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# Antiviral activity *in vitro* of two preparations of the herbal medicinal product Sinupret® against viruses causing respiratory infections

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ABSTRACT

Sinupret®, a herbal medicinal product made from Gentian root, Primula flower, Elder flower, Sorrel herb, and Verbena herb is frequently used in the treatment of acute and chronic rhinosinusitis and respiratory viral infections such as common cold. To date little is known about its potential antiviral activity. Therefore experiments have been performed to measure the antiviral activity of Sinupret® oral drops (hereinafter referred to as "oral drops") and Sinupret® dry extract (hereinafter referred to as "dry extract"), *in vitro* against a broad panel of both enveloped and non-enveloped human pathogenic RNA and DNA viruses known to cause infections of the upper respiratory tract: influenza A, Chile 1/83 (H1N1) virus (FluA), Porcine Influenza A/California/07/2009 (H1N1) virus (pFluA), parainfluenza type 3 virus (Para 3), respiratory syncytial virus, strain Long (RSV), human rhinovirus B subtype 14 (HRV 14), coxsackievirus subtype A9 (CA9), and adenovirus C subtype 5 (Adeno 5).

Concentration-dependent antiviral activity ($EC_{50}$ between 13.8 and 124.8 μg/ml) of Sinupret® was observed against RNA as well as DNA viruses independent of a viral envelope. Remarkable antiviral activity was shown against Adeno 5, HRV 14 and RSV in which dry extract was significantly superior to oral drops. This could be ascertained with different assays as plaque-reduction assays in plaque forming units (PFU), the analyses of a cytopathogenic effect (CPE) and with enzyme immunoassays (ELISA) to determine the amount of newly synthesised virus.

Our results demonstrate that Sinupret® shows a broad spectrum of antiviral activity *in vitro* against viruses commonly known to cause respiratory infections.

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## Introduction

A number of antiviral therapies have evolved that may be effectively administered to treat respiratory viral diseases, thus providing the physician with a range of therapy alternatives including amantadine (Hay et al. 1985), neuraminidase inhibitors (Calfee and Hayden 1998) and nucleoside analogues (Fyfe et al. 1978; Hruska et al. 1990). On one hand, these therapies are of limited effectiveness and on the other hand, side-effects and systemic toxicity may limit their application, particularly in paediatric, geriatric and compromised patients (Reusser 1996; Cassady and Whitley 1997; Bacon et al. 2003; Hayden et al. 1983; Janai et al. 1990; Englund et al. 1990). As a result, there is great interest in developing efficacious antiviral compounds of herbal origin with low toxicity that are well tolerated.

Herbal medicinal plant extracts have been widely used in traditional medicine (De Clercq 2004) due to their antimicrobial and antiviral effects. Interestingly, about 10% of the more than 4000 species studied showed a significant antiviral activity *in vitro* (Che 1991). Innumerable potentially useful medicinal plants and herbs are waiting to be evaluated and exploited for therapeutic applications against genetically and functionally diverse virus families (Jassim and Naji 2003). Several hundred already investigated plant and herb species have potential as novel antiviral agents (Jassim and Naji 2003). However, for the majority of species studied so far, systematic investigation of their activity against a broad panel of viruses still remains to be done.

Summerfield et al. described the *Acanthospermum hispidum* (Summerfield et al. 1997) activity against animal pathogenic herpes viruses, pseudorabiesvirus (PRV) and bovine herpesvirus 1 (BHV-1). More recently, Glatthaar-Saalmüller et al. (2001) were able to show antiviral activity of an extract from *Eleutherococcus senticosus* against human rhinovirus (HRV), human respiratory syncytial virus (RSV) and influenza A virus, which was discussed as RNA-virus specific reactivity. In addition, a very broad antiviral activity

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of the homeopathic preparation of Gripp-Heel was demonstrated to act against human herpesvirus (HSV-1), human adenovirus (HAV), human parainfluenza virus, human coxsackievirus, influenza A virus, HRV, and RSV, which might be based on the induction of type 1 interferons (Glatthaar 2007) by the respective substances. Nevertheless, no detailed information is yet available on the exact mode of antiviral action of plant extracts.

Just recently, Michaelis et al. (2011) investigated the influence of a standardised extract of *Pelargonium sidoides* (EPs® 7630), on the market as herbal medicinal product Umckaloabo® for the treatment of acute bronchitis, on replication of a panel of respiratory viruses. The authors were able to show that EPs® 7630 at concentrations up to 100 μg/ml interfered with replication of seasonal influenza A virus strains (H1N1 and H3N2), respiratory syncytial virus, human coronavirus, parainfluenza virus, and coxsackievirus but did not affect replication of highly pathogenic avian influenza A virus (H5N1), adenovirus, or rhinovirus.

An *in vitro* antiviral potential of the herbal medicinal product Sinupret®, widely used in phytotherapeutical treatment of acute and chronic rhinosinusitis, common cold and infections of the upper respiratory tract has been observed by Glatthaar and Christoffel (1998). The authors could show that Sinupret® drops and 2 of 5 extracts that constitute the combination, i.e. extracts of Verbena herb and Primula flower, reduced the spreading of three viruses (influenza A, respiratory syncytial virus and parainfluenza type 1). An antiviral effect of Sinupret® *in vivo* could be demonstrated in a murine virus model (Sendai virus), in which the survival time of mice could be increased by Sinupret® tablets therapeutically (März et al. 1999) and by Sinupret® drops if given prophylactically (Schmolz et al. 2001).

In the present study the antiviral effect of two Sinupret® preparations, oral drops (hereinafter referred to as "oral drops") and Sinupret® dry extract (hereinafter referred to as "dry extract"), have been investigated. These two herbal extracts of Gentian root, Primula flower, Elder flower, Sorrel herb and Verbena herb have been tested against a broad panel of viruses responsible for infections of the upper respiratory tract. The objective of this study was to evaluate *in vitro* the antiviral characteristics of both Sinupret® preparations against human pathogenic RNA- and DNA-viruses.

## Materials and methods

### *Test substances*

#### *Sinupret oral drops*

Commercially available Sinupret® was supplied by the manufacturer as ethanolic solution (19% (V/V), Sinupret oral drops). 100 g of oral drops contained 29 g of an aqueous-ethanolic extract (extracting agent: ethanol 59% (V/V); drug/extract ratio 1:11) from Gentianae radix, Primulae flos cum calycibus, Sambuci flos, Rumicis herba (sorrel) and Verbenae herba; in the fixed ratio of 1:3:3:3:3. Concentrations of oral drops are given as equivalents of the dry extract, using the known drug/extract ratios.

#### *Sinupret dry extract*

In addition Sinupret® was tested as native dry extract (special extract BNO 1011). The dry extract was delivered by the manufacturer; the composition was identical to that of the drops regarding the herbs. The dry extract was prepared with 59% ethanol as extracting agent (V/V) (with a final drug/extract ratio of 4.2:1). For use in the tests dry extract was prepared as a stock solution of 5 mg/ml in 29.5% ethanol or 100 mg/ml in 50% ethanol (repetition of FluA and pFluA).

#### *Quality of Sinupret® preparations*

Quality of herbal drugs (starting material) is specified according to the relevant EMA-Guidelines for herbal medicinal products. The preparations are manufactured in a validated production process according to GMP. Comprehensive specifications and standardised production processes guarantee high batch-to-batch consistency.

In all experiments, the stock solutions of the test substances were diluted in cell-culture medium before they were added to the cell cultures for the respective tests.

### *Reference drugs*

For all virus strains with the exception of CA9 positive controls were included in a single concentration close to their $IC_{50}$. Ribavirin (Virazole®, ICN Pharmaceuticals, Frankfurt, Germany, 6 μg/ml) was used in infections with RSV (Hruska et al. 1990) and amantadine (amantadine hydrochloride, Ratiopharm, Ulm, Germany) was used in infections with human and porcine influenza A virus (Hay et al. 1985; 5 μg/ml and 6 μg/ml, respectively). Laboratory internal standards were used as positive control for Para 3, HRV 14 and Adeno 5 (10 μg/ml, 20 μg/ml and 7.5 μg/ml, respectively). The effectiveness of the reference substances was confirmed in the tests. All reference substances showed a 50–65% reduction of viral plaques for FluA, pFluA, Para 3 and HRV 14 and of CPE for Adeno 5.

### *Cells and viruses*

Human rhinovirus B subtype 14 (HRV 14) was obtained from the Institute for Virology of the Friedrich-Schiller-University, Jena, Germany. Influenza A, Chile 1/83 (H1N1) virus (FluA), respiratory syncytial virus, strain Long (RSV), parainfluenza type 3 virus (Para 3), cocksackievirus subtype A9 (CA9), and adenovirus C subtype 5 (Adeno 5) were obtained from the Department of Medical Virology and Epidemiology of Virus Diseases of the Hygiene Institute of the University of Tübingen, Germany. Porcine Influenza A/California/07/2009 (H1N1, pFluA) was obtained from the National Institute for Biological Standards and Control (NIBSC), Hertfordshire, UK. All viruses were identified and characterised with a panel of monoclonal antibodies (BioWhittacker Products, Walkersville, MD).

RSV, Para 3 and Adeno 5 were propagated on Human Epithelial Cells (HEp-2); HRV 14 on HeLa cells and CA9 on buffalo-green-monkey (BGM) cells, in Hank's/Earle's minimal essential medium (MEM) containing 2% foetal calf serum, 25 mM $MgCl_2$, 2 mM of L-glutamine, 100 U/ml of penicillin, and 0.1 mg/ml of streptomycin. FluA and pFluA were grown on Madin-Darby-Canine-Kidney (MDCK) cells with serum-free MEM containing 1 μg/ml of trypsin, 2 mM of L-glutamine, 100 U/ml of penicillin, and 0.1 mg/ml of streptomycin.

In order to determine the virus titres, the respective cells were incubated with serially diluted serum-free virus stock solutions for 1 h at 34 °C. After removal of the virus inoculum, cell cultures were overlaid with the respective virus-specific medium containing agarose or carboxymethylcellulose. The analyses of the plaques (plaque forming unit = PFU) and the cytopathogenic effect (CPE) were performed 3–7 days later. The respective virus titres were calculated as PFU/ml or with the Spearman–Kärber method by the mean infectious concentration (log 10 $TCID_{50}$)/ml.

### *Virus assays*

Plaque assays or assays for the CPE were performed with MDCK, HEp-2, BGM and HeLa-cell cultures using standard procedures for the detection of infectious particles. For the quantification of RSV- and Adeno 5 antigens, enzyme immunoassays (Virion/Serion,

**Table 1**
Cytotoxicity of Sinupret® preparations (oral drops and dry extract). Inhibitory concentration ($IC_{50}$, μg/ml).

| | MTT-assay | | | | |
|---|---|---|---|---|---|
| | MDCK | HEp-2 | HeLa | BGM | Highest concentration used in the antiviral tests |
| $IC_{50}$ | | | | | |
| Oral drops | 543 | 930 | 827 | 516 | 120 |
| Dry extract | >500 | >500 | >500 | >500 | 100 (500[a]) |

The cytotoxicity on the respective cells (MDCK, HEp-2, HeLa and BGM) cultivated with different concentrations of the Sinupret® preparations (range: 1240–0.1 μg/ml) and the corresponding ethanol (ethanol range: 3.8–0.0003%) solutions were quantified using an MTT-assay. The relative cytotoxicity of the Sinupret® preparations was standardised by the medium control representing 100% viability. The table shows the concentration-dependent intoxication of Sinupret® calculated as a 50% inhibitory concentration ($IC_{50}$). All data represent four replicates.

[a] Concentration used in the additional tests with FluA and pFluA.

Würzburg, Germany; Merlin Diagnostika GmbH, Bornheim-Hersel, Germany) were used.

*Cytotoxicity tests*

Analyses of the *in vitro* cytotoxicity of Sinupret® preparations were performed with physiologically active cells and an enzymatic assay (MTT-assay; Mosmann 1983) which is capable of quantifying the activity of mitochondrial enzymes in active and dividing cells showing a direct correlation between viability and enzyme activity. Additionally, the cytotoxicity of test substances on the respective cells was monitored by microscopic examination of the cell cultures for altered cell morphology. For the determination of the limits of the toxic concentrations MDCK, HEp-2, BGM and HeLa cells were cultivated in their growth period together with different dilutions (log 2-dilutions from 1240 μg/ml to 0.1 μg/ml) of the test substances at 37 °C and 5% $CO_2$ for at least 5 days. Respective cell culture media without any test component were used as control (medium control).

*Assays for antiviral activity*

*Plaque-reduction assay (PFU), cytopathogenic effect (CPE) and immunoassay (ELISA)*

The antiviral activity of oral drops and dry extract was measured with plaque-reduction assays (Cooper 1955) in plaque forming units (PFU) for FluA and pFluA, Para 3, RSV, HRV 14, CA9 or with the analyses of a cytopathogenic effect (CPE) for Adeno 5. Cell monolayers were infected with a multiplicity of infection (M.O.I.) of 0.0004 (FluA, Para 3, RSV, HRV 14, CA9), 0.0008 (pFluA) or 0.008 (Adeno 5) without or in the presence of different non-toxic dilutions of the test substances (ranging from 0.031 to 500 μg/ml) and in addition the respective ethanol controls. Cells were infected for 1 h at 34 °C. The cell monolayers were then washed and overlayed with medium containing different concentrations of the test substances (0.031–500 μg/ml). Subsequently, the infected cell cultures were cultivated for three days (MDCK: FluA; BGM: CA9), four days (HeLa: HRV 14), five days (MDCK: pFluA, Para 3), six days (HEp-2: RSV) or seven days (HEp-2: Adeno 5) until lesions were visible in the cell monolayer (plaques or CPE) of the virus infected control group cultivated in medium alone. At this time point, the cells were fixed with paraformaldehyde and the remaining cell monolayers were stained with a crystal violet solution. Non-stained lesions in the cell monolayer (plaques, CPE) were quantified by employing an optical evaluation system. In case of RSV and Adeno 5, in addition to the analysis of the lesions (plaque and CPE), the amount of newly synthesised virus was determined by enzyme immunoassays (ELISA).

*Calculation of antiviral activity*

The quantification of the antiviral activity was carried out either by analysing the number of plaques (PFU: FLuA, Para 3, HRV 14, CA9, RSV, pFluA), the lesions of viral CPE (Adeno 5) or by the amount of viral proteins (ELISA: Adeno 5 and RSV). The calculation was based on mean values of two (CPE: Adeno 5; PFU: RSV, pFluA, repetition of FluA) or three (PFU: FLuA, Para 3, HRV 14, CA9; ELISA: Adeno 5, RSV) replicates derived from two independent experiments. Because ethanol control values did not differ significantly from those of water control, the values have been normalised to the mean values of the corresponding cell culture medium control. The concentration of oral drops has been calculated as the equivalent dry extract concentration based on the known drug/extract ratios. The results of the non-treated virus control groups were defined as 100% infection (0% inhibition) and *in vitro* effects of the substances standardised as relative inhibitory effects.

The calculation of $EC_{50}$, $R^2$ and significance values has been performed by nonlinear regression using GraphPad Prism's "log (agonist) vs. normalised response – variable slope" function with integrated comparison between calculated $EC_{50}$ values ("extra sum of squares *F*-test", GraphPad Prism Version 5.01 for Windows, GraphPad Software Inc.). In cases of maximal virus inhibition of less than 50% apparent $EC_{50}$ values are given as >100 μg/ml and >120 μg/ml, respectively.

## Results

*Cytotoxicity of different preparations of Sinupret®*

Cytotoxicity results of both Sinupret® preparations are presented in Table 1. The MTT-assay demonstrated a low toxicity of the plant extract. The 50% inhibitory concentration ($IC_{50}$) for the ethanolic plant composition (oral drops) calculated from several experiments was less than 930 μg/ml, i.e. 543 μg/ml for MDCK cells, 930 μg/ml for HEp-2 cells, 827 μg/ml for HeLa cells and 516 μg/ml for BGM cells. The $IC_{50}$ of the dry extract preparation was comparable to that of oral drops with toxicity limits for all cell lines being higher than 500 μg/ml. No further test substance-related metabolic impairment could be detected in both groups at a concentration of 240 μg/ml. Ethanol controls used in the assays for toxicity at a final concentration of 3.8% (V/V) showed slightly reduced metabolism. Dilutions of less than 1.9% (V/V) ethanol in the cell culture medium showed no cytotoxic effects. Consequently, the preparation of oral drops used for the evaluation of the antiviral activity was adjusted to an ethanol content of less than 0.38% (V/V) corresponding to the highest concentration of 120 μg/ml. Accordingly Sinupret dry extract was adjusted to a concentration of 100 μg/ml with a maximum ethanol content of 0.59% (V/V) as the highest concentration used in the antiviral tests with the exception of the additional tests on FluA and pFluA where 500 μg/ml with a maximum ethanol content of 0.25% was used as the highest concentration.

*Antiviral activity of different preparations of Sinupret®*

The data of the antiviral activity of the two preparations of Sinupret® are demonstrated in Figs. 1–3 and are summarised in

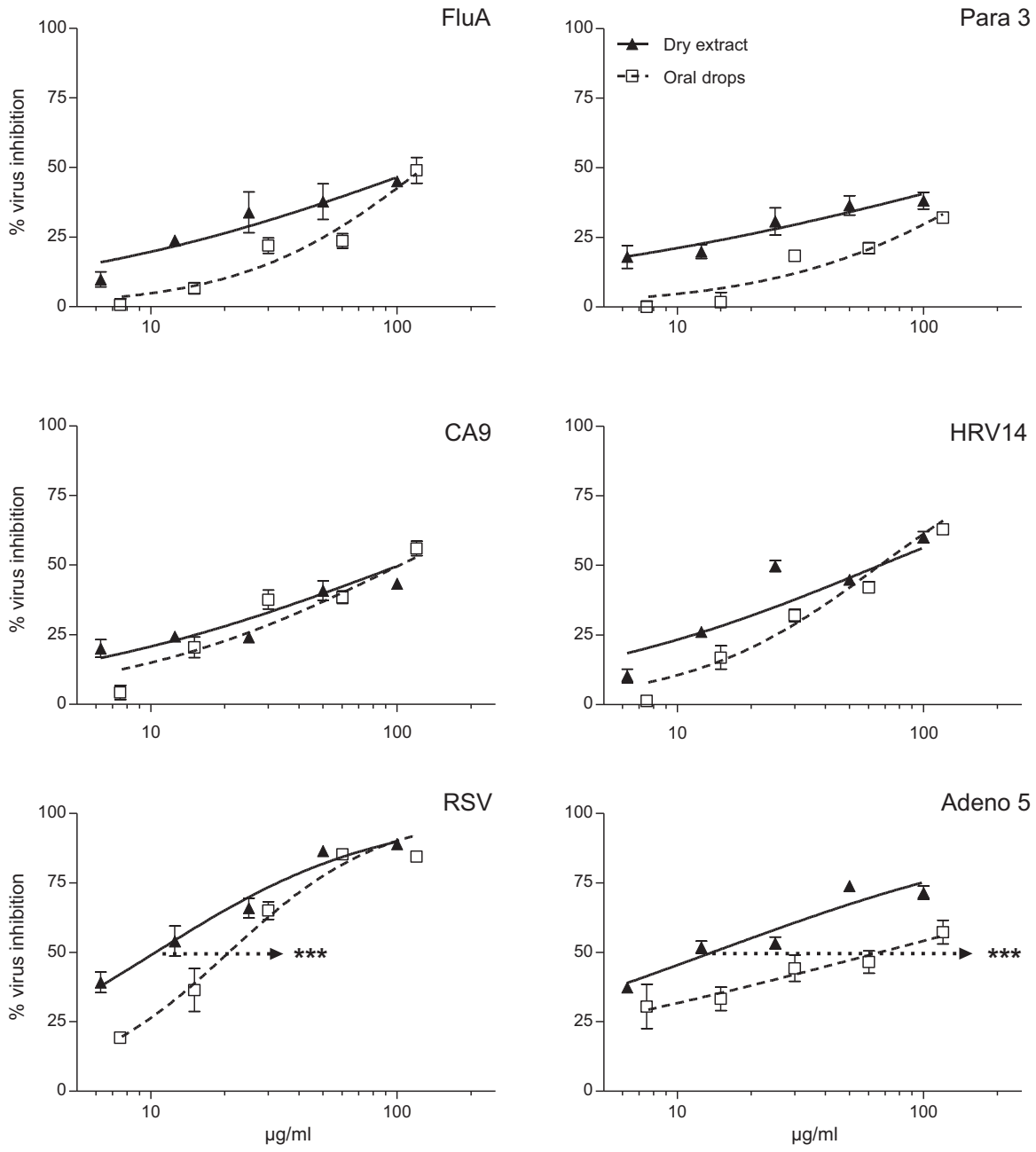


**Fig. 1.** Antiviral activity of two Sinupret® preparations against a broad panel of viruses. To test the efficacy of the two Sinupret® preparations – oral drops and dry extract – on virus replication, virus susceptible cells (MDCK, HEp-2, HeLa and BGM) were infected with a multiplicity of infection (M.O.I.) of 0.0004 (FluA, Para 3, RSV, HRV and CA9) or 0.008 (Adeno 5), without or in presence of five descending non-cytotoxic concentrations of the test substances oral drops (open squares) and dry extract (closed triangles). The antiviral activity (*y*-axis, % virus inhibition) of the test candidates (*x*-axis, concentration in µg/ml) was determined in plaque-reduction assays (PFU) for FluA, Para 3, RSV, HRV 14, and CA9 or in analyses of a cytopathogenic effect (CPE) for Adeno 5. The relative inhibitions (% inhibition, ordinate) were calculated by analysing the number of plaques or lesions of the viral CPE of the respective groups and standardised by the virus control representing 100% infectivity (0% inhibition). Positive controls confirmed the procedure (FluA, 5 µg/ml amantadine, 58% inhibition; Para 3, laboratory standard 10 µg/ml, 57% inhibition; HRV 14, laboratory standard 20 µg/ml, 54% inhibition; Adeno 5, laboratory standard 7.5 µg/ml, 57% inhibition; RSV, 6 µg/ml ribavirin, 60% inhibition). All data represent means and SEM from two independent experiments with two (Adeno 5, RSV) or three (FLuA, Para 3, HRV 14 and CA9) replicates. Stars indicate statistically significant differences for the $EC_{50}$ values between dry extract and oral drops (***$p < 0.001$).

Table 2, presenting the effective concentrations with 50% reduction of the virus replication ($EC_{50}$, µg/ml).

The treatment (addition of substance 1 h after virus infection) of cell cultures infected with enveloped RNA viruses – FluA and Para 3 – with the Sinupret® preparations showed clear concentration-dependent effects on the virus replication *in vitro* (Fig. 1).

Regarding the influence on FluA-specific virus plaques, oral drops (open squares) produced 49.0% reduction in the highest concentration of 120 µg/ml. Dry extract (closed triangles) showed comparable results (45.1% plaque reduction at 100 µg/ml). In both groups the antiviral effects were diminished within two dilution steps, demonstrating a concentration-dependent effect. Comparing the antiviral effects of dry extract against pFluA and FluA, the $EC_{50}$ were 43.4 µg/ml and 124.8 µg/ml, respectively (Fig. 3 and Table 2), whereas the $EC_{50}$ value of oral drops against FluA was >120 µg/ml (Fig. 1 and Table 2). The reference substance amantadine reduced FluA growth by 58% at a concentration of 5 µg/ml and pFluA growth by 65% at a concentration of 6 µg/ml.

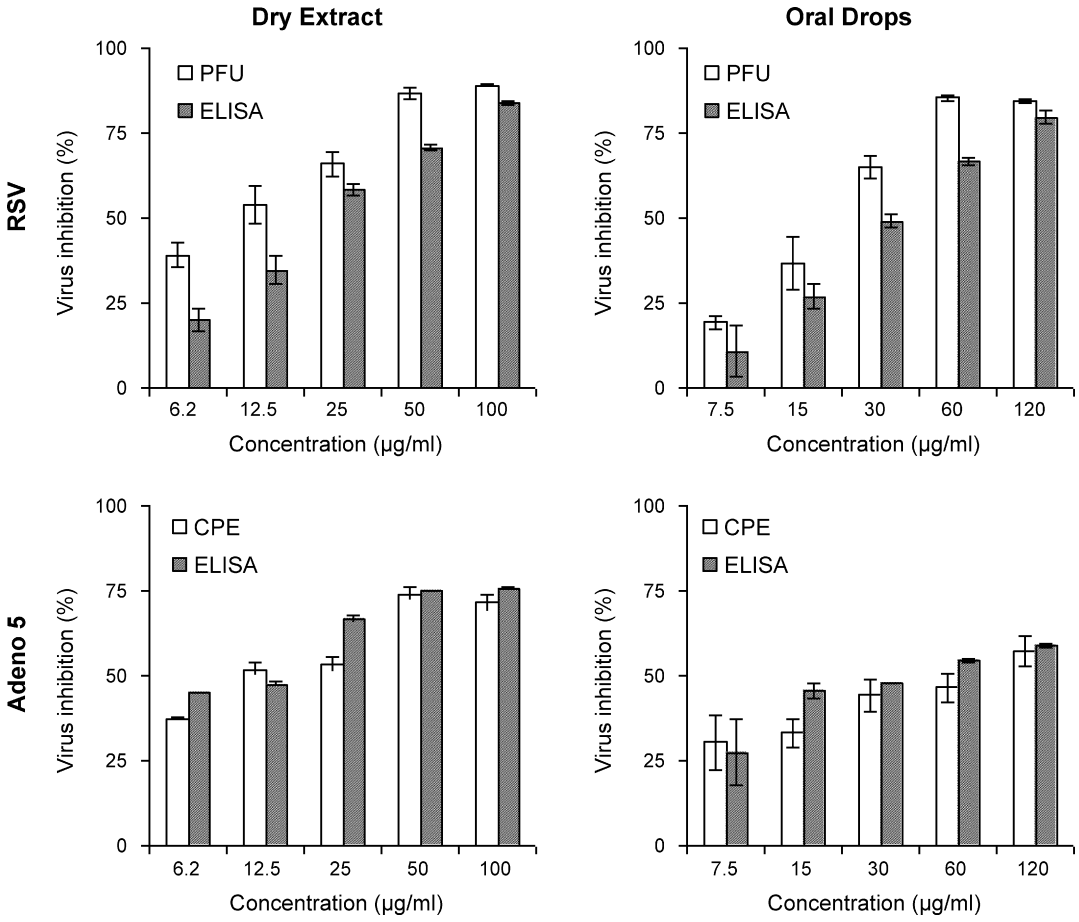


**Fig. 2.** Ascertainment of antiviral activity of two Sinupret® preparations with different assays. To test the efficacy of the two Sinupret® preparations – oral drops and dry extract – HEp-2 cells were infected with RSV (M.O.I. of 0.0004) or with Adeno 5 (M.O.I. of 0.008). After infection, cell monolayers were incubated without (medium-control) or in the presence of five descending non-cytotoxic concentrations (*x*-axis, µg/ml) of the test substances oral drops and dry extract. The antiviral activity of Sinupret® against the viruses was determined with plaque reduction assays in plaque forming units (PFU) for RSV or by analyses of a cytopathogenic effect (CPE) for Adeno 5 (open columns) and with the quantification of the amount of newly synthesised virus by enzyme immunoassays (ELISA; striped columns). The relative inhibition (*y*-axis, % inhibition) was calculated by analysing the number of plaques, the lesions of the viral CPE or the amount of the viral proteins of the respective groups and standardised by the virus control representing 100% infectivity (0% inhibition). The results of the antiviral activity against RSV are shown in the upper, against Adeno 5 in the lower panels. Positive controls confirmed the procedure (Adeno 5, laboratory standard 7.5 µg/ml, CPE 57% and ELISA 53% inhibition; RSV 6 µg/ml Ribavirin, PFU 60% and ELISA 57% inhibition). All data represent means and SEM from two independent experiments with two (PFU and CPE) to three (ELISA) replicates.

**Table 2**
Activity of Sinupret ® preparations (oral drops and dry extract) against DNA and RNA viruses.

| Virus | Virus assay | Cell culture | Oral drops | Dry extract | *p* |
|---|---|---|---|---|---|
| | | | $EC_{50}$ (µg/ml) | | |
| **RNA-virus (enveloped)** | | | | | |
| Influenza A, Chile 1/83 (H1N1) virus (FluA) | PFU | MDCK | >120 | 124.8[a] | n.c. |
| Porcine Influenza A/California/07/2009 (pFluA) | PFU | MDCK | n.t. | 43.4[a] | n.c. |
| Parainfluenza type 3 virus (Para 3) | PFU | MDCK | >120 | >100 | n.c. |
| Respiratory syncytial virus, strain long (RSV) | PFU | HEp-2 | 20.7 | 10.4 | <0.001 |
| | ELISA | | 34.0 | 21.0 | <0.001 |
| **RNA-virus (non-enveloped)** | | | | | |
| Rhinovirus B subtype 14 (HRV 14) | PFU | HeLa | 73.1 | 50.5 | >0.05 |
| Coxsackievirus subtype A9 (CA9) | PFU | BGM | 86.6 | >100 | n.c. |
| **DNA-virus (non-enveloped)** | | | | | |
| Adenovirus C subtype 5 (Adeno 5) | CPE | HEp-2 | 66.4 | 13.8 | <0.001 |
| | ELISA | | 40.6 | 10.0 | <0.001 |

$EC_{50}$, concentration that inhibits the viral acitivity by 50%; n.c., not calculated (Inhibition in the maximum concentration for at least one preparation lower than 50%); n.t., not tested; *p*, significance (dry extract vs. oral drops).
Relative to virus addition to cells different concentrations of Sinupret® were added 1 h after infection and left on throughout the incubation period. The antiviral activity was determined in plaque-reduction assays (PFU) for FluA, pFluA, Para 3, RSV, HRV 14 and CA9 or with the analyses of a cytopathogenic effect (CPE) for Adeno 5. In addition for RSV and Adeno 5 data were ascertained by ELISA. The relative inhibition by Sinupret® was standardised by the virus control representing 100% infectivity (0% inhibition). The table shows the concentration-dependent anti-viral effect of Sinupret® by using a therapeutic protocol calculated as a 50% effective concentration ($EC_{50}$). All data are based on means of two (Adeno 5 CPE, RSV PFU, pFluA, repetition of FluA) or three (FLuA, Para 3, HRV 14, CA9, Adeno 5 ELISA and RSV ELISA) replicates derived from two independent experiments.

[a] Additional test with higher concentrations of dry extract used.

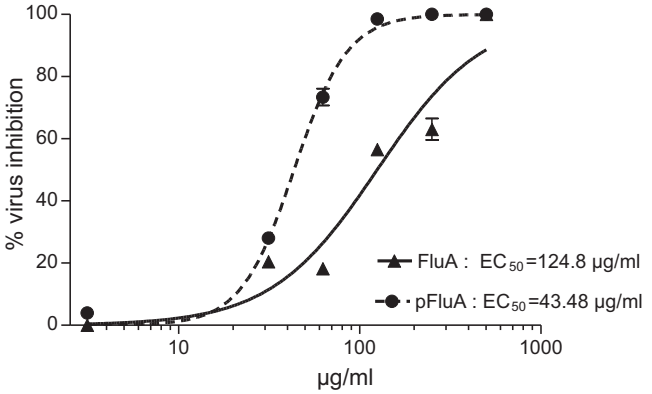


**Fig. 3.** Antiviral activity of a Sinupret® preparation against human and porcine influenza virus. To test the efficacy of one Sinupret® preparation – dry extract – on virus replication, virus susceptible cells (MDCK) were infected with a multiplicity of infection (M.O.I.) of 0.0004 (FluA) or 0.0008 (pFluA), without or in presence of six descending non-cytotoxic concentrations of the test substance dry extract. The antiviral activity (*y*-axis, % virus inhibition) of the test candidate (*x*-axis, concentration in µg/ml) was determined in plaque-reduction assays (PFU) for FluA (closed triangles) and pFluA (closed circles). The relative inhibitions (% inhibition, ordinate) were calculated by analysing the number of plaques of the respective groups and standardised by the virus control representing 100% infectivity (0% inhibition). Positive controls confirmed the procedure (FluA, amantadine 5 µg/ml, 58% inhibition; pFluA, amantadine 6 µg/ml, 65% inhibition). All data represent means and SEM from two independent experiments with two to three replicates.

The effects of the Sinupret® preparations on Para 3 infected cells (HEp-2) were less prominent. Both preparations showed only some concentration-dependent plaque reduction by 32.1% and 38.2% at their highest concentration (120 µg/ml, oral drops and 100 µg/ml, dry extract, respectively (Fig. 1)). Considering the concentration response curves, the efficacy of dry extract was slightly higher than that of oral drops. The laboratory internal reference compound inhibited Para 3 to 57% at a concentration of 10 µg/ml.

In two non-enveloped RNA viruses, CA9 and HRV 14, oral drops and dry extract produced significant viral plaque reduction:

In CA9 virus infected cells (BGM cell cultures) dry extract induced about 43% viral plaque reduction at 100 µg/ml whereas oral drops showed about 56% viral plaque reduction at 120 µg/ml. The effects of both Sinupret® preparations were concentration-dependent and showed comparable concentration response curves (Fig. 1). $EC_{50}$ values were >100 µg/ml for dry extract and 86.6 µg/ml for oral drops (Table 2).

Oral drops (120 µg/ml) induced 63.0% and dry extract (100 µg/ml) induced 60.1% concentration-dependent inhibition of HRV 14 plaques in HeLa cell cultures (Fig. 1). Dry extract ($EC_{50}$ 50.5 µg/ml) had the tendency ($p = 0.087$) to be more effective than Sinupret oral drops ($EC_{50}$ 73.1 µg/ml) (Fig. 1 and Table 2). The laboratory internal reference compound inhibited HRV 14 to 54% at a concentration of 20 µg/ml.

The strongest antiviral effects of the two Sinupret® preparations could be detected against the two other human pathogenic viruses RSV, an enveloped RNA-virus, and against Adeno 5, a non-enveloped DNA-virus (Figs. 1 and 2 and Table 2).

At the highest concentration of 120 µg/ml oral drops induced 84.5% reduction in RSV virus plaques. Dry extract showed comparable effects (89.1% plaque reduction at 100 µg/ml) against RSV (Fig. 1). These data could be confirmed by RSV-specific ELISA for the detection of newly synthesised viral proteins with 79.7% reduction for oral drops and 83.8% reduction for dry extract (Fig. 2). The suppressive activity of both Sinupret® preparations against RSV was concentration dependent. Again, the activity of dry extract was in all dilution steps higher ($p < 0.001$) compared to the activity of oral drops with an $EC_{50}$ value of 10.4 µg/ml for dry extract and 20.7 µg/ml for oral drops (Table 2). The reference substance ribavirin produced approximately 60% reduction of RSV plaques and 57% reduction of virus protein measured by ELISA at a concentration of 6 µg/ml.

Similar concentration-dependent antiviral effects were observed against the non-enveloped DNA virus Adeno 5 (Fig. 1). At the highest concentration both Sinupret® preparations reduced adenovirus infectivity measured in CPE assays by about 57.3% (oral drops, 120 µg/ml) and 71.4% (dry extract, 100 µg/ml). This antiviral effect could be verified in the virus-specific ELISA, where oral drops showed 58.8% and dry extract 75.4% reduction of viral protein production (Fig. 2). In the CPE assays as well as in ELISA, again, dry extract was more active compared to oral drops (Fig. 2). Higher activity of dry extract was also observed ($p < 0.001$) when comparing the $EC_{50}$ values of 13.8 µg/ml for the dry extract and 66.4 µg/ml for oral drops against Adeno 5 (Table 2). The laboratory internal reference compound produced approximately 57% reduction of the cytopathogenic effect (CPE) of Adeno 5 and 53% reduction of virus proteins measured by ELISA at a concentration of 7.5 µg/ml.

In summary, the present data demonstrate that both preparations of Sinupret® – oral drops and dry extract – inhibit a broad spectrum of viruses including enveloped as well as non-enveloped viruses of both DNA and RNA varieties *in vitro*. The inhibitory effect of dry extract is higher compared to that of oral drops.

## Discussion

The antiviral activity of two preparations of Sinupret® – oral drops and dry extract – against a wide panel of human pathogenic viruses causing infections of the upper respiratory tract could be demonstrated. Both preparations showed a very similar concentration-dependent activity with a higher potency of dry extract.

Interestingly, the treatment with the compositions did not affect all viruses equally, e.g. Para 3 and CA9 were only to a limited degree reduced by the highest concentrations of Sinupret® used in the assays. Stronger effects were visible against HRV 14, RSV and Adeno 5, with a unique antiviral effect against RSV. Relevant activity against FluA was only seen in higher concentrations of the dry extract whereas it showed a stronger effect against pFluA. This heterogeneous activity might indicate some specificity against distinct types of viruses but a clear border between e.g. activity against DNA and/or RNA viruses, enveloped and/or non enveloped viruses was not observed. RNA viruses with (RSV) or without envelope (HRV 14) were affected significantly whereas other enveloped RNA viruses (FluA and Para 3) as well as non-enveloped RNA viruses (CA9) were rather insensitive.

On the other hand, replication of the DNA virus Adeno 5 could be significantly suppressed by the test substances. This virus was the only DNA virus tested so far and this causes the question whether similar sensitivity could be also found in other DNA viruses e.g. viruses belonging to the herpesvirus family, such as Herpes Simplex Virus (HSV), Human Cytomegalovirus (HCMV), and Varicella Zoster virus (VSV).

It is obvious that besides the activity against a non-enveloped DNA virus there is no common feature of the less sensitive and high sensitive RNA viruses with or without envelope. Therefore, additional experiments might give further explanations. The non-selective antiviral activity of the Sinupret® preparations is in contrast to the antiviral activity of other plant-derived substances which is very often characterised by a clear border in the activity against DNA or RNA viruses. For *Acanthospermum hispidum* Summerfield et al. (1997) described a significant *in vitro* activity

against animal pathogenic DNA viruses, e.g. pseudorabies virus (PRV) and bovine herpesvirus I (BHV I) whereas RNA viruses, which are responsible for classical swine fever (CSF) and foot-and-mouth disease (FMD), were not impaired by this substance. On the other hand, Glatthaar-Saalmüller et al. described a clear activity of an extract of *Eleutherococcus senticosus* against RNA viruses (Glatthaar-Saalmüller et al. 2001).

The above few comprehensive studies using different viruses might give a hint to the mechanism of antiviral activity. For other plant-derived substances only data on single viruses were published, e.g. Schnitzler et al. reported on antiviral activity of *Melissa officinalis* against herpes viruses (Schnitzler et al. 2008), but nothing is known on its behaviour against other DNA as well as RNA viruses, with or without envelope.

In the present study it could be shown that Sinupret® produces clear antiviral effects when added to the tissue cultures directly after the infection. This represents a therapeutic treatment and it confirms early therapeutic effects. More detailed analyses including the time course should advance our understanding of the effect on the complex virus-cell interactions and on the viral replication cycles. Of particular interest could be the question: "Is it possible to inhibit intracellular virus amplification with the anti-viral active test substances?" or "Can we measure direct effects of Sinupret® on viral particles?"

Our present data can be interpreted as a hint to indirect antiviral mechanisms: an induction of type I interferons could be responsible for the observed antiviral activity (Dorr 1993). In this case the test substances would be capable of inducing the cellular interferon synthesis in cells used for the propagation of the viruses and leading to an inhibition of the virus replication. However, one might argue that sensitive and non-sensitive viruses had been grown on the same cell lines (e.g. RSV, Adeno 5 and Para 3 on HEp-2 cells). On the other hand, it is known that type I interferons show a different behaviour against different viruses.

There are lots of open questions and many approaches for a better understanding of Sinupret® preparations, the antiviral activity of the sinlge components and possible synergistic effects (Wagner and Ulrich-Merzenich 2009). Nevertheless, our data demonstrate an antiviral effect of two preparations of Sinupret – dry extract and oral drops – against a broad panel of viruses.

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