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### Antiviral Action of *Euphorbium compositum*<sup>®</sup> and Its Components

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## Key Words

Antiviral activity · *Euphorbium compositum*<sup>®</sup> SN · *Euphorbium resinifera* · *Pulsatilla pratensis* · *Luffa operculata* · Plant extract

## Summary

**Introduction:** *Euphorbium compositum*<sup>®</sup> SN (Biologische Heilmittel Heel GmbH, Baden-Baden, Germany, a homeopathic combination preparation available in form of drops, nasal spray, and injection solution), is prescribed for inflammation of the mucosae of the nose and sinuses. Infections in these areas are primarily of viral origin although bacterial superinfections are also common. **Objective:** The main question was whether or not this homeopathic remedy shows an activity against viruses responsible for infections of the respiratory tract. **Methods:** This in vitro study using virus plaque reduction assays examined the effect of *Euphorbium compositum* SN against pathogens causing various viral infections: influenza A virus, respiratory syncytial virus (RSV), human rhinovirus (HRV) and herpes simplex virus type 1 (HSV-1). **Results:** Analysis of virus production after treatment of the infected cells with the remedy showed an antiviral activity of *Euphorbium compositum* SN against RSV and HSV-1. In addition, an antiviral effect against influenza A virus and HRV, though minimal, was also noted. Analyses of the plant-derived components of *Euphorbium compositum* SN, e.g. *Euphorbium resinifera*, *Pulsatilla pratensis* and *Luffa operculata* for their antiviral activity revealed a clear activity of *Euphorbium resinifera* and *Pulsatilla pratensis* against RSV. In contrast, no effect was detected using the same protocol with *Luffa operculata*. **Conclusions:** *Euphorbium resinifera* and *Pulsatilla pratensis* as components of *Euphorbium compositum* SN are responsible for its antiviral activity.

## Schlüsselwörter

Antivirale Aktivität · *Euphorbium compositum*<sup>®</sup> SN · *Euphorbium resinifera* · *Pulsatilla pratensis* · *Luffa operculata* · Pflanzenextrakt

## Zusammenfassung

**Einleitung:** *Euphorbium compositum*<sup>®</sup> SN (Biologische Heilmittel Heel GmbH, Baden-Baden, Deutschland), das als Tropfen, Nasenspray oder Injektionslösung verabreicht wird, stellt derzeit ein Präparat auf dem homöopathischen Arzneimittelmarkt dar, welches in seiner Darreichungsform bei Entzündungen der Nasenschleimhaut und den Nebenhöhlen verwendet wird. Infektionen in diesen Bereichen sind primär viraler Natur und nehmen erst sekundär einen bakteriellen Verlauf. **Fragestellung:** Eine bedeutende Frage war nun, ob *Euphorbium compositum* SN eine Hemmung der Vermehrung der Viren, die für Infektionen des Respirationstrakts verantwortlich sind, bewirkt. **Methoden:** Der Nachweis einer antiviralen Aktivität von *Euphorbium compositum* SN wurde im Rahmen einer In-vitro-Untersuchung mit dem Plaque-Reduktions-Assay bei Erregern von Virusinfektionen, dem Influenza-A-Virus, dem Respiratory syncytial virus (RSV), dem humanen Rhinovirus (HRV) und dem Herpes-simplex-Virus Typ 1 (HSV-1) durchgeführt. Geprüft wurde in allen Tests die antivirale Wirkung von *Euphorbium compositum* SN nach erfolgter Virusinfektion. **Ergebnisse:** *Euphorbium compositum* SN zeigte eine deutliche antivirale Wirkung bei Infektionen mit RSV und HSV-1. Im Falle von HRV und Influenza-A-Virus konnte keine eindeutige antivirale Aktivität nachgewiesen werden. Bei der Untersuchung der Einzelkomponenten *Euphorbium resinifera*, *Pulsatilla pratensis* und *Luffa operculata* konnte eine deutliche antivirale Wirkung von *Euphorbium resinifera* und *Pulsatilla pratensis* gegen RSV gezeigt werden. *Luffa operculata* zeigte dagegen keine Aktivität gegen RSV. **Schlussfolgerung:** *Euphorbium resinifera* und *Pulsatilla pratensis* als Bestandteile des *Euphorbium compositum* SN sind für dessen antivirale Aktivität verantwortlich.

## Introduction

From the medical perspective, viral illnesses have increased in importance in the recent years. In fighting bacterial infections there is a broad palette of antibiotics to choose from, but only a few effective antiviral drugs are available. Most of the few antiviral medicaments are nucleoside analogue inhibitors (antimetabolites), e.g. aciclovir, which show selective activity against virus synthesis [8]. Ribavirin, which also serves as an analogue for nucleotides in viral replication, is highly effective against a large number of RNA and DNA viruses [11]. However, the numerous side effects of this substance restrict its use to severe indications [13]. Amantadine, a cyclic amine, has been widely used in prophylaxis and therapy of influenza virus infections due to its unique antiviral activity [9]. Recently, important progress in the therapy of influenza was made with zanamivir and oseltamivir, inhibitors of the viral neuraminidase [1].

These drugs are highly effective against viruses but may also cause severe adverse effects in humans [7, 10, 13]. Thus, the search for antiviral substances with high efficacy and minor side effects must continue.

Plants and plant-derived extracts might represent an approach to overcome this problem, and for several plant-derived substances an antiviral activity could be demonstrated [3]. *Euphorbium compositum*<sup>®</sup> SN, a complex remedy containing three plant-derived components – *Euphorbium resinifera*, *Pulsatilla pratensis* and *Luffa operculata* –, was used since 1984 in the treatment of inflammations of the nasal mucosa. In patients with rhinitides as well as chronic sinusitides, clinical studies showed a palliation of the symptoms without any side effects [4, 15–17].

The present study investigates the antiviral action of *Euphorbium compositum* SN and its plant-derived components *Euphorbium resinifera*, *Pulsatilla pratensis*, and *Luffa operculata* under in vitro conditions, with special emphasis on their effects against a panel of human pathogenic viruses. This study included both RNA and DNA viruses: influenza A virus (Orthomyxoviridae) and respiratory syncytial virus (RSV, Paramyxoviridae), two enveloped, single-stranded RNA viruses; human rhinovirus (HRV, Picornaviridae), a nonenveloped single-stranded RNA virus; herpes simplex type 1 virus (HSV-1) which belongs to the enveloped double-stranded DNA viruses (Herpesviridae).

## Material and Methods

*Euphorbium compositum* SN – consisting of 6 ingredients (table 1) – was used in physiological NaCl solution (batch 911050); *Euphorbium resinifera* (D3), *Pulsatilla pratensis* (D3), and *Luffa operculata* (D4) were used as dilutions of ethanol (30% (v/v)). All agents were supplied by Biologische Heilmittel Heel GmbH (Baden-Baden, Germany).

In the first screening, coded samples of the substances were used. Further investigations were done without blinding of the samples.

In all experiments the test substances were diluted in cell culture medium before adding to the cell cultures. In order, to exclude the possibility of

**Table 1.** Composition of *Euphorbium compositum* SN (batch 911050)

<i>Euphorbium resinifera</i> (D4)
<i>Pulsatilla pratensis</i> (D2)
<i>Luffa operculata</i> (D2)
Mercurius bijodatus (D8)
Hepar sulfuris (D10)
Argentum nitricum (D10)

ethanol-based toxic effects, dilutions of ethanol in medium served as controls.

### Reference Drugs

In order to verify our in vitro systems for testing the antiviral activity of the substances indicated above, aciclovir (Zovirax<sup>®</sup>; Glaxo Wellcome, Burgwedel, Germany), ribavirin (Virazole<sup>®</sup>; ICN Pharmaceuticals, Frankfurt/M., Germany), and amantadine (amantadine hydrochloride; Ratiopharm, Ulm, Germany) were used as references (positive controls).

Aciclovir was used as a positive control in infections with HSV-1 [8], ribavirin in infections with RSV [11], and amantadine in infections with influenza A virus [9]. The drugs were diluted according to their in vitro cytotoxicities and used in concentrations of 10 µg/ml for aciclovir, of 6.25 µg/ml for ribavirin, and of 5 µg/ml for amantadine in the respective in vitro tests.

### Cells and Viruses

All viruses used for the analyses were isolated from nasopharyngeal secretion or secretions from the upper respiratory tract. HRV-14 was obtained from the Institute for Virology of the Friedrich Schiller University, Jena, Germany. RSV (strain Long), influenza A/Chile 1/83 (H1N1) virus, and HSV-1 (strain Thea) were obtained from the Department of Medical Virology and Epidemiology of Virus Diseases of the Hygiene Institute of the University of Tübingen, Germany. All viruses were identified and characterized with a panel of monoclonal antibodies (BioWhittaker, Walkersville, MD, USA) [6]. RSV and HSV-1 strains were propagated on human epidermoid carcinoma cells (HEp-2), HRV 14 on HeLa cells in Hank's/Earle's minimal essential medium (MEM) containing 2% fetal calf serum (FCS), 25 mM MgCl<sub>2</sub>, 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Influenza A virus was grown on Madin-Darby canine kidney (MDCK) cells with serum-free MEM containing 1 µg/ml of trypsin, 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. In order to determine the virus titers, the respective cells were incubated in 12- or 24-well tissue culture dishes 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. Plaques were counted several days later, and the virus titers were calculated as plaque-forming units (PFU) per ml.

### Virus Assays

Hemagglutination assays, enzyme immunoassays, and plaque assays were carried out using standard procedures. Influenza A surface antigens were quantified by hemagglutination assays performed in microtiter plates using chicken erythrocytes diluted in PBS (0.5%). For the detection of RSV antigens, enzyme immunoassays (Virion, Würzburg, Germany) were used. Plaque assays for the detection of infectious particles were performed with MDCK, HEp-2 and HeLa cell cultures.

### Cytotoxicity Tests

Analyses of the in vitro cytotoxicity of the test substances were performed on physiologically active cells by an enzymatic assay (MTT assay) [14] which enabled the quantification of the activity of mitochondrial enzymes in active and dividing cells, showing a direct correlation between vitality and enzyme activity. Furthermore, the functional activity of cells and cell

**Table 2.** Determination of the cytotoxicity of the test substances

	Dilution of the test substances, 1/x					
	Euphorbium compositum SN			<i>Euphorbium resinifera</i> (D3)	<i>Pulsatilla pratensis</i> (D3)	<i>Luffa operculata</i> (D4)
	HeLa	HEp-2	MDCK	HEp-2	HEp-2	HEp-2
IC <sub>10</sub>	3,5	3	<2	35	33	15
IC <sub>50</sub>	<4	<4	<4	24	18	8
Lowest dilution used in the antiviral tests	8	8	8	40	40	20

The cytotoxicity of *Euphorbium compositum* SN and its plant-derived ingredients *Euphorbium resinifera*, *Pulsatilla pratensis* and *Luffa operculata* on cell cultures was measured 5 days after the addition of the substances. The viability of the cells (HeLa, HEp-2, MDCK) cultivated with different concentrations of test substances was quantified either using an MTT test or by determination of the ratio FDA- versus EtBr-positive cells. The relative cytotoxicity was standardized by the medium control representing 100% viability. The inhibitory concentrations determine the dilutions of the substances showing 10% (IC<sub>10</sub>) and 50% (IC<sub>50</sub>) viable cells after the cultivation period. All data represent 6 replicates derived from 2 experiments. Standard deviations were in all experiments less than 9%.

membranes was tested by fluorochrome labelling using fluorescein diacetate (FDA) and ethidium bromide (EtBr) [12]. Only active cells are able to incorporate FDA and to hydrolyze it into its fluorescent subunit. In contrast EtBr stains selectively cells with injured cell membrane. Intoxication of cell cultures leads to an increase of the FDA/EtBr ratio. Additionally, the cytotoxicity of the test substances on the respective cells was monitored by microscopic examination of the cell cultures for altered cell morphology.

MDCK, HEp-2 and HeLa cells were incubated after 3 days of precultivation for additional 5 days with 6 descending concentrations of the test substances (6 replicates/dilution). The controls used in the assays were the respective cell culture media without any component (medium control) and dilutions of ethanol to the cell culture medium corresponding to the ethanol concentration in the assays for antiviral activity (ethanol controls). The quantification of the cytotoxicity on treated cell cultures was performed with the assays mentioned above.

#### Antiviral Activity

Analyses for antiviral activity against influenza A virus, RSV, HRV and HSV-1 were performed using plaque reduction assays [5]. With regard to the determination of the antiviral activity against HRV, RSV, influenza A virus and HSV-1, confluent monolayers of MDCK, HEp-2 or HeLa cells, cultivated in 6- or 12-well plates (MEM containing 10% FCS) were infected with a defined multiplicity of infection (M.O.I.). The titer of the respective virus solutions calculated in M.O.I. had been determined in previous experiments. Cells were infected at 34 °C for 1 h. Then the cell monolayers were washed and overlaid with a semisolid medium containing the different concentrations of the respective test substances. Cell cultures were then cultivated until lesions were visible in the cell monolayer (plaques) of the control group cultivated in medium alone. After removal of the overlays, cells were fixed with formalin (5%) and labelled with Giemsa solution. Then the plaques, which were visible as transparent dots, could be counted.

#### Quantification of the Antiviral Activity in vitro

The quantification of the antiviral activity of the test substances in influenza A virus-, RSV-, HRV- and HSV-1-infected cell cultures is based on the number of plaques of 6 replicates from 2 experiments. The number of plaques of the nontreated virus controls was estimated as 100% infection, and the percentages of infection of the cultures treated were calculated from the reduction in the number of the respective plaques [2].

## Results

### Cytotoxic Effects

Our objective was to ascertain the in vitro cytotoxicity of *Euphorbium compositum* SN and its three plant-derived components *Euphorbium resinifera*, *Pulsatilla pratensis*, and *Luffa operculata* to select the highest possible concentration that could be used in antiviral tests to determine dosage-dependent antiviral effects.

Analyses of the cytotoxicity of these substances were performed with an enzymatic assay (MTT assay) [14] and FDA exclusion or EtBr incorporation, measuring the mitochondrial activity and the number of physiologically active cell membranes of the specific cell lines, respectively [12]. The results of the analyses shown in table 2 demonstrated that these substances in a dilution of 1:8 for *Euphorbium compositum* SN, of 1:40 for *Pulsatilla pratensis* (D3) and *Euphorbium resinifera* (D3), and of 1:20 for *Luffa operculata* (D4) did not affect the viability of the cell lines. The IC<sub>50</sub> calculated from several experiments were for *Euphorbium compositum* SN on HeLa, HEp-2 and MDCK cells a dilution of less than 1:4, for *Euphorbium resinifera* a dilution of 1:24, for *Pulsatilla pratensis* of 1:18, and for *Luffa operculata* of 1:8. In order to exclude the possibility of any concentration-dependent mistakes, dilutions of 1:8 (*Euphorbium compositum* SN), of 1:40 (*Euphorbium resinifera* (D3) and *Pulsatilla pratensis* (D3)) and of 1:20 (*Luffa operculata* [D4]) were used as highest concentrations in assays for antiviral activity.

### Determination of the Antiviral Activity

In order to test the effects of the substances on the replication of the viruses, parallel groups of virus-specific cells (MDCK, HEp-2, HeLa) were infected with a M.O.I. of 0.0001 PFU/cell

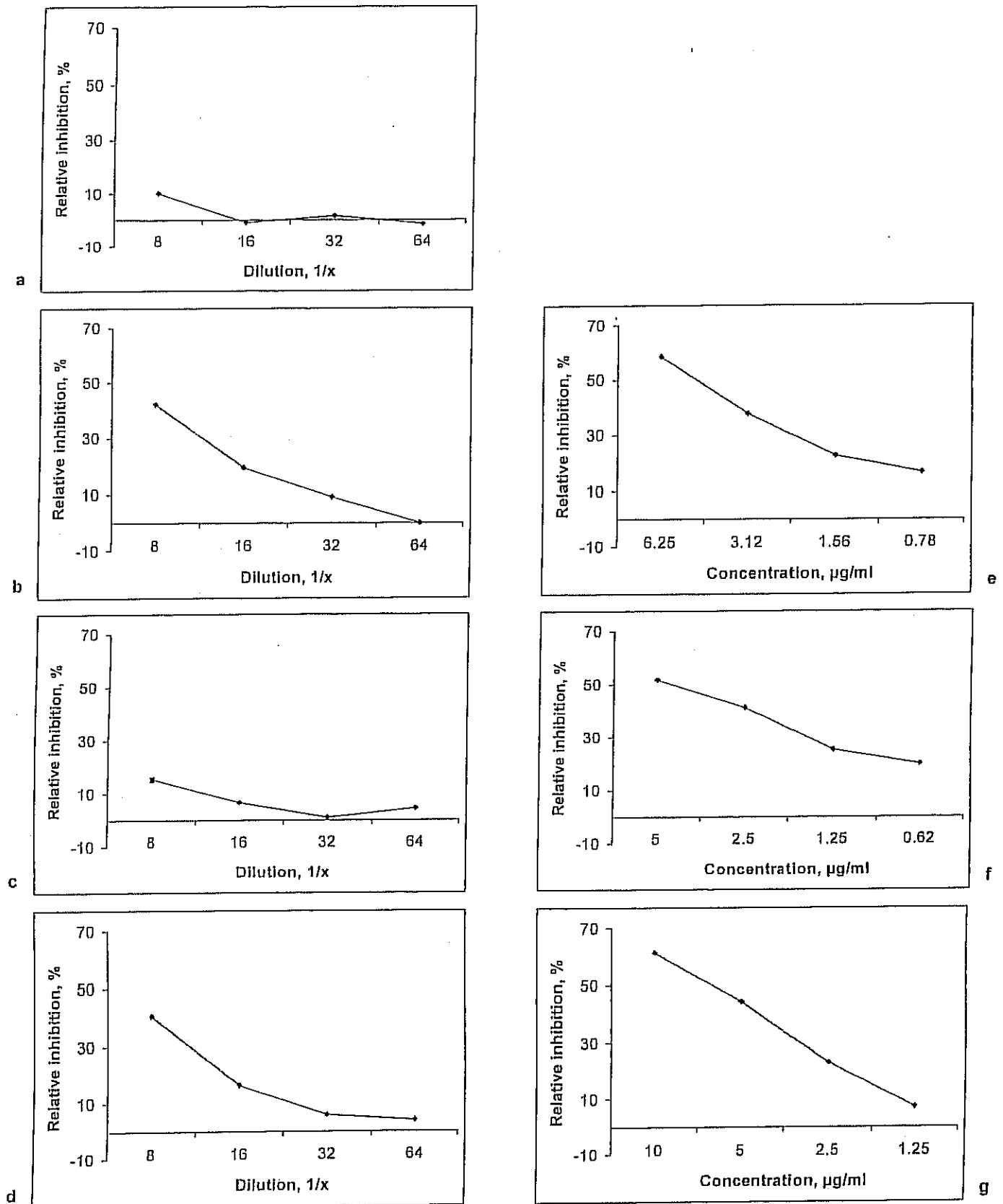


Fig. 1. Activity of *Euphorbium compositum* SN against a HRV-14, b RSV, c influenza A virus, and d HSV-1. MDCK cells were infected with influenza A virus (M.O.I. 0.0001); HEp-2 cells with RSV or HSV-1 (M.O.I. 0.0001) and HeLa cells with HRV-14 (M.O.I. 0.0001). After infection, cell monolayers were incubated without (controls) or in the presence of different concentrations of the test substance (dilution (1/x)).

The antiviral activities of the synthetic drugs e ribavirin against RSV, f amantadine hydrochloride against influenza A virus and aciclovir against HSV-1 were confirmed. The antiviral activity was determined in plaque reduction assays. All single points represent data from 6 replicates derived from 2 experiments. Standard deviations were about 10%.

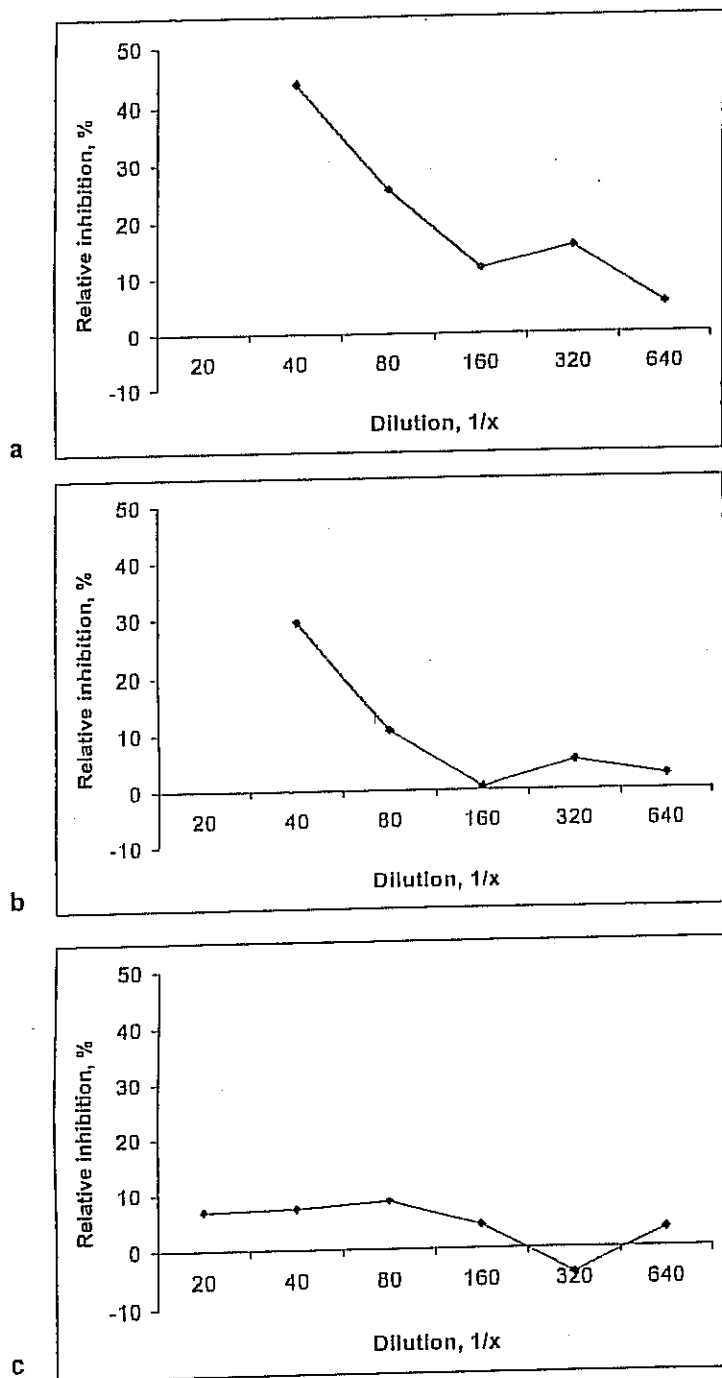


Fig. 2. Activity of a *Euphorbium resinifera*, b *Pulsatilla pratensis* and c *Luffa operculata* against RSV. HEP-2 cells were infected with RSV (M.O.I. 0.0001). After infection, cell monolayers were incubated without (controls) or in the presence of different concentrations of the test substances (dilution (1/x)). The antiviral activity of the test substances against RSV was determined in plaque reduction assays. The points in the graphs represent data of 6 replicates derived from 2 experiments. Standard deviations were about 13%.

(influenza A virus, RSV, HSV-1, HRV) without (controls) or in the presence of different concentrations of the test substances. The antiviral activities of the substances against influenza A virus, RSV, HRV, and HSV-1 were determined in plaque reduction assays. Thus, cell cultures were cultivated for 5 days

(MDCK; influenza A virus), 8 days (HEp-2; RSV), 3 days (HeLa; HRV) or 7 days (HEp-2; HSV-1) until lesions (virus plaques) were visible in the controls.

#### Antiviral Activity of *Euphorbium compositum* SN against Influenza A Virus, RSV, HRV, and HSV-1

Antiviral effects of *Euphorbium compositum* SN could be detected against RSV- (fig. 1b) and HSV-1-infected cell cultures (fig. 1d). At a dilution of 1:8 a clear reduction of virus plaques could be shown (RSV 42%; HSV-1 40%). The antiviral activity of ribavirin (fig. 1e) and aciclovir (fig. 1g) enables a partial quantification of the antiviral effect of *Euphorbium compositum* SN. In a dilution of 1:8 it showed similar effects against RSV as about 3.12 µg/ml ribavirin and against HSV-1 as about 4.5 µg/ml aciclovir, resulting in a reduction of the viral activity by about 40%. In comparison to the controls, *Euphorbium compositum* SN did not show any significant activities against influenza A virus (fig. 1c) and HRV (fig. 1a) although minimal effects could be surmised at the lowest dilution of 1:8 (relative inhibition of influenza A virus = 17% and of HRV = 6%). The efficacy of the synthetic molecule amantadine hydrochloride against influenza A virus was confirmed in the in vitro test system (fig. 1f).

#### Antiviral Activity of *Euphorbium resinifera*, *Pulsatilla pratensis* and *Luffa operculata* against RSV

On the basis of the results presented in figure 1, the antiviral activities against RSV of three plant-derived components of *Euphorbium compositum* SN (*Euphorbium resinifera*, *Pulsatilla pratensis* and *Luffa operculata*) were further studied. With a 1:40 dilution of *Euphorbium resinifera*, a 44% reduction of the RSV-caused virus plaques could be achieved, the next higher dilution of 1:80 induced a 25% reduction of these plaques (fig. 2a). Also *Pulsatilla pratensis* showed an inhibitory effect on RSV (fig. 2b). Using a 1:40 dilution, a reduction of the virus plaques of about 29% was achieved, but this inhibitory activity decreased very rapidly with diminishing concentration (fig. 2b). No RSV-specific antiviral activity could be detected for *Luffa operculata* in all dilutions used in the experiments (fig. 2c).

#### Discussion

In the study presented here we demonstrate the antiviral activity of a plant-derived remedy used in homeopathic medicine. These data confirm results derived from clinical trials which were performed earlier [4, 15, 16, 17]. These studies produced evidence of efficacy of *Euphorbium compositum* SN against inflammations of the nasal mucosa. Furthermore, this remedy could be shown to be well-tolerated. Here, we were now able to support the in vivo data derived from these trials by demonstrating the antiviral activity of *Euphorbium compositum* SN in vitro.

Our experiments showed an antiviral effect against RSV and HSV-1 when *Euphorbium compositum* SN was added to tissue cultures after the infection with the viruses had taken place. In contrast, using the same protocol, only a minimal effect against influenza A virus and HRV could be detected. Because *Euphorbium compositum* SN represents a mixture of several agents and plant-derived substances (table 1), it was interesting to analyze also the antiviral capacity of the single components and furthermore to clarify which of the component(s) is (are) responsible for the antiviral activity of this remedy. Based on screening experiments of plant-derived substances [3], it was obvious to analyze in a first step the plant-derived components of *Euphorbium compositum* SN (*Euphorbium resinifera*, *Pulsatilla pratensis*, *Luffa operculata*) for their antiviral activities against RSV. A clear effectiveness of *Euphorbium resinifera* and *Pulsatilla pratensis* against RSV could be demonstrated, showing that the antiviral activity of these plant-derived substances was comparable to the activity of the complex remedy *Euphorbium compositum* SN. In contrast, no RSV-specific antiviral activity could be detected for *Luffa operculata*. In conclusion, it could be shown that the components *Euphor-*

*bium resinifera* and *Pulsatilla pratensis* are responsible for the antiviral activity of *Euphorbium compositum* SN. It has to be mentioned that the activity of this homeopathic remedy is not as strong as that of the synthetic drugs aciclovir, ribavirin, and amantadine hydrochloride. However, *Euphorbium compositum* SN showed clear advantages compared with the synthetic drugs with regard to its low toxicity and its effects in vivo.

Further studies are needed to clarify if other viruses responsible for infections of the respiratory tract are affected by this remedy, too. Moreover, the action of *Euphorbium compositum* SN and its components on the molecular level as well as chemical aspects of the plant extract components should be identified in the future.

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