In vitro antiviral activity of Echinaforce®, an *Echinacea purpurea* preparation, against common cold coronavirus 229E and highly pathogenic MERS-CoV and SARS-CoV

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Abstract:

Coronaviruses (CoVs) were long thought to only cause mild respiratory and gastrointestinal symptoms in humans but recent outbreaks of Middle Eastern Respiratory Syndrome (MERS)-CoV, Severe Acute Respiratory Syndrome (SARS)-CoV, and the newly identified SARS-CoV-2 have cemented their zoonotic potential and their capacity to cause serious morbidity and mortality, with case fatality rates ranging from 2 to 35%. Currently, no specific prophylaxis or treatment is available for CoV infections and therefore we investigated the antiviral potential of *Echinacea purpurea* (Echinaforce®) against human coronavirus (HCoV) 229E and the highly pathogenic MERS- and SARS-CoVs in vitro. We found that HCoV-229E was irreversibly inactivated when exposed to Echinaforce at 3.2µg/ml IC_{50}. Pre-treatment of cell lines, however, did not inhibit infection with HCoV-229E and post-infection treatment had only a marginal effect on virus propagation at 50 µg/ml. However, we did observe a protective effect in an organotypic respiratory cell culture system by exposing pre-treated respiratory epithelium to droplets of HCoV-229E, imitating a natural infection. Finally, antiviral activity was not restricted to common cold coronaviruses, as the highly pathogenic SARS- and MERS-CoVs were inactivated at comparable concentrations. These results suggest that *Echinacea purpurea* preparations, such as Echinaforce, could be effective as prophylactic treatment for all CoVs, including newly occurring strains, such as SARS-CoV-2.

1. Introduction

Coronaviruses (CoVs) are believed to be responsible for 10-15% of all upper respiratory tract infections in humans and were mainly thought to be responsible for the common cold until 2002 (1). Currently, seven CoVs have been found to cause disease in humans. Four of those, HCoV-229E, HCoV-OC43, HCoV-NL63 and HCoV-HKU1, are non-zoonotic and cause worldwide outbreaks predominantly in the winter period. These HCoVs replicate in the nasopharynx and generally cause mild, self-limited upper respiratory tract infections with short incubation periods, although lower tract respiratory infections and pneumonia have occasionally been described (2-5). The more virulent coronaviruses, Middle East respiratory syndrome (MERS)-CoV and Severe Acute Respiratory Syndrome (SARS)-CoV have animal reservoirs with proposed origins in bats (6) and can cause severe pneumonias with longer incubation periods and often fatal outcome (7). SARS-CoV was introduced into the human species in 2002 causing a worldwide epidemic in 2003 culminating in 8422 infections and 916 deaths (8). MERS-CoV is heavily endemic in dromedary camels and leads to lower respiratory tract infections in humans with a current case-fatality rate of 35.5% (9). As of December 31st 2019, a pneumonia outbreak originating from a live seafood market in Wuhan, China, has resulted in an increasing number of fatal severe respiratory tract infections and, so far unprecedented, travel bans (10). To date, there is a lack of established and clinically tested antiviral compounds against coronaviruses in general and, more distressingly, the zoonotic betacoronaviruses (11). Given their increasing incidence and burden, an inexpensive, accessible and effective treatment for HCoVs is of utmost importance.

Echinacea plants have traditionally been used in North America for the prevention and treatment of cold and flu symptoms and are now one of the most widely used medical plants in both North America and Europe (12). Several different products are on the market, not only varying in the *Echinacea* species and the parts of the plant used but also in manufacturing procedures, which, unfortunately, results in a large variability in quality and activity (13, 14). Echinaforce® is a standardized preparation extracted from herb and roots of freshly harvested *Echinacae purpurea* plants with a 65% alcoholic solution.

Echinaforce as prevention and treatment of respiratory tract infections has been investigated in both pre-clinical and clinical studies and its beneficial effects documented (15-18). Specific mechanism of

action is not fully understood but in vitro studies indicate that Echinaforce inhibits membranous respiratory viruses including influenza A and B, respiratory syncytial virus (RSV) or parainfluenza virus, through direct interaction with virus particles and viral envelope proteins (19, 20). Intracellular activity of *Echinacea* has been observed for some viruses (e.g. influenza and herpes simplex virus) but not others (e.g. RSV), and only at higher concentrations required for extracellular inactivation. Furthermore, *Echinacea* has been shown to interfere with virus mediated cytokine release (21, 22) and since typical symptoms of the common cold, sneezing, coughing and runny nose, are the results of the stimulation of pro-inflammatory cytokines, the reduction of cytokine release might help to ease symptoms. In a randomized, double-blind, multi-center, non-inferiority clinical trial Echinaforce was demonstrated to be non-inferior to Oseltamivir in patients with influenza-like illness and confirmed influenza infection with a trend for lower incidence of complications with Echinaforce Hot Drink as with Oseltamivir (16).

The antiviral activity of *Echinacea* has been investigated in vitro for most of the respiratory viruses associated with common colds and flu, but as of yet, not for coronaviruses. HCoV-229E is a typical representative of a coronavirus strain causing a seasonal common cold. By using HCoV-229E as a model, we investigated the anti-coronaviral activity of Echinaforce extract, thereby closing the knowledge gap on the antiviral effects of *Echinacea purpurea* on typical common cold viruses. Furthermore, we expanded our analysis to the highly pathogenic SARS- and MERS-CoVs and other viruses that cause disease in humans. Additionally, we utilized an organotypic respiratory cell culture system (MucilAir™) of nasal origin to investigate the protective effect of Echinaforce against coronaviruses in a culture system that closely mimics in vivo human airway epithelium. In the current study, we observed an irreversible inhibition of the infectivity of three coronavirus strains upon direct contact with Echinaforce. Furthermore, a protective effect was observed upon pre-treatment in an organotypic airway model.

2. Material and Methods

2.1 Echinacea preparation

Echinaforce® (A.Vogel AG, Roggwil, Switzerland) is derived from hydroethanolic extraction (65% v/v ethanol) of freshly harvested *Echinacea purpurea* herb and roots (95:5). The composition of typical marker compounds such as caftaric acid, chlorogenic acid, echinacoside and alkylamide derivates has been previously described elsewhere (20). The final concentration of ethanol in the extract was 65% v/v with 16 mg/ml dry mass *Echinacea*. Experimental concentrations are expressed as dry mass of Echinaforce extract.

2.2. Cell lines and viruses

Table 1: Overview of cell lines used in the current study.

Name	Animal	Tissue	Medium*	Procured from
Huh-7	Human	Liver	DMEM+10%FBS, 2mM Glutamine, non-essential amino acids, Pen/strep, HEPES (Biochrom, Germany)	Prof. Volker Thiel, University of Bern, Switzerland
Vero (CRL 81 TM)	African Green Monkey	Kidney	MEM+10%FBS, 2mM Glutamine, non-essential amino acids, Pen/strep, HEPES (Biochrom, Germany)	ATCC (Manassas, VA, 20110 USA)
A9 (85011426)	Mouse	Areolar adipose tissue	DMEM+10%FBS, 2mM Glutamine, non-essential amino acids, Pen/strep, HEPES (Biochrom, Germany)	ECACC (Public Health England, Salisbury, UK)

*Dulbecco's Modified Eagle Medium (DMEM), Minimum Essential Medium (MEM), Fetal Bovine Serum (FBS), Penicillin/Streptomycin (Pen/Strep, 100U/mL).

All cells were cultured at 37°C without CO_2.

Table 2: Overview of viruses used in the current study.

Name	Strain	Propagated in	Medium*	Procured from
HCoV	229E	Huh-7, 33°C	DMEM+5%FBS, 2mM Glutamine, non-essential amino acids,	Prof. Volker Thiel, University of Bern,

			Pen/strep, HEPES (Biochrom, Germany)	Switzerland (23, 24)
MERS-CoV	EMC	Vero, 37°C	DMEM+2%FBS, 2mM Glutamine, non-essential amino acids, Pen/strep, HEPES (Biochrom, Germany)	
SARS-CoV	Frankfurt-1			
Mouse parvovirus	MVM Prototype, ATCC-1346	A9, 37°C		The National Collection of Pathogenic Viruses, UK
Yellow Fever virus	17D, NCPV-0507	Vero, 37°C		
Vaccinia virus	Elstree (Lister Vaccine), ATCC-VR-1549			

All viruses were cultured without CO_2 in non-vented flasks, 24 well-, or 96 well-plates covered with sealing foil (Biorad, microseal B-film, MSB 1001) for the duration of experiments.

2.3 In vitro reconstituted human airway epithelia (MucilAir™)

Reconstituted human airway epithelia (MucilAir™) from nasal epithelial cells were purchased from Epithelix Sàrl, Geneva, Switzerland. Cells from three different healthy donors were used in all experiments to account for donor variability and experiments were conducted four times, in duplicates. During maintenance, basal culture medium (MucilAir™, 500 µl/24-well) was exchanged every 2-3 days while the apical side was washed gently (2-4 times) with 200 µl of media to remove residual mucus.

2.4 Cell toxicity

Cell toxicity was determined by exposing 80% confluent Huh-7 and Veroells to serial dilutions of Echinaforce and measuring cell viability by MTT assay (Vybrant® MTT Cell Proliferation Assay Kit, ThermoFisher, Rheinach, Switzerland) according to the manufacturer's protocol. Briefly, Echinaforce was diluted in corresponding cell culture medium to 100, 50, 20, 10, 1 and 0 µg/ml and added to 80% confluent Huh-7, Vero or Vero E6 cells in 96 well plates (200 µl/well). Cells were covered with sealing foil and incubated at 33°C for 5 days (Huh-7) or 7 days (Vero and Vero E6). For analysis, medium was

exchanged with fresh cell culture medium (200 µl/well), 10 µl of MTT stock solution added per well and cells incubated for 4 h at 37°C. Following the incubation, 100 µl SDS-HCL solution was added per well and incubated for 18 h at 37°C. Absorbance was read in a photometer (SpectraMax Plus, Bucher Biotec, Basel, Switzerland) at 570 nm.

2.5 Antiviral activity against HCoV-229E in cell cultures

2.5.1 Pre-treatment of virus particles

$4x10^4$ $TCID_{50}$/ml HCoV-229E were incubated with Echinaforce diluted to 0, 2, 5, 10, 20, 40, 500 and 100 µg/ml in 2%-FBS DMEM and incubated for 1 hour at room temperature (RT) on a rocking platform. To estimate residual infectivity, treated virus dilutions were washed four times with 15-17 ml wash buffer (1:100 PBS, pH 7.4, in dH_2O, Biochrom, Germany) and filtered through Vivaspin® 20 Ultrafiltration Units (Sartorius AG, Goettingen Germany) at 800 g for 15 min. Viruses were recovered from the Ultrafiltration Unit with glycine buffer (3750 mg/l glycine, 10 g/l beef extract, 14.6 g/l NaCl, pH 9.5, Sigma-Aldrich, Germany), and diluted in 1:10 in 5%-FBS DMEM. Residual virus infectivity was determined by a limiting dilution assay ($TCID_{50}$) according to Spearman-Karber (25).

2.5.2 Pre-treatment of cells

Huh-7 cells were incubated with 0, 1, 10 or 50 µg/ml *Echinaforce* in cell culture medium for 3 days at 33°C. Thereafter, Echinaforce-containing medium was removed and cells infected with 100 $TCID_{50}$ HCoV-229E for 1h at 33°C. Medium was replaced and cells further incubated for 48 h at 33°C and virus titer determined by limiting dilution assay.

2.5.3 Post-infection-treatment of cells

Huh-7 cells were infected with 100 $TCID_{50}$ HCoV-229E for 1 h at 33°C and after washing the cells twice with complete culture medium; medium containing 0, 1, 10 or 50 µg/ml Echinaforce was added. Cells were incubated at 33°C for 72 h and virus titer determined at 24 and 72 hours post infection by limiting dilution assay.

2.6 Antiviral activity against HCoV-229E on re-differentiated respiratory epithelium

Prior to treatment, the mucus layer was removed from the apical surface of MucilAir™ respiratory cultures (Epithelix Sàrl, Geneva, Switzerland) by washing it three times with 200 µl Hank's Balanced Salt Solution (HBSS, Cat N° 14175095, Thermo Fisher Scientific, Rheinach, Switzerland). Thereafter, the epithelium was pre-treated by incubating the inserts with 100µl MucilAir™ culture medium containing 1, 10 or 50 µg/ml *Echinaforce* for 1 h at 33°C before re-establishing air-liquid interface. The following day, 50µl HBSS buffer containing 1, 10 or 50 µg/ml Echinaforce was added to the apical surface, followed by another 50 µl of HBSS containing 100 $TCID_{50}$ HCoV-229E, added dropwise, and incubating for 1 h at 33°C. Subsequently, air-liquid interface was re-established and cultures further incubated at 33°C. Progeny virus was collected from the apical side by washing inserts with 200 µl HBSS on 24.48.and 72 hours post infection. Virus titers were determined by limiting dilution assay.

2.7 Antiviral activity against MERS-CoV, SARS-CoV, YFV, VACV and MVM

To evaluate the antiviral activity of Echinaforce against MERS-CoV and SARS-CoV, yellow fever virus (YFV), vaccinia virus (VACV) and mouse parvovirus (minute virus of mice, MVM), we incubated MERS-CoV ($5x10^4$ pfu/ml), SARS-CoV ($2x10^5$ pfu/ml), YFV ($4x10^5$ pfu/TCID50) and VACV ($8x10^4$ pfu) and MVM ($8x10^4$ TCID50) with of 0, 1, 10 and 50 µg/ml Echinaforce in cell culture media for 60 minutes at RT on a rocking platform. Residual infectivity was determined by standard plaque assay on Vero cells (MERS-CoV, SARS-CoV, YFV and VACV) or in a limiting dilution assay on A9 cells (MVM) as described below.

2.8 Virus quantification

Tissue culture infectious dose ($TCID_{50}$) for HCoV-229E and MVM was determined by limiting dilution assay. Briefly, the samples of interest were serially diluted 1:10 in 2%-FBS MEM. From each dilution 100 µl were applied to 10 separate wells of a 96-well plate containing 80% confluent Huh-7 cells (HCoV-229E) or A9 cells (MVM). After 7 days of incubation at 33°C (HCoV-229E) or 13 days at 37°C (MVM) plates were stained with crystal violet (1% aqueous solution, Merck, Zug, Switzerland) and $TCID_{50}$ calculated using the Spearman-Karber Method (25). Plaque forming units (pfu) for MERS-CoV

and SARS-CoV, YFV, VACV were determined by standard plaque assay. Serially diluted samples (100 µl/well) were titrated on Vero cells in 24-well plates, overlaid with 2% FBS MEM medium containing 1.2% methylcellulose (90HG 4000cP, Sigma Aldrich, Switzerland) and incubated at 37°C until plaques were clearly visible. For visualization, plates were stained with Crystal Violet for 15 minutes and washed with PBS.

3. Results

3.1 Echinaforce reduces the infectivity of HCoV-229E in a dose dependent manner

To assess the direct antiviral activity of Echinaforce against human coronaviruses, $4x10^4$ $TCID_{50}$/ml HCoV-229E was exposed to increasing concentrations of extract and the effect on virus infectivity determined by quantifying infectious virus particles by a limiting dilution assay. Exposure to Echinaforce for 60 minutes led to a dose dependent reduction of infectious HCoV-229E virus particles (Figure 1). Complete inhibition of replicating virus was observed at 50-100 µg/ml extract, with mean inhibitory concentration (IC_{50}) 3.2 ug/ml. Parallel incubation of cells with Echinaforce showed stable cell viability at all tested concentrations (Figure 1).

3.2 Echinaforce affects infectivity through stable interactions with HCoV-229E virus particles

Since little is known about the mode of action of *Echinace*a extracts we aimed to determine whether Echinaforce exerts its antiviral activity exclusively through direct interaction with virus particles or also intracellularly during virus replication. To this end, Echinaforce was introduced at different stages of HCoV-229E infection. First, HCoV-229E virus particles were pre-treated prior to infection. Second, cells were pre-treated for 3 days prior to infection. Third, Echinaforce was added to cells one hour post-infection (hpi). Results show, that upon contact with the extract, a permanent alteration of virus infectivity occurred, as the inhibitory effect could not be reversed through extensive washing of treated virus particles (Figure 2a). In contrast, pre-treatment of cells had no influence on HCoV-229E infectivity or replication (Figure 2b). In cells treated post-infection, a small reduction in virus titer was observed after treatment with the highest dose of 50 µg/ml (Figure 2c).

3.3 Echinaforce inhibits HCoV-229E infection of respiratory epithelial cells

To evaluate how Echinaforce may exert its antiviral activity in a more natural setting, we utilized a re-differentiated, pseudostratified respiratory epithelial cell culture model. The reconstituted epithelium is functional, produces mucus and exhibits active ciliary-beating and mucociliary clearance much like in vivo epithelium. To simulate daily usage of the extract, cultures were pre-treated with 0, 10 and 50 µg/ml Echinaforce for one day. Virus exposure, reflecting common cold transmission, was simulated

by dropwise application of 100 $TCID_{50}$ HCoV-229E virus suspension onto the apical surface of the epithelium, covered with 0, 10 and 50 µg/ml Echinaforce (Figure 3a). Virus infection and replication was analyzed 24, 48 and 72 hpi by measuring infectious virus particles in apical secretions. In non-treated respiratory epithelium (0 µg/ml), HCoV-229E replicated efficiently; an elevation of virus titer could be observed as early as 24 hours after infection and virus titers increased over 72h to a mean of $2x10^6$ $TCID_{50}$/ml. In respiratory epithelium pre-treated with 50 µg/ml Echinaforce, viral titers remained below detection level in most cultures at 48 hours (7/8) and 72 hours (5/8) post infection (Figure 3b). When virus was not completely neutralized (3/8), the increase of viral titer started later and eventually reached titers that remained 2-3 logs below controls at 72 hpi, indicating a protective effect in the absence of total inactivation. Pre-treatment of respiratory epithelium with 10 µg/ml Echinaforce was less effective; it did nonetheless result in delayed and reduced increase of viral titers, but completely inhibited virus growth in only 1 out of 8 cultures.

3.4 Echinaforce exerts antiviral effects on MERS and SARS coronaviruses

Since *Echinacea* preparations have shown an antiviral effect against HCoV-229E and other membranous respiratory viruses (12, 26), we expected to see a similar effect on the related, highly pathogenic coronaviruses MERS-CoV and SARS-CoV. To this end, we evaluated the antiviral activity of Echinaforce against these viruses and found that the antiviral effects against MERS-CoV (Figure 4a) and SARS-CoV (Figure 4b) were comparable with the effect observed for HCoV-229E. Interestingly, MERS-CoV was even more sensitive than HCoV-229E to pre-treatment with the lower concentration (10 µg/ml) of Echinaforce.

3.5 Echinaforce reduces infectivity other membranous viruses in vitro

Similar antiviral activity was observed for yellow fever virus, another enveloped RNA virus (Figure 5a). In contrast, Echinaforce showed no effect at all on the infectivity of vaccinia virus (Figure 5b) and the minute virus of mice (Figure 5c), which are DNA viruses, with and without an envelope, respectively.

4. Discussion:

Broadly active antiviral therapeutics are of great interest to medicine, as drugs with too high of a specificity rely on quick and accurate pathogen identification and may fail to target genetic variants or newly emerging viruses. Due to the sheer number of different viruses capable of causing respiratory disease and the speed at which symptoms can develop, readily available and broadly effective therapeutics would be highly desirable for both prophylaxis and treatment of respiratory infections. However, for most respiratory viruses, no specific antiviral therapy is available. Effective broad-spectrum antivirals would reduce the severity of illness, reduce transmission and prevent secondary infections, thereby lessening the general burden and morbidity of these viruses. Given their penchant for zoonotic transmission, antiviral treatments against highly pathogenic coronaviruses are of particular interest and the current SARS-CoV-2 outbreak further illustrates the need for accessible, fast-acting anti-coronavirals.

Herbal preparations of *Echinacea* have traditionally been used to prevent and treat symptoms of colds and flu and are still widely used (9, 12). Echinaforce, an *Echinacea purpurea* extract, has been shown to broadly inhibit the infectivity of influenza A and B, RSV, parainfluenza virus, and herpes simplex virus in-vitro and to interfere with cytokine production induced upon viral infection (19-21). Results from the current study complement these previous findings by demonstrating a direct antiviral activity of Echinaforce both against common cold coronavirus 229E (HCoV-229E) and highly pathogenic coronaviruses (SARS-CoV and MERS-CoV). We observed a dose dependent inactivation of HCoV-229E upon direct exposure to the extract and 50% reduction of HCoV-229E infectivity (IC_{50}) was achieved at 3.2 µg/ml. As previously seen for RSV, limited intracellular effect was observed for HCoV-229E, as virus replication was not affected by the addition of Echinaforce prior to infection. This observation, along with the fact that treatment of cell cultures with the extract post infection has only a limited effect at the highest concentration (50µg/ml), suggests that the observed antiviral effects against coronaviruses are primarily restricted to the extracellular phases, i.e prior to viral entry into the cell and/or during progeny virus release. Furthermore, this antiviral activity is not strain-specific since the related coronaviruses SARS-CoV and MERS-CoV were inactivated in a comparable manner.

Interestingly, even unrelated enveloped RNA viruses such as yellow fever virus were sensitive to Echinaforce treatment indicating a broad antiviral activity against enveloped viruses.

Mechanism of action of different *Echinacea* extracts are currently unclear, however, for most viruses, Echinaforce seems to exert its antiviral effect upon direct contact, leading to a permanent inactivation of the virus particles. In the current study, inhibition of HCoV-229E infectivity after direct exposure could not be reverted by washing. This observed effect is likely due to a stable alteration of viral components, presumably, the viral membrane, or membrane proteins. Although specific inhibition has been suggested for Influenza (19), the heterogeneity of the envelope proteins and cell receptors used by all the different viruses susceptible to *Echinacea* treatment strongly argues against a specific mechanism of action. Rather, the broad antiviral activity of *Echinacea* on various membranous RNA viruses points to a more general inhibitory effect. Non-enveloped rhinoviruses are sensitive to Echinaforce at high concentrations while adenoviruses and mouse parvovirus are not (20). Interestingly, Echinacea does not inhibit vaccinia virus, a large, enveloped DNA virus. So far, it is the only enveloped virus found to be resistant to treatment with *Echinaforce*.

We investigated whether a protective effect in the upper-respiratory tract could be reproduced in-vitro, in re-constituted three-dimensional nasal epithelium, i.e air-liquid interface (ALI) cell cultures, where the apical side is exposed to air resembling the human airways in-vivo. This cell culture system recapitulates many of the characteristics of the human respiratory tract, including ciliary beating and mucus production (27, 28). Regular intake of Echinaforce was simulated by overlaying cells with a thin layer of the extract and this treatment was sufficient to either prevent or reduce infection with HCoV-229E in respiratory epithelium. Almost complete protection was observed in respiratory epithelium treated with 50 µg/ml. At a lower concentration (10 µg/ml), the protection was less efficient but detectable. These results are in agreement with observations made in clinical studies investigating the effect of Echinaforce on the incidence of respiratory tract infections in 755 volunteers. In this randomized, double blind, placebo controlled, clinical study the numbers of cold episodes were significantly lower in the volunteers receiving Echinaforce. While the placebo group had 188 cold episodes, with a collective duration of 850 days, the Echinaforce-treated group had 149 with a duration of 672 days. Throughout the whole study period, 54 viral infections, of which 21 were caused by

298 coronaviruses (9: 229E, 11: HKU1, 1: OC43) were detected in the treated group and 74, of which 33
299 were coronaviruses (15: 229E, 17: HKU1, 1: OC43) in the placebo group. The same study found that
300 the infection rates of membranous respiratory viruses (including HCoV-229E, NL-63 and OC-43) could
301 be reduced in adults by approximately 50% (p=0.0114) during a 4-month prophylactic treatment with
302 Echinaforce (15). Furthermore, very similar results were recently obtained in a pediatric study where
303 similar reduction in infection rates was observed in 203 children, aged 4-12 years (p=0.0218) after
304 Echinaforce treatment (Ogal M, unpublished data).

305 These studies indicate a clinically relevant protection against coronaviruses with prophylactic
306 Echinaforce treatment at tolerable and safe dosages. Furthermore, we have also observed partial
307 protection at lower concentrations. In vivo, this might be due to insufficient dosage. A better protection
308 may be achieved by ingesting higher doses of the extract or a more directed distribution of Echinaforce
309 in the airways, e.g. by aerosol delivery. Furthermore, isolation and concentration of the active
310 compounds in *Echinacea* products could result in smaller daily doses and increased activity.

311 As previously mentioned, in addition to direct inactivation of viral particles, *Echinacea* also
312 inhibits cytokine secretions during virus infection. Excessive production of interleukin-6 (IL-6) or IL-
313 8 have been associated with symptomatic development of viral infections and such responses, i.e. a
314 cytokine storm, are likely responsible for many of cold-associated symptoms such as runny nose,
315 coughing, sneezing et cetera (29). During certain viral infections (e.g. influenza), the heightened
316 immune response may actually contribute to the destruction of respiratory epithelium and may even be
317 the dominant reason for symptoms in absence of virus-mediated cytopathicity (30, 31). In these cases,
318 the inhibition of virus-induced cytokine production by Echinaforce may be beneficial by limiting the
319 damage of the respiratory epithelium provoked by the immune system (13). For many other viruses,
320 including coronaviruses, no direct cell destruction is observed during infection. This is in accordance
321 with the fact that coronaviruses, in general, do not elicit a pronounced cytokine response upon infection
322 (32). Despite severe symptoms and pulmonary pathology, the highly pathogenic MERS-CoV does not
323 elicit an overwhelming cytokine response in primary respiratory epithelial cells in the early course of
324 infection. However, later on, a marked induction of the pro-inflammatory cytokines/chemokines IL-1β,
325 IL-8 and IL-6 was observed (33). Even if the anti-inflammatory action of Echinaforce is less relevant

for coronaviruses, treatment with 50µg/ml Echinaforce inactivated both MERS-CoV and SARS-CoV particles to similar levels as observed for HCoV-229E.

In the current study, we have shown that human coronaviruses are readily inhibited by Echinaforce in vitro, further strengthening its use as a prophylactic treatment against a wide range of respiratory viruses causing either serious pulmonary disease or the common cold. Furthermore, a broadly acting antiviral compound suitable for long-term prophylaxis upon exposure could potentially reduce the high mortality rates associated with MERS- and SARS-CoV infections. Due to its general mode of action, novel zoonotic coronaviruses, such as SARS-CoV-2, could also be sensitive to Echinaforce, potentially providing an accessible and inexpensive prophylactic treatment for emerging coronavirus infections.

Author contributions:

J.S, R.Z, S.R, R.A.G, N.L and D.S performed experiments and analyses. R.S and A.S provided material and expertise. W.C.A and M.S provided expert counsel. H.R.J performed experiments, wrote and revised the manuscript. O.B.E supervised the study and wrote the manuscript. This manuscript has been read and approved by all authors.

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Conflicts of interest:

R. Schoop and A. Suter are employees of A. Vogel AG and W. C. Albrich has been the recipient of grants and fees from A. Vogel AG. The remaining authors declare no conflict of interest.

Data availability:

All relevant source data is available from the corresponding author upon request.

References:

1. Elliot AJ, Fleming DM. Common respiratory infections diagnosed in general practice. In: Eccles R, Weber O, editors. Common Cold. Basel: Birkhäuser Basel; 2009. p. 47-75.
2. Dominguez SR, Robinson CC, Holmes KV. Detection of four human coronaviruses in respiratory infections in children: a one-year study in Colorado. Journal of medical virology. 2009;81(9):1597-604.
3. Pene F, Merlat A, Vabret A, Rozenberg F, Buzyn A, Dreyfus F, et al. Coronavirus 229E-Related Pneumonia in Immunocompromised Patients. Clinical Infectious Diseases. 2003;37(7):929-32.
4. Woo PCY, Lau SKP, Chu C-m, Chan K-h, Tsoi H-w, Huang Y, et al. Characterization and Complete Genome Sequence of a Novel Coronavirus, Coronavirus HKU1, from Patients with Pneumonia. Journal of Virology. 2005;79(2):884-95.
5. Su S, Wong G, Shi W, Liu J, Lai ACK, Zhou J, et al. Epidemiology, Genetic Recombination, and Pathogenesis of Coronaviruses. Trends in microbiology. 2016;24(6):490-502.
6. Cui J, Li F, Shi ZL. Origin and evolution of pathogenic coronaviruses. Nature reviews Microbiology. 2019;17(3):181-92.
7. de Wit E, van Doremalen N, Falzarano D, Munster VJ. SARS and MERS: recent insights into emerging coronaviruses. Nature reviews Microbiology. 2016;14(8):523-34.
8. Peiris JS, Guan Y, Yuen KY. Severe acute respiratory syndrome. Nature medicine. 2004;10(12 Suppl):S88-97.
9. Clarke TC, Black LI, Stussman BJ, Barnes PM, Nahin RL. Trends in the use of complementary health approaches among adults: United States, 2002-2012. National health statistics reports. 2015(79):1-16.
10. Paules CI, Marston HD, Fauci AS. Coronavirus Infections-More Than Just the Common Cold. Jama. 2020.
11. Zumla A, Chan JF, Azhar EI, Hui DS, Yuen KY. Coronaviruses - drug discovery and therapeutic options. Nature reviews Drug discovery. 2016;15(5):327-47.
12. Barrett B. Medicinal properties of Echinacea: a critical review. Phytomedicine : international journal of phytotherapy and phytopharmacology. 2003;10(1):66-86.
13. Vimalanathan S, Schoop R, Suter A, Hudson J. Prevention of influenza virus induced bacterial superinfection by standardized Echinacea purpurea, via regulation of surface receptor expression in human bronchial epithelial cells. Virus research. 2017;233:51-9.
14. Osowski S, Rostock M, Bartsch HH, Massing U. [Pharmaceutical comparability of different therapeutic Echinacea preperations]. Forschende Komplementarmedizin und klassische Naturheilkunde = Research in complementary and natural classical medicine. 2000;7(6):294-300.
15. Jawad M, Schoop R, Suter A, Klein P, Eccles R. Safety and Efficacy Profile of Echinacea purpurea to Prevent Common Cold Episodes: A Randomized, Double-Blind, Placebo-Controlled Trial. Evidence-based complementary and alternative medicine : eCAM. 2012;2012:841315.
16. Raus K, Pleschka S, Klein P, Schoop R, Fisher P. Effect of an Echinacea-Based Hot Drink Versus Oseltamivir in Influenza Treatment: A Randomized, Double-Blind, Double-Dummy, Multicenter, Noninferiority Clinical Trial. Current therapeutic research, clinical and experimental. 2015;77:66-72.
17. Schapowal A. Efficacy and safety of Echinaforce(R) in respiratory tract infections. Wiener medizinische Wochenschrift (1946). 2013;163(3-4):102-5.
18. Schapowal A, Klein P, Johnston SL. Echinacea reduces the risk of recurrent respiratory tract infections and complications: a meta-analysis of randomized controlled trials. Advances in therapy. 2015;32(3):187-200.
19. Pleschka S, Stein M, Schoop R, Hudson JB. Anti-viral properties and mode of action of standardized Echinacea purpurea extract against highly pathogenic avian influenza virus (H5N1, H7N7) and swine-origin H1N1 (S-OIV). Virology journal. 2009;6:197.

20. Sharma M, Anderson SA, Schoop R, Hudson JB. Induction of multiple pro-inflammatory cytokines by respiratory viruses and reversal by standardized Echinacea, a potent antiviral herbal extract. Antiviral research. 2009;83(2):165-70.
21. Sharma M, Schoop R, Hudson JB. Echinacea as an antiinflammatory agent: the influence of physiologically relevant parameters. Phytotherapy research : PTR. 2009;23(6):863-7.
22. Sharma M, Schoop R, Hudson JB. The efficacy of Echinacea in a 3-D tissue model of human airway epithelium. Phytotherapy research : PTR. 2010;24(6):900-4.
23. Kindler E, Jonsdottir HR, Muth D, Hamming OJ, Hartmann R, Rodriguez R, et al. Efficient replication of the novel human betacoronavirus EMC on primary human epithelium highlights its zoonotic potential. mBio. 2013;4(1):e00611-12.
24. Thiel V, Herold J, Schelle B, Siddell SG. Infectious RNA transcribed in vitro from a cDNA copy of the human coronavirus genome cloned in vaccinia virus. The Journal of general virology. 2001;82(Pt 6):1273-81.
25. Ramakrishnan MA. Determination of 50% endpoint titer using a simple formula. World journal of virology. 2016;5(2):85-6.
26. Hudson J, Vimalanathan S. Echinacea—A Source of Potent Antivirals for Respiratory Virus Infections. Pharmaceuticals (Basel). 2011;4(7):1019-31.
27. de Jong PM, van Sterkenburg MA, Hesseling SC, Kempenaar JA, Mulder AA, Mommaas AM, et al. Ciliogenesis in human bronchial epithelial cells cultured at the air-liquid interface. American journal of respiratory cell and molecular biology. 1994;10(3):271-7.
28. de Jong PM, van Sterkenburg MA, Kempenaar JA, Dijkman JH, Ponec M. Serial culturing of human bronchial epithelial cells derived from biopsies. In vitro cellular & developmental biology Animal. 1993;29a(5):379-87.
29. Doyle WJ, Skoner DP, Gentile D. Nasal cytokines as mediators of illness during the common cold. Current allergy and asthma reports. 2005;5(3):173-81.
30. Lee S, Hirohama M, Noguchi M, Nagata K, Kawaguchi A. Influenza A Virus Infection Triggers Pyroptosis and Apoptosis of Respiratory Epithelial Cells through the Type I Interferon Signaling Pathway in a Mutually Exclusive Manner. J Virol. 2018;92(14).
31. Van Reeth K. Cytokines in the pathogenesis of influenza. Veterinary microbiology. 2000;74(1-2):109-16.
32. Thiel V, Weber F. Interferon and cytokine responses to SARS-coronavirus infection. Cytokine & growth factor reviews. 2008;19(2):121-32.
33. Lau SK, Lau CC, Chan KH, Li CP, Chen H, Jin DY, et al. Delayed induction of proinflammatory cytokines and suppression of innate antiviral response by the novel Middle East respiratory syndrome coronavirus: implications for pathogenesis and treatment. The Journal of general virology. 2013;94(Pt 12):2679-90.

Figure legends:

Figure 1

Dose-dependent inactivation of HCoV-229E by *Echinaforce*

Direct exposure of virus particles to Echinaforce lead to a dose-dependent inactivation of HCoV-229E. Mean inhibitory concentration, IC_{50}, was calculated as 3.2 µg/ml and complete virus inactivation was achieved at a concentration of 50 µg/ml, while no effect was observed on cell viability (right y-axis). The data shown are representative of three independent experiments (mean ± sd).

Figure 2:

Treatment of cells with *Echinaforce* does not inhibit HCoV-229E replication

(a) Direct exposure of virus particles to Echinacea led to a permanent inactivation that could not be reverted by extensive washing. (b) Three day pre-treatment of Huh-7 cells with Echinaforce does not inhibit virus replication. (c) Treatment of Huh-7 cells one-hour post infection (hpi) only resulted in lower viral titers at the highest concentration (50 µg/ml). Dashed line: detection limit, 10 $TCID_{50}$/ml, n.d: not detected at detection limit. The data shown are representative of three independent experiments (mean ± sd).

Figure 3:

***Echinaforce* inhibits infection of HCoV-229E in organotypic airway cultures**

(a) To simulate natural infection, organotypic nasal epithelial cultures were infected with droplets of HCoV-229E from the apical side. (b) Viral titer in apical secretions was determined at 24, 48 and 72 hpi. Pre-treatment with 50 µg/ml lead to complete inhibition of virus replication in 5/8 cultures, while 10 µg/ml showed complete inhibition only in 1/8 cultures. In both pre-treatment groups, a reduction of mean titer was observed when compared to non-treated controls. Dashed line: detection limit, 10 $TCID_{50}$/ml.

Figure 4:

MERS-CoV and SARS-CoV are inactivated upon direct contact with Echinaforce®.

(a) MERS-CoV is highly sensitive to direct Echinaforce treatment, with significant reduction in viral titer observed at 10µg/ml and complete inactivation at 50µg/ml. (b) SARS-CoV is completely inactivated at the highest concentration with a slight reduction in viral titer after exposure to 10µg/ml. No effect was observed on cell viability (right y-axis). The data shown are representative of two independent experiments (mean ± sd).

Figure 5:

Antiviral effect of Echinaforce on other viruses.

(a) Exposure to 50 µg/ml *Echinacea* extract leads to complete inactivation of yellow fever virus. (b,c) Vaccinia virus and mouse parvovirus (MVM) were not sensitive to Echinaforce. Data shown are representative of two independent experiments (mean ± sd).