication. Rosmarinic acid is, at a concentration of 9.75 μg/ml, the predominant phenolic compound in Melissa extract. It is the main contributor for the virucidal effect and significantly inhibits herpes viral attachment. The IC_{50} value of 10 μg/ml for rosmarinic acid corresponds well to the actual concentration in the Melissa extract of 9.75 μg/ml. The rather low concentrations of caffeic acid and *p*-coumaric acid in the tested extract might not contribute to the antiviral effect of the extract, since IC_{50} values of these compounds are quite higher. Similar results for caffeic acid which is also present in *Plantago major* extracts have been reported previously, but the plant extract itself did not show antiherpetic activity [31]. However, other yet unidentified compounds of Melissa extract might enhance the antiviral effect.

Antiherpetic activity has been shown for aqueous plant extracts from *Echinacea pallidum* [32], *Rumex acetosa* [33] and *Rhododendron ferrugineum* [34], for polysaccharides derived from marine algae [35], for steroidal compounds against adenovirus [36] and an ethylacetate-soluble fraction from the lichen *Ramalina farinacea* against HIV [37]. Although all analyzed drugs in our study demonstrated virucidal activity, Melissa extract demonstrated a higher virucidal activity when compared to single compounds. Since all compounds tested in our study contribute to virucidicity against HSV, these compounds might act together or synergistically in the plant extract. Complex interactions between phytochemicals and a multitarget therapeutic concept of phytotherapy have been demonstrated recently [38]. Similar results have been shown for the antiherpetic effect of monoterpenes and sesquiterpenes [39]. The complex mixture of the Melissa extract revealed a higher antiviral activity and SI of 875, whereas analyzed single constituents demonstrated lower SIs. Cos et al. [40] recommended IC_{50} values for natural products against infectious diseases, e.g. for extracts below 100 μg/ml. Melissa extract revealed an IC_{50} value of 0.4 μg/ml in our study which is far below the recommended cutoff.

HSV-1 entry involves complex ligand-receptor interactions and has been shown to be an ideal target for antiviral compounds [33, 41]. Melissa extract highly inhibits HSV attachment to host cells. In contrast, among the tested phenolic compounds, only rosmarinic acid revealed some activity against attachment. Inhibition of adsorption and penetration of HSV by an aqueous extract from *Rhododendron ferrugineum* has been reported previously [34]. Similar results for inhibition of HSV attachment have been obtained with oligomeric proanthocyanidins from *Rumex acetosa* [33]. Garozzo et al. [42] investigated the antiviral activity of the essential oil from *Melaleuca alternifolia* (tea tree oil), and found that all the main components, i.e. terpinen-4-ol, α-terpinene, γ-terpinene, *p*-cymene, terpinolene and α-terpineol, were ineffective

against HSV-1 and HSV-2 and only a slight virucidal effect was observed for tea tree oil against these herpes viruses.

In conclusion, the phenolic compound rosmarinic acid in the Melissa extract contributed mainly to the virucidal activity, but revealed a lower SI when compared to the complex plant extract. In contrast to single phenolic compounds, Melissa extract additionally inhibited viral attachment and thus Melissa extract is preferable as a topical prophylactic/therapeutic agent for HSV infections. Our results demonstrate an antiherpetic effect of *M. officinalis* extract in vitro and support its traditional use in the topical treatment of recurrent herpes infection.

Acknowledgements

This work was supported by a grant to A. Astani from the Iranian Ministry of Health. We are very grateful to Dr. T. Bamdad, Tarbiat Modares University, Tehran, Iran for helpful discussions and M. Heidary Navid for critically reading the manuscript.

References

1 Whitley RJ, Roizman B: Herpes simplex virus infections. Lancet 2001;357:1513–1518.
2 Whitley RJ, Levin M, Barton N, Hershey BJ, Davis G, Keeney RE, Welchel J, Diethelm AG, Kartus P, Soong SJ: Infections caused by herpes simplex in the immunocompromised host: natural history and topical acyclovir therapy. J Infect Dis 1984;150:323–329.
3 Brady RC, Bernstein DI: Treatment of herpes simplex virus infections. Antiviral Res 2004; 61:73–81.
4 De Clercq E: Antiviral drugs in current clinical use. J Clin Virol 2004;30:115–133.
5 Efferth T: Natural products pave their way in cancer therapy. Cancer Biol Ther 2009;8: 1869–1870.
6 Wölbling RH, Leonhardt K: Local therapy of herpes simplex with dried extract from *Melissa officinalis*. Phytomedicine 1994;1:25–31.
7 Saller R, Buechi S, Meyrat R, Schmidhauser C: Combined herbal preparation for topical treatment of herpes labialis. Forsch Komplementmed 2001;8:373–382.
8 Bozin B, Mimica-Dukic N, Samojlik I, Jovin E: Antimicrobial and antioxidant properties of rosemary and sage (*Rosmarinus officinalis* L. and *Salvia officinalis* L., Lamiaceae) essential oils. J Agric Food Chem 2007;55:7879–7885.
9 Moreno S, Scheyer T, Romano CS, Vojnov AA: Antioxidant and antimicrobial activities of rosemary extracts linked to their polyphenol composition. Free Radic Res 2006;40: 223–231.
10 Fu Y, Zu Y, Chen L, Efferth T, Liang H, Liu Z, Liu W: Investigation of antibacterial activity of rosemary essential oil against *Propionibacterium acnes* with atomic force microscopy. Planta Med 2007;73:1275–1280.
11 Vanden Berghe DA, Vlietinck AJ, Van Hoof L: Plant products as potential antiviral agents. Bull Inst Pasteur 1986;84:101–147.
12 Koytchev R, Alken RG, Dundarov S: Balm mint extract (Lo-701) for topical treatment of recurring herpes labialis. Phytomedicine 1999;6:225–230.
13 Gaby AR: Natural remedies for herpes simplex. Altern Med Rev 2006;11:93–101.
14 Debiaggi M, Tateo F, Cereda PM, Pagani L, Landini P, Romero E: Effect of a *Rosmarinus officinalis* L. fraction on uninfected and herpes simplex virus infected cells. Ann Microbiol Enzimol 1989;39:181–194.
15 Carnat P, Carnat A, Fraisse D, Lamaison J: The aromatic and polyphenolic composition of lemon balm (*Melissa officinalis* L. subsp. *officinalis*) tea. Pharma Acta Helv 1998;72: 301–305.
16 Nolkemper S, Reichling J, Stintzing FC, Carle R, Schnitzler P: Antiviral effect of aqueous extracts from species of the Lamiaceae family against herpes simplex virus type 1 and type 2 in vitro. Planta Med 2006;72:1378–1382.
17 Geuenich S, Goffinet C, Venzke S, Nolkemper S, Baumann I, Plinkert P, Reichling J, Keppler OT: Aqueous extracts from peppermint, sage and lemon balm leaves display potent anti-HIV-1 activity by increasing the virion density. Retrovirology 2008;5:27.
18 Oruga J, Vlase L, Toiu A, Benedec D, Duda M: Evaluation of phenolic acid derivatives and essential oil content in some *Melissa officinalis* L. varieties. Farmacia 2008;58:764–769.
19 Fecka I, Turek S: Determination of water-soluble polyphenolic compounds in commercial herbal teas from Lamiaceae: peppermint, Melissa, and sage. J Agric Food Chem 2007;55:10908–10917.
20 Agata I, Kusakabe H, Hatano T, Nishibe S, Okuda T: Melitric acids A and B, new trimeric caffeic acid derivatives from *Melissa officinalis*. Chem Pharm Bull 1993;41:1608–1611.
21 Patora J, Klinek B: Flavonoids from lemon balm (*Melissa officinalis* L., Lamiaceae). Acta Pol Pharm 2002;59:39–143.
22 Schnitzler P, Koch C, Reichling J: Susceptibility of drug-resistant clinical herpes simplex virus type 1 strains to essential oils of ginger, thyme, hyssop, and sandalwood. Antimicrob Agents Chemother 2007;51:1859–1862.
23 Söderberg T, Johannson A, Gref R: Toxic effects of some conifer resin acids and tea tree oil on human epithelial and fibroblast cells. Toxicology 1996;107:99–109.
24 Cheng HY, Lin CC, Lin TC: Antiherpes simplex virus type 2 activity of casuarinin from the bark of *Terminalia arjuna* Linn. Antiviral Res 2002;55:447–455.
25 Su CT, Hsu JT, Hsieh HP, Lin PH, Chen TC, Kao CL, Lee CN, Chang SY: Anti-HSV activity of digitoxin and its possible mechanisms. Antiviral Res 2008;79:62–70.
26 Cheng HY, Lin TC, Yang CM, Wang KC, Lin LT, Lin CC: Putranjivain A from *Euphorbia jolkini* inhibits both virus entry and late stage replication of herpes simplex virus type 2 in vitro. J Antimicrob Chemother 2004;53: 577–583.
27 Khan MT, Ather A, Thompson K, Gambari R: Extracts and molecules from medicinal plants against herpes simplex viruses. Antiviral Res 2005;67:107–119.
28 Akhtar J, Shukla D: Viral entry mechanisms: cellular and viral mediators of herpes simplex virus entry. FEBS J 2009;276:7228–7236.
29 Halle W, Göres E: Prediction of LD_{50} values by cell culture. Pharmazie 1987;42:245–248.
30 Mazzanti G, Battinelli L, Pompeo C, Serrilli AM, Rossi R, Sauzullo I, Mengoni F, Vullo V: Inhibitory activity of *Melissa officinalis* L. extract on herpes simplex virus type 2 replication. Nat Prod Res 2008;22:1433–1440.
31 Chiang LC, Chiang W, Chang MY, Ng LT, Lin CC: Antiviral activity of *Plantago major* extracts and related compounds in vitro. Antiviral Res 2002;55:53–62.
32 Schneider S, Reichling J, Stintzing FC, Messerschmidt S, Meyer U, Schnitzler P: Antiherpetic properties of hydroalcoholic extracts and pressed juice from *Echinacea pallida*. Planta Med 2010;76:265–272.
33 Gescher K, Hensel A, Hafezi W, Derksen A, Kühn J: Oligomeric proanthocyanidins from *Rumex acetosa* L. inhibit the attachment of herpes simplex virus type-1. Antiviral Res 2011;89:9–18.

34 Gescher K, Kühn J, Hafezi W, Louis A, Derksen A, Deters A, Lorentzen E, Hensel A: Inhibition of viral adsorption and penetration by an aqueous extract from *Rhododendron ferrugineum* L. as antiviral principle against herpes simplex virus type-1. Fitoterapia 2011;82:408–413.
35 Bandyopadhyay SS, Heidary Navid M, Ghosh T, Schnitzler P, Ray B: Structural features and in vitro antiviral activities of sulfated polysaccharides from *Sphacelaria indica*. Phytochemistry 2011;72:276–283.
36 Romanutti C, Bruttomesso AC, Castilla V, Galagovsky LR, Wachsman MB: Anti-adenovirus activity of epiandrosterone and dehydroepiandrosterone derivatives. Chemotherapy 2010;56:158–165.
37 Esimone CO, Grunwald T, Nworu CS, Kuate S, Proksch P, Überla K: Broad spectrum antiviral fractions from the lichen *Ramalina farinacea* (L.) Ach. Chemotherapy 2009;55:119–126.
38 Efferth T, Koch E: Complex interactions between phytochemicals. The multi-target therapeutic concept of phytotherapy. Curr Drug Targets 2011;12:122–132.
39 Astani A, Reichling J, Schnitzler P: Screening for antiviral activities of isolated compounds from essential oils. Evid Based Complement Alternat Med DOI: 10.1093/ecam/nep187.
40 Cos P, Vlietinck AJ, Berghe DV, Maes L: Anti-infective potential of natural products: how to develop a stronger in vitro 'proof-of-concept'. J Ethnopharmacol 2006;106:290–302.
41 Bergefall K, Trybala E, Johansson M, Uyama T, Naito S, Yamada S, Kitagawa H, Sugahara K, Bergstrom T: Chondroitin sulfate characterized by the E-disaccharide unit is a potent inhibitor of herpes simplex virus infectivity and provides the virus binding sites on gro2C cells. J Biol Chem 2005;280:32193–32199.
42 Garozzo A, Timpanaro R, Bisignano B, Furneri PM, Bisignano G, Castro A: In vitro antiviral activity of *Melaleuca alternifolia* essential oil. Lett Appl Microbiol 2009;49:806–808.