Prevention of influenza virus induced bacterial superinfection by standardized *Echinacea purpurea*, via regulation of surface receptor expression in human bronchial epithelial cells

Selvarani Vimalanathan [1,*], Roland Schoop [2], Andy Suter [2], James Hudson [1]

[1] Department of Pathology & Laboratory Medicine, University of British Columbia, Vancouver, BC, Canada V6T 2B5
[2] Medical Department, A. Vogel Bioforce AG, Roggwil TG, Switzerland

ABSTRACT

Viral infections may predispose the airways to secondary bacterial infections that can lead to unfavorable progression of principally self-limiting illnesses. Such complicated respiratory infections include pneumonia, bronchitis, sinusitis, acute otitis media, and sepsis, which cause high morbidity and lethality. Some of the pathogenic consequences of viral infections, like the expression of bacterial adhesion receptors and the disturbance of physical barrier integrity due to inflammation, may create permissive conditions for co-infections. Influenza virus A (H3N2) is a major pathogen that causes secondary bacterial infections and inflammation that lead to pneumonia. The herbal medicine *Echinacea purpurea*, on the other hand, has been widely used to prevent and treat viral respiratory infections, and recent clinical data suggest that it may prevent secondary infection complications as well. We investigated the role of standardized *E. purpurea* (Echinaforce® extract or EF) on H3N2-induced adhesion of live nontypeable *Haemophilus influenzae* (NTHi) and *Staphylococcus aureus*, along with the expression of bacterial receptors, intracellular adhesion molecule-1 (ICAM-1), fibronectin, and platelet activating factor receptor (PAFr), by BEAS-2B cells. Inflammatory processes were investigated by determining the cellular expression of IL-6 and IL-8 and the involvement of Toll-like receptor (TLR-4) and NFκB p65. We found that influenza virus A infection increased the adhesion of *H. influenzae* and *S. aureus* to bronchial epithelial cells via upregulated expression of the ICAM-1 receptor and, to some extent, of fibronectin and PAFr. Echinaforce (EF) significantly reduced the expression of ICAM-1, fibronectin, and PAFr and consequently the adhesion of both bacterial strains. EF also effectively prevented the super-expression of inflammatory cytokines by suppressing the expression of NFκB and possibly TLR-4. These results indicate that *E. purpurea* has the potential to reduce the risk of respiratory complications by preventing virus-induced bacterial adhesion and through the inhibition of inflammation super-stimulation (cytokine storms).

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1. Introduction

The WHO estimates that annual influenza epidemics result in 3 to 5 million cases of severe illness worldwide as well as in 250 000 to 500 000 deaths (World Health Organization, 2014). Every year, throughout late fall to early spring, in temperate regions, influenza infections are a leading cause of hospitalization, with high morbidity and mortality, many cases of which are now attributable to co-infections with commensal bacteria. It has been proposed that the severe tissue pathology of some of the more severe influenza pandemics, such as the "Spanish 'flu" in 1918, was due to secondary infections by *Staphylococcus aureus, Haemophilus*

influenzae, and *Streptococcus pneumoniae* (Pneumococcus), which led to lethal pneumonia (Morens et al., 2008; McCullers, 2011; Kash and Taubenberger, 2015). Co-infection of influenza with these bacteria may produce cytokine storms that change typically self-limited 'flu episodes into highly severe and often lethal illnesses. Notably, the annual incidence of community-acquired pneumonia in the elderly is estimated to be up to 44 cases per 1000 people, and represents the single most common cause of death from infectious diseases (Hoyert et al., 2005; Janssens and Krause, 2004). *H. influenzae* and *S. pneumoniae* are the most common pathogens associated with community-acquired pneumonia in the elderly, and antibiotic resistance is often encountered with these pathogens (Torres et al., 2014).

Bacterial superinfections however are not exclusive to influenza and pneumonia, or to bronchitis in the lungs; they can also affect the sinuses, leading to bacterial (rhino-) sinusitis, bacterial otitis media

* Corresponding author.
  E-mail address: vimrani1@mail.ubc.ca (S. Vimalanathan).

in the middle ear, tonsillo-pharyngitis, epiglottitis, and Strep-A positive angina (Heikkinen et al., 2004; Peltola et al., 2006, 2005; Putto, 1987; Welte et al., 2012). Every year more than 400 000 children in the US are admitted to the hospital due to complicated viral respiratory tract infections, as are a similar number of elderly patients (Henrickson et al., 2004; Mizgerd, 2006). The incidence of complicated infections including pneumonia increases with known risk factors such as smoking, a weak immune system, and underlying illnesses, such as chronic obstructive pulmonary disease (COPD) (Baik et al., 2000; Koivula et al., 1994; Stampfli and Anderson, 2009; Welte et al., 2012).

The pathophysiological mechanisms by which initial viral infections can predispose the airways to subsequent bacterial superinfections involve the ability of most respiratory agents to upregulate the expression of surface receptors on epithelial cells that mediate bacterial adhesion. Influenza virus, for example, strongly increases the expression of intracellular adhesion molecule 1 (ICAM-1) and platelet-activating factor receptor (PAFr) (Matsukura et al., 1996; Othumpangat et al., 2016). ICAM-1 is a transmembrane glycoprotein receptor that is a member of the immunoglobulin superfamily and that is expressed at low levels in various cell types, including nasal (Winther et al., 2002) and respiratory epithelial cells (Bella et al., 1998). ICAM-1, fibronectin, and PAFr serve as ligation structures for *H. influenzae*, *S. aureus*, and *S. pneumoniae* (Avadhanula et al., 2006; Chavakis et al., 2002; Ishizuka et al., 2003; Watson and Eisenhut, 2013). Importantly, in addition to influenza virus, rhinovirus, respiratory syncytial virus, and parainfluenza viruses also stimulate the expression of ICAM-1 resulting in enhanced binding of, for example, *H. influenzae*. Our experiments focused on the influenza virus, since it is one of the most notorious inducers of complicated respiratory infections.

We previously reported the ability of a standardized *Echinacea purpurea* extract (Echinaforce® or EF) to inactivate various human and avian influenza viruses, as well as rhinoviruses and other respiratory viruses, in human epithelial cell lines (Pleschka et al., 2009; Vimalanathan et al., 2013). The standardized EF extract contains a mixture of potent antiviral compounds. The extract also acts as an immune modulator in various cell culture systems and can reverse the stimulatory effects of a number of inflammatory cytokines and chemokines that are induced by influenza and other respiratory viruses (Sharma et al., 2009).

A recent meta-analysis encompassing a total of 2548 patients found that Echinacea had important effects on infection recurrences and complications, including pneumonia, bronchitis, and sinusitis. The overall risk for developing complications was significantly reduced by approximately 50%. The authors hypothesized that the beneficial effects of Echinacea on illness progression might be attributed to its antiviral and immune supportive effects, but no conclusive molecular mode of action was given (Schapowal et al., 2015).

Accordingly, the present study examined whether and how Echinacea impacted the virally induced adhesion of pathogenic bacteria. In principal, a carefully standardized broad-spectrum and multifunctional anti-viral herbal medicine could be useful not only in treating influenza infection but also its complications.

Here we investigated the protective effects of *E. purpurea* against the influenza-induced adhesion of live *H. influenzae* (NTHi) and *S. aureus*, and correlated this effect with the expression of the relevant bacterial receptors ICAM-1, fibronectin, and PAFr. This *in vitro* study used BEAS-2B cells of bronchial origin, since the lungs are the main location of influenza replication. In addition, we evaluated the effects of *E. purpurea* on the expression of NFκB and TLR4 and on the subsequent production of IL-8 and IL-6 in response to lipopolysaccharide (LPS), a bacterial trigger of inflammation.

2. Materials and methods

2.1. Cell lines, viruses, and bacterial strains

Madin-Darby canine kidney (MDCK) cells and BEAS-2B normal human bronchial epithelial cells were acquired originally from ATCC (American Type Culture Collection, Rockville, MD) and cultured in cell culture flasks in Dulbecco's MEM (DMEM) supplemented with 5% gamma-irradiated heat-inactivated fetal bovine serum (HyClone Fetal Bovine Serum, U.S. Origin, Fisher Scientific, Canada), at 37 °C in a 5% CO_2 atmosphere. Cell culture reagents were obtained from Invitrogen, Ontario, Canada. No antibiotics or antimycotic agents were used.

Influenza virus A/Victoria/H3N2 was acquired from the BC Centre for Disease Control, Vancouver. Influenza virus (H3N2) was grown in MDCK cells with TPCK (L-1-tosylamide-2-phenylethyl chloromethyl ketone, Sigma Chemical Co., Oakville, ON, Canada) treated trypsin (2 μg/mL). The titer of the influenza virus was measured quantitatively by plaque formation in MDCK cells.

The bacterial strains used in this study were *S. aureus* ATCC 25923 and NTHi, which were obtained from Dr. Michael Noble, Clinical Microbiology Proficiency Testing Lab, University of British Columbia. *S. aureus* was cultured on blood agar plates (Hardy Diagnostics, Santa Maria, CA, USA). NTHi was cultured on chocolate agar at 37 °C (Hardy Diagnostics) in 5% CO_2. The bacteria were grown overnight in 5 mL of liquid broth containing BHI media supplemented with 5% FILDES extract (Oxoid, ON, Canada). The next day, the culture was mixed well, and 1 mL was place in a 1.5-mL Eppendorf tube, pelleted, and the pellet was washed three times with phosphate-buffered saline (PBS) and then diluted to 1×10^7 CFU/mL in fresh cell culture medium (DMEM) for the bacterial adhesion assay. The number of bacterial colonies (CFU/mL) was determined by plating 10-fold dilutions on agar plates.

2.1.1. Standardized Echinacea preparation

Echinaforce® (A. Vogel Bioforce AG) is a 65% ethanolic extract of freshly harvested aerial parts and roots of *E. purpurea* at a ratio of 95:5. The phytochemical characterization of Echinaforce® has been described earlier (Sharma et al., 2009).

2.1.2. Cytotoxicity assay

Cytotoxicity of EF was determined by MTS assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] (Promega, Madison, WI).

Briefly, BEAS-2B cells were seeded at a density of 1×10^4/well, in 96-well plates and allowed to adhere for 24 h at 37 °C in a 5% CO_2 incubator. All media was removed from the wells and replaced with 100 μL of serum-free medium containing increasing concentrations of EF (1:100–1:1600). In addition, negative/vehicle controls were included. Cells were cultured for 48 h, after which 20 μL of MTS solution was added to each well and further incubated for 1 h. The optical density was measured at 490 nm using a spectrophotometer and data presented as a percentage of vehicle treated cells cultured under the same conditions. The percentage viability was calculated using the following formula:

Viable cell number (%) = OD_{490} EF treated cells/OD_{490} (vehicle-treated control cells) × 100. Each assay was carried out in quadruplicate and the results were expressed as the mean ± standard deviation (SD).

2.2. Viral infection and the bacterial adhesion assay

2.2.1. Bacterial adhesion (CFU)

The bacterial adhesion assay was performed as described (Avadhanula et al., 2006) with some modifications. BEAS-2B cells (2.5×10^5 cells/well) were grown to 90% confluent monolayers in

6-well trays. Cells were infected with influenza virus A at a multiplicity of infection (MOI) of 1.0 PFU/cell for 1 h at 37 °C in 5% CO$_2$. Following adsorption inocula were removed, and the cells were washed once with PBS to remove unbound virus. In the control group, cells were cultured in medium containing vehicle alone (ethanol final concentration 0.325% v/v). In the H3N2-infection group, cells were infected with H3N2 a multiplicity of infection (MOI) of 1.0 PFU/cell for 1 h at 37 °C. Following adsorption for 1 h, inoculums were removed, and the cells were washed once with PBS to remove unbound virus, and incubated for 48 h at 37 °C in DMEM without serum.

To assess the effect of EF, it was added to the wells of virus-infected wells at various concentrations (1:200, 1:400, and 1:800). Cells were cultured for 48 h at 37 °C in 5% CO$_2$. Viral infection was confirmed by plaque assay. The monolayers were washed with PBS, 1 mL of DMEM without serum was added to each well, and either *S. aureus* or NTHi was added to each well at an MOI of 10. Cells were incubated with bacteria at 37 °C for 1 h and washed 4 times with PBS containing 6% FBS to remove loosely adherent bacteria. Cells were then detached using 0.5 mL of 0.25% trypsin-EDTA (Invitrogen, Ontario, CA, USA), and the number of cells per well were determined with a hemocytometer. The detached cells were serially diluted, and *S. aureus*-and NTHi-treated cells were plated on blood agar plates and chocolate agar plates, respectively, to quantitate the colonies. Each assay was run in triplicate and repeated three times, and the number of adherent bacteria was normalized to the number of epithelial cells.

2.2.2. FITC labeling of bacteria and confocal microscopy imaging

For FITC labeling, *S. aureus* bacteria were grown overnight in 5 mL Mueller-Hinton broth without shaking at 35 °C, collected by centrifugation, washed twice with PBS, and resuspended in 1 mL of phosphate-buffered saline (Sigma Aldrich, MO, USA). Bacteria (1 × 10^8) were transferred to 1 mL of freshly prepared FITC buffer (0.1 M sodium bicarbonate pH 9.0) containing FITC (1 mg/mL). The bacterial cells were incubated at room temperature for 1 h at 22 °C in the dark. The stained bacterial cells were washed extensively (6 times) with PBS and re-suspended at a final concentration of 10^6 CFU/mL using the method as previously described (Krut et al., 2003) with minor modifications.

To study the adhesion of bacterial cells to epithelial cells, 1 × 10^4 BEAS-2B cells were grown on glass chamber slides (Fisher Scientific, ON, Canada) in 24-well plates. At 90% confluence, the cells were infected with virus and with EF as described for the bacterial adhesion assay (Section 2.2.1). The *S. aureus* adhesion assay was performed as described (Min et al., 2012). Briefly, fluorescein-isothiocyanate- (FITC-) labeled bacteria (4000 CFU) were added to each well along with 200 μl of DMEM without serum. After incubation for 1 h at 37 °C in a 5% CO$_2$ incubator, the cells were washed 4 times with PBS and fixed with 1% paraformaldehyde for 20 min at 4 °C then washed once with PBS. The slides were mounted with DAPI (Sigma Aldrich, St. Louis, MO). The cells were visualized using a Zeiss LSM 510 Meta confocal microscope (CarlZeiss, Obertochen, AG Germany). The fluorescence intensity was quantified using the ImageJ image analysis platform (Jensen, 2013).

2.3. Expression of ICAM-1, fibronectin, PAFr, TLR 4, and NFκB p65

2.3.1. Confocal microscopy and immunocytochemical staining

BEAS-2B cells were cultured on glass chamber slides in 24-well culture plates at a density of 1 × 10^4 cells/well. After 24 h, the cells were infected with influenza virus (H3N2) for 1 h at 37 °C. Subsequent infection with virus and treatment with EF were carried out as for the bacterial adhesion assay (Section 2.2.1). Immunostaining was performed after 24 h for TLR4 and NFκB p65 and after 48 h for ICAM-1 and PAFr. Briefly, the cells were fixed with

1% paraformaldehyde for 15 min at 4 °C and washed twice with cold PBS. For NFκB p65 staining, cells were permeabilized with 0.5% saponin in PBS (Sigma Aldrich, St. Louis, MO) for 10 min at room temperature and then cells were washed three times in PBS. Non-specific binding sites were blocked with 10% goat serum (Cedarlane, Burlington, ON, Canada) in PBS for 30 min at 22 °C. The blocking buffer was removed, and the primary antibody was added after dilution in 1% BSA in PBST (200 μL/well at 5 μg/mL) and incubated overnight at 4 °C. The following primary antibodies were obtained from Abcam (Cambridge, MA, USA): mouse monoclonal antibodies to ICAM-1 (ab2213) and TLR4 (ab22048); rabbit polyclonal antibody to PAFr (ab104162) and chicken polyclonal antibody to NFκB p65 (ab140751). The next day, the cells were washed three times (5 min each) in PBS. The cells were incubated for 1 h at room temperature in the dark with the following secondary antibodies that were specific to the primary antibodies (diluted 1:500 in 1% BSA): goat anti-mouse IgG Alexa Fluor 488 for ICAM-1 and TLR4 (ab150117); goat anti-rabbit IgG H&L Alexa Fluor 594 (ab150080) for PAFr; and goat anti-chicken IgY H&L Alexa Fluor 647 (ab150171)for NFκB p65. After the secondary antibody solution was removed, the cells were washed three times (5 min each) in PBS in the dark. Fluorescence imaging of the fixed cells was carried out with a Zeiss Axio Observer Z1 inverted fluorescence microscope equipped with a 20× lens using Zeiss AxioVision Software release 4.8 package. The mean fluorescent intensity was calculated using the ImageJ platform.

2.3.2. The effect of anti-human ICAM-1 antibody on the adhesion of NTHi to BEAS-2B cells

BEAS-2B cells were infected with influenza virus A and cultured for 48 h, then the virus-induced cell surface ICAM-1 receptor was blocked by incubating the cells at 37 °C for 2 h with different concentrations (0–5 μg/mL) of purified anti-ICAM-1 rabbit monoclonal antibody (Abcam, USA). Cells were washed and then incubated with NTHi for 1 h at 37 °C in 5% CO$_2$ as described in Section 2.2.1.

### 2.3.3. ICAM-1 and protein quantitation of cell lysate

Virus-infected cells, uninfected cells, and virus-infected/EF-treated BEAS-2B cells were lysed in ice-cold RIPA lysis buffer (100 μL/10^6 cells) that contained 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), protease inhibitor cocktail (1:100) (Abcam), and phosphatase inhibitors. The ICAM-1 protein was detected using the Human ICAM-1/CD54 DuoSet ELISA (DY720-05; R&D Systems, Minneapolis, MN, USA) following the manufacturer's protocol.

2.3.4. Fibronectin quantification

The fibronectin protein was quantified in cell culture supernatants and cell lysates using the Human Fibronectin PicoKine ELISA Kit (Boster Biological Technology, Pleasanton, CA, USA). Protein levels were normalized to cell number and expressed as pg/10^5 cells. We measured ICAM and PAFr using fluorescence microscopy and fibronectin via ELISA because the prior receptors represent membrane-bound structures, while fibronectin is secreted in cell culture medium also and thereby necessitates alternative measuring methods like ELISA.

2.4. Cytokine assays for IL-8 and IL-6

BEAS-2B cells were grown in 6-well plates to 90% confluence and then infected with H3N2 0.1 MOI/cell for 1 h at 37 °C. The inoculum was removed and replaced either with media (in virus control wells) or with media containing various concentrations of EF (in treatment wells) and incubated for 24 h. At 24-h intervals, culture medium was removed and 5 μg/mL LPS from *Pseudomonas aeruginosa* (Sigma Aldrich; L9143) was added to H3N2 infected
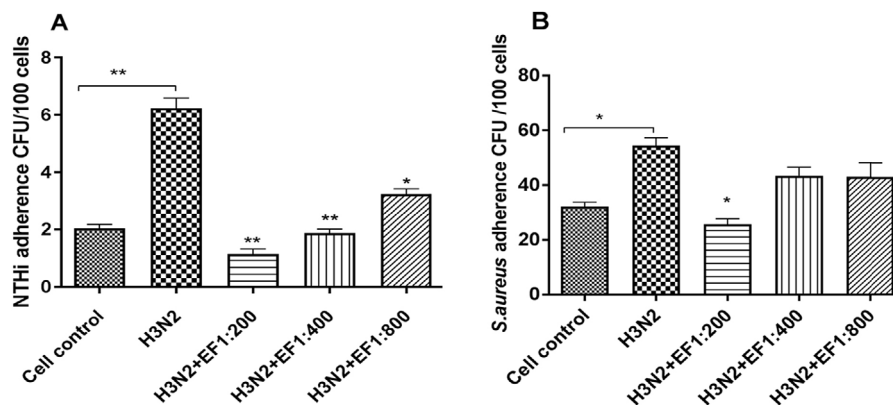
Fig. 1. (A) The effect of Echinaforce® extract (EF) on the adhesion of NTHi to H3N2-infected BEAS-2B cells. Adhesion is expressed as the number of colony forming units (CFU) bound to 100 cells. EF inhibited NTHi adhesion in a dose-dependent manner, *$p < 0.05$, **$p < 0.01$. (B). Adhesion of *S. aureus* is expressed as the number of CFU bound to 100 cells. EF inhibited *S. aureus* adhesion to infected BEAS 2B cells in a concentration-dependent manner. Assays were carried out in triplicate for three independent experiments, and the mean and standard errors of three independent experiments are shown.

wells and to non-infected cells. Cells were incubated for 1 h at 37 °C, and then the LPS was removed and replaced with 2 mL of media or media with EF at different concentrations. In a parallel experiment, EF was added to only H3N2 infected or to only LPS stimulated cells. Supernatants were collected from each sample after 24 h, centrifuged briefly to remove residual cells, and the concentrations of IL6 and IL8 were measured using DuoSet enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN, USA). All assays were performed according to the manufacturer's instructions.

2.5. Statistical analysis

Results were expressed as means ± SEM. One-way analysis of variance (ANOVA) was used followed by the Dunnett's *t*-test for statistical analysis of the anti-inflammatory activity. A *p* value less than 0.05 ($p < 0.05$) was considered statistically significant.

3. Results

3.1. Cytotoxicity test

Cytotoxicity test revealed that none of the tested EF concentrations showed a statistically significant difference in cell viability compared to cell controls. After 48 h of exposure to BEAS-2B cells, EF 1:100 showed 86 ± 9% viability and EF 1:200 to 1:1600 displayed 100% cell viability compared to untreated cell.

3.2. E. purpurea inhibits influenza virus-induced adhesion of H. influenzae and S. aureus to BEAS-2B cells

Infection of BEAS-2B cells with H3N2 increased the adhesion of live nontypeable *H. influenzae* (NTHi) (Fig. 1A) significantly by 3.08-fold (±0.16) compared to control cells ($p < 0.01$). Virus-infected cells that were incubated with EF showed significantly reduced adhesion of NTHi from 3.08-fold (±0.16) to 0.55-fold (±0.18) for the 1:200 EF dilution (81.9% reduction compared to H3N2 infected cells); 0.94-fold (±0.17) for the 1:400 EF dilution (69.5% reduction); and 1.6-fold (±0.14) for the 1:800 EF dilution (48.3% reduction) ($p < 0.05$). A dilution of 1:800 corresponds to a total EF concentration of 20 μg/mL.

Next, we examined the adhesion of live *S. aureus* to H3N2-infected BEAS-2B cells compared to uninfected control cells (Fig. 1B). The addition of EF significantly decreased *S. aureus* binding in a dose-dependent manner: H3N2-infected BEAS-2B cells showed a 1.70-fold (±0.11) increase in *S. aureus* binding compared to uninfected BEAS-2B cells. Virus-infected cells that were incubated with

EF showed significantly reduced adhesion of *S. aureus* from 1.70-fold to 0.86-fold (±0.16) for the 1:200 EF dilution ($p < 0.05$), which represented a 49.2% reduction compared to H3N2 infected cells. At higher EF dilutions of 1:400 and 1:800, which corresponded to EF concentrations of 40 and 20 μg/mL, there was 1.37-fold (±0.24) and 1.44-fold (±0.35) decrease which represents a 19.6% reduction; this did not reach statistical significance.

These results were confirmed by confocal microscopy using FITC-labeled *S. aureus* (Fig. 2A and B). Using this method, the viral stimulation of binding as measured by fluorescence intensity was more evident, as was the extent of decreased binding by EF treatment. The decrease in binding in response to EF treatment was dose-dependent. Taken together, these findings indicate that EF inhibits influenza virus-induced adhesion of NTHi and *S. aureus* to bronchial epithelial cells.

### 3.3. The effect of EF on influenza virus-induced expression of the cell surface receptor ICAM-1

We next investigated whether Echinacea had an effect on the expression of ICAM-1, which is a recognized NTHi receptor. First, we used flow cytometry to confirm influenza virus-induced ICAM-1 expression on BEAS-2B cells. However, we could not measure the effect of Echinacea using this technique because EF-treated cell samples showed increased fluorescence (auto-fluorescence), leading to false-positive results (data not shown).

As an alternative approach, we used an immunocytochemistry staining method that involves confocal fluorescence microscopy. Initially, to confirm that EF-treated samples did not have increase auto-fluorescence using this method, we stained EF-treated cells with all the secondary antibodies used in this study (Alexa 488, Alexa 598, and Alexa 647).

Our experiments revealed that influenza virus infection upregulated ICAM-1 expression in BEAS-2B cells (Fig. 3A) and that EF decreased ICAM-1 expression significantly (Fig. 3A and B). To confirm our immunocytochemistry results, we quantified the membrane-bound ICAM-1 protein in cell lysate using ELISA. The results clearly indicated that EF had a significant effect on ICAM-1 expression (Fig. 3C).

### 3.4. Blocking ICAM-1-mediated bacterial adhesion

To determine the extent to which the observed NTHi adhesion was mediated specifically by ICAM-1, we evaluated whether NTHi adhesion to influenza-virus infected cells could be blocked by an ICAM-1 antibody. Blocking with a monoclonal anti-ICAM-
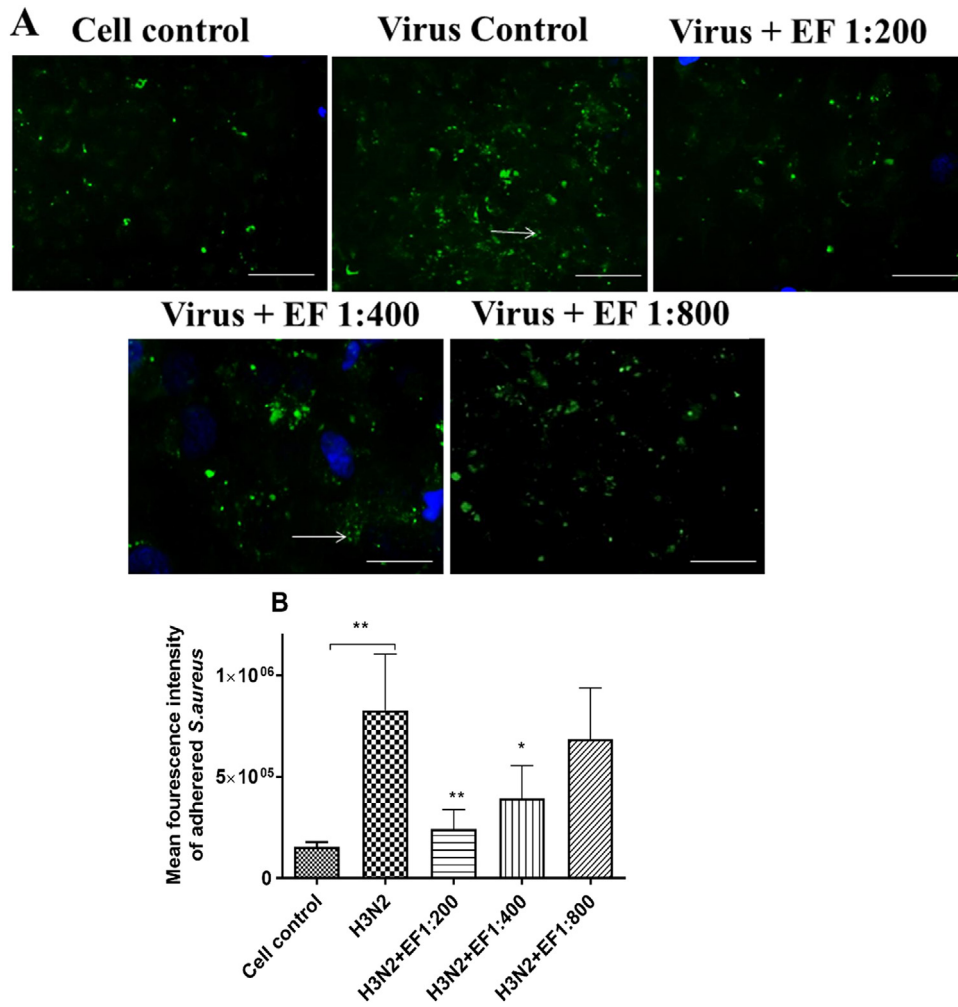
Fig. 2. (A). Adhesion of fluorescein-isothiocyanate- (FITC-) labeled *S. aureus* to H3N2-infected BEAS-2B cells in the presence or absence of Echinaforce® extract (EF) at various concentrations. Adhesion was analyzed using confocal immunofluorescence microscopy. (**B**). The mean fluorescence intensity was calculated using ImageJ. *$p < 0.05$, **$p < 0.01$. Scale bar = 100 μm.
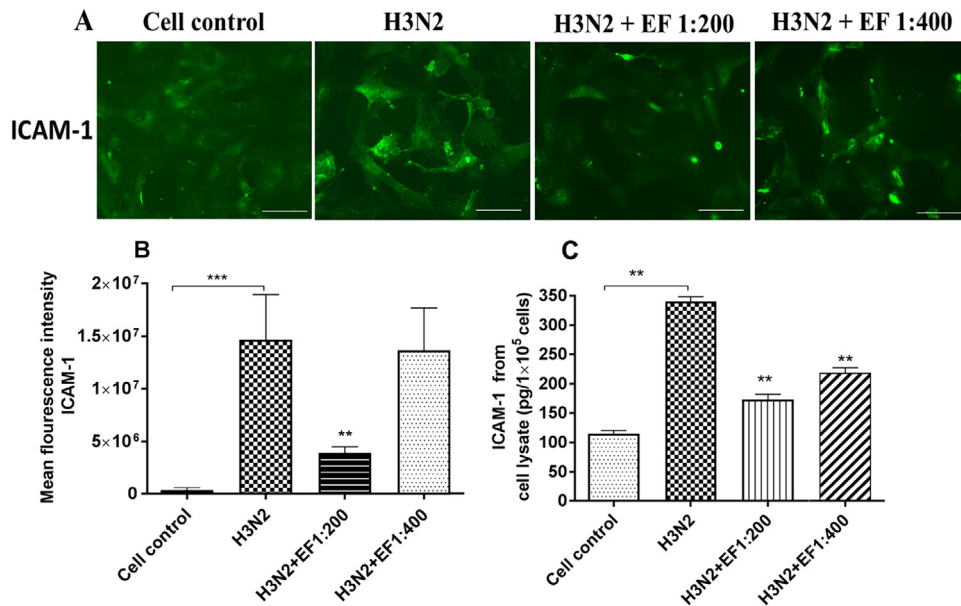
Fig. 3. The inhibitory effect of Echinaforce® extract (EF) on H3N2-induced ICAM-1 expression by BEAS-2B cells. (A). Surface ICAM-1 was measured by fluorescence microscopy; cells were cultured with medium alone (control), with influenza virus A (H3N2), or were treated with EF (H3N2 + EF). Cells were immunostained for ICAM-1 after 48 h. (B). Mean fluorescent intensity was calculated for ICAM-1 using ImageJ. (C). ICAM-1 protein levels were also determined from cell lysates using ELISA; **$p < 0.01$, ***$p < 0.001$. Scale bar = 100 μm.
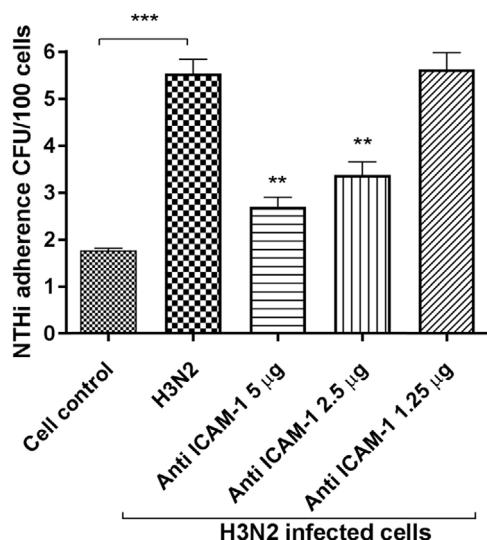
Fig. 4. Adhesion of NTHi to H3N2-infected BEAS-2B cells was blocked by purified rabbit monoclonal anti-ICAM-1 antibody in a dose-dependent manner (**$p < 0.01$, ***$p < 0.001$). Data are expressed as the means ± SD for three samples.

1 antibody significantly reduced the number of adherent bacteria in a dose-dependent manner (Fig. 4). The anti-ICAM-1 antibody inhibited adhesion from 5.5 (±0.5) to 2.26 (±0.3) bacteria/100 cell ($p < 0.01$) at 5 µg/mL and from 5.5 (±0.5) to 3.36 (±0.46) bacteria per cell at 2.5 µg/mL antibody ($p < 0.01$). These results show that the adhesion of NTHi to BEAS-2B cells was to a great extent specific to the ICAM-1 receptor (Fig. 4).

3.5. Influenza virus-induced PAFr and fibronectin expression

Several investigators have reported that further ligation receptors such as PAFr or fibronectin may play an important role in the invasion of NTHi and *S. aureus* and that both are upregulated after infection by a variety of respiratory viruses (Fowler et al., 2000; Li et al., 2014; Massey et al., 2001; Sugiyama et al.,

2015). The immunocytochemical analysis showed that H3N2 moderately induced PAFr expression on the surface of BEAS-2B cells, though this increase was not statistically significant. Nevertheless, EF treatment reversed this increase, and the difference compared to non-EF-treated, virus-infected cells was statistically significant for the highest concentration tested 80 µg/mL EF (Fig. 5A and B). Culture supernatants and cell lysates were collected after 48 h of virus infection, and the fibronectin levels were normalized to the cell number and expressed as pg/10^5 cells (Fig. 5C). The results showed that virus-infected cells produced a significant amount of fibronectin in the supernatants but not in the cell lysates (data not shown) compared to the control cells ($p < 0.01$). The addition of EF after virus infection reversed and normalized the fibronectin levels in the supernatants ($p < 0.05$). The effects of EF on PAFr and fibronectin expression were only significant for higher EF concentrations of 80 µg/mL EF.

3.6. The effect of EF on virus and LPS-enhanced cytokine release

Influenza (H3N2) and bacterial LPS (from *P. aeruginosa*) each induced the release of the cytokines IL-6 and IL-8 by BEAS-2B and A549 cells, as shown previously (Sharma et al., 2009). However, when LPS was added to influenza virus-infected cells, those cells produced 1.5- to 2.0-fold higher levels of IL-6 and IL-8 compared to cells that were only virus-infected or to only LPS-stimulated. In order to evaluate the role of EF on cytokine production, we added EF to cells which were both virus-infected and LPS stimulated at a variety of concentrations. Fig. 6 shows the dose-dependent responses of IL-6 production to EF in BEAS-2B cells. EF reversed superstimulation by both agents in a dose-dependent manner. In a parallel experiment, EF was added to only H3N2 infected or LPS stimulated cells and the results revealed that EF down-regulated cytokine productions in both conditions (data not shown).

Finally, we wanted to investigate whether the modulation of cytokine expression by EF involved TLR-4 (the cognate receptor of LPS) and NFκB p65 (The most prevalent transcription factor). Both flow cytometry and confocal microscopy staining confirmed that infection with the influenza virus induced TLR-4 expression
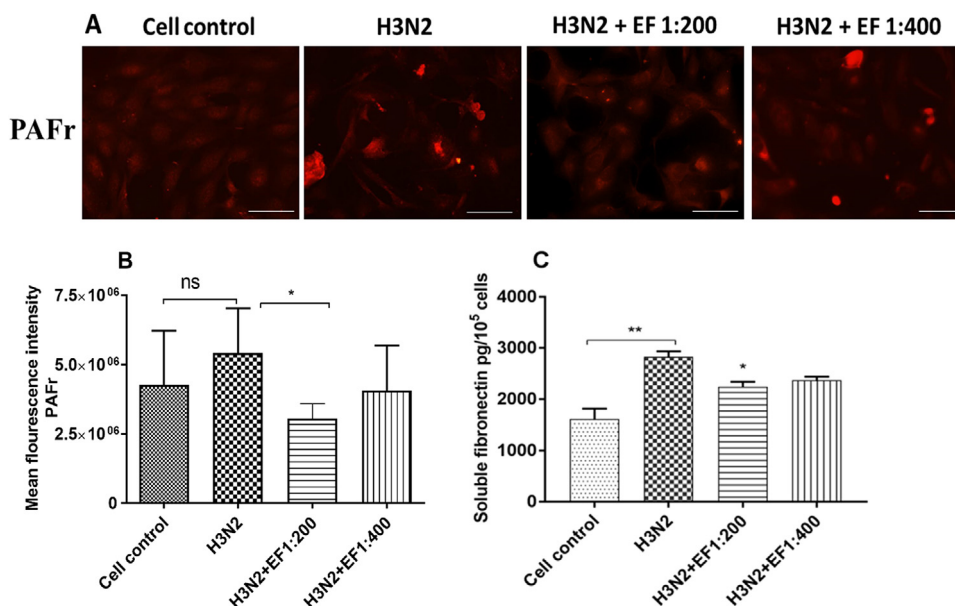
Fig. 5. Effect of Echinaforce® extract (EF) on the expression of H3N2-induced platelet activating factor receptor (PAFr) and fibronectin by BEAS-2B cells. (A) Surface PAFr expression was measured by fluorescence microscopy. The results are shown for one representative experiment (of three independent experiments), magnification 20×. (B) Graphs show mean fluorescence values ± SD of three independent experiments done in triplicates and fluorescent intensity was calculated using ImageJ; *$p < 0.05$, **$p < 0.01$, ns, not significant. (C) Fibronectin levels were determined using ELISA and normalized to cell number and expressed as pg/10^{5}cells. Scale bar = 100 µm.
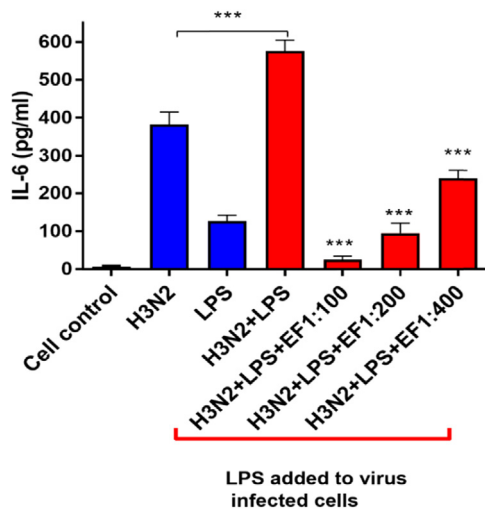
Fig. 6. BEAS-2B cells were inoculated with H3N2 or control medium was added and the cells were incubated for 60 min followed by stimulation with LPS (5 μg/mL) (H3N2 + LPS) for 60 min. Echinaforce® extract (EF) was added to the cells at different concentrations after virus infection and LPS stimulation. After a further 24 h, the supernatants were assayed to determine the IL-6 content. Data represent the values ± SD of three independent experiments; ***p < 0.001.

(Fig. 7A and B) and that EF downregulated virus-induced TLR4 expression however it was not statistically significant. NFκB was induced by H3N2 almost in parallel to the TLR-4 receptor, and again, EF reversed this induction almost completely at a concentration of 40 μg/mL (p < 0.05; Fig. 7A and C).

4. Discussion

Respiratory tract infections like colds and 'flu are usually self-limited viral infections that last an average of 7 to 10 days. Early clearance of the causative agent and limitation of the inflammatory response by the host are preconditions for an uncomplicated course of illness (Johnston, 1995). Virtually all known respiratory viruses have the potential to disrupt the physical barrier function of airway epithelium and to enhance the expression of membranous receptors that serve as targets for bacteria ligation and colonization (Folkerts et al., 1998). In these circumstances, the initial viral infections predispose the host airways to subsequent bacterial superinfection. Even the common rhinovirus is thought to multiply and hamper the function of the tissue in the lower respiratory tract (Sajjan et al., 2008). The clinical consequences include the immediate exacerbation of initially mild coughing or nasal complaints into severe bronchitis, pneumonia, or sinusitis, which are often accompanied by high fever. Sometimes, bacterial complications may occur even during convalescence and after the principal cold or flu is resolved. In this case, it can be difficult to distinguish bacterial infections from recurrent viral infections, which can also occur following increased expression of their binding receptors. Thus, prevention of bacterial superinfections is a major goal in the successful treatment of respiratory tract infections. Some practitioners are tempted to prescribe antibiotics to prevent bacterial colonization and complications even during the viral phase of cold episodes. As a consequence, respiratory infections are the most frequent reason for antibiotic misuse with the known consequences on development of resistance. Rigorous symptomatic treatment and suppression of inflammation on the other hand appear to increase the risk of complications, so additional pharmacological treatments are urgently needed.

Superinfections have their origin in viral respiratory infections, and they often occur in individuals with reduced immune func-

tion (Kash and Taubenberger, 2015; McCullers, 2014). Accordingly, interventions that offer an alternative to antibiotics are needed, possibly for use upstream of antibacterial treatment. This study investigated whether EF, a standardized extract prepared from freshly harvested *E. purpurea*, could prevent bacterial adhesion to virus-infected bronchial epithelial cells and whether it could limit cytokine superstimulation.

Our results showed that infection of BEAS-2B cells for 48 h with the influenza virus strongly induced the expression of the ICAM-1 receptor by the bronchial epithelial cells, thereby facilitating the adhesion of *H. influenzae*. This interaction could be blocked by preincubation of anti-ICAM monoclonal antibody with influenza virus infected epithelial cells. The results demonstrated that ICAM-1 is an important receptor for *H. influenzae* binding, which is in accordance with previous findings (Avadhanula et al., 2007). EF down-regulated ICAM-1 expression in a dose-dependent manner, as shown by immunocytochemistry, ELISA, and Western blot analysis (results not shown). As a consequence, the influenza-induced adhesion of *H. influenzae* was prevented even by an EF dilution of 1:800, which corresponds to 20 μg/mL of extract (dry mass).

Similar effects were seen for fibronectin and PAFr expression, which are additional putative bacterial receptors that are expressed by the airway epithelium. In contrast to ICAM-1, PAFr and fibronectin were constitutively expressed by the BEAS-2B cells, and there were only moderate increases in their expression following infection. Treatment of virus-infected bronchial cells with EF led to decreased expression of fibronectin and to decreased binding of *S. aureus*, but the effects were not as obvious as for ICAM-1-mediated *H. influenzae* binding. Intriguingly, very similar effects were observed for the level of fibronectin expression as for the binding of *S. aureus* to the epithelium. It has been proposed that fibronectin binding protein A (FnBPA) of *S. aureus* binds to fibronectin and then to α5β1 cell receptor, thereby fibronectin acts as a bridging molecule between *S. aureus* and host cell receptor (Sinha et al., 1999). The observed correlation suggests that soluble fibronectin may act as a bridging molecule for *S. aureus* and the host cell receptor.

Co-infection by viruses and bacteria induces dramatic production of inflammatory cytokines (cytokine storm), which can be lethal due to high fever, multi-organ failure, and sepsis. We did not use live bacteria for stimulation; rather we used LPS extracted from *P. aeruginosa*, which is the antigenic component of gram-negative bacteria. LPS induces an intracellular cascade by binding to TLR-4, leading to the expression of inflammatory cytokines (Shirey et al., 2013). As expected, the *in vitro* co-stimulation of virus-infected cells with LPS led to additive production of IL-6 and IL-8. The addition of EF reversed this excessive production of both cytokines in a dose-dependent manner.

NFκB is involved in the regulation not only of inflammation but also in the expression of many surface receptors, like TLRs and ICAM (Jang et al., 2009; Marr and Turvey, 2012; Min et al., 2012; Papi and Johnston, 1999). Interestingly, NFκB p65 was significantly down-regulated by EF in virus infected cells which in turn may modulate the expression of inflammation and also the expression of bacterial adhesion molecule including ICAM-1. Our results suggest NFκB as a therapeutic target for the prevention of secondary bacterial infection by EF. This study partly clarifies how EF exerts effects on bacterial superinfection. Inhibition of virus replication, virally induced changes in the host cells or a principal inhibition of bacterial binding are possible. Preliminary experiments have shown a basic down-regulation of EF on bacterial adhesion even in absence of viral infection (data not shown). Viral replication is less likely to be influenced because replication and spread are not affected by EF when given post infection (Pleschka et al., 2009). Finally, our cytokine and NFκB expression data indicate that virally induced changes in the host cell are affected by EF. For multicomponent
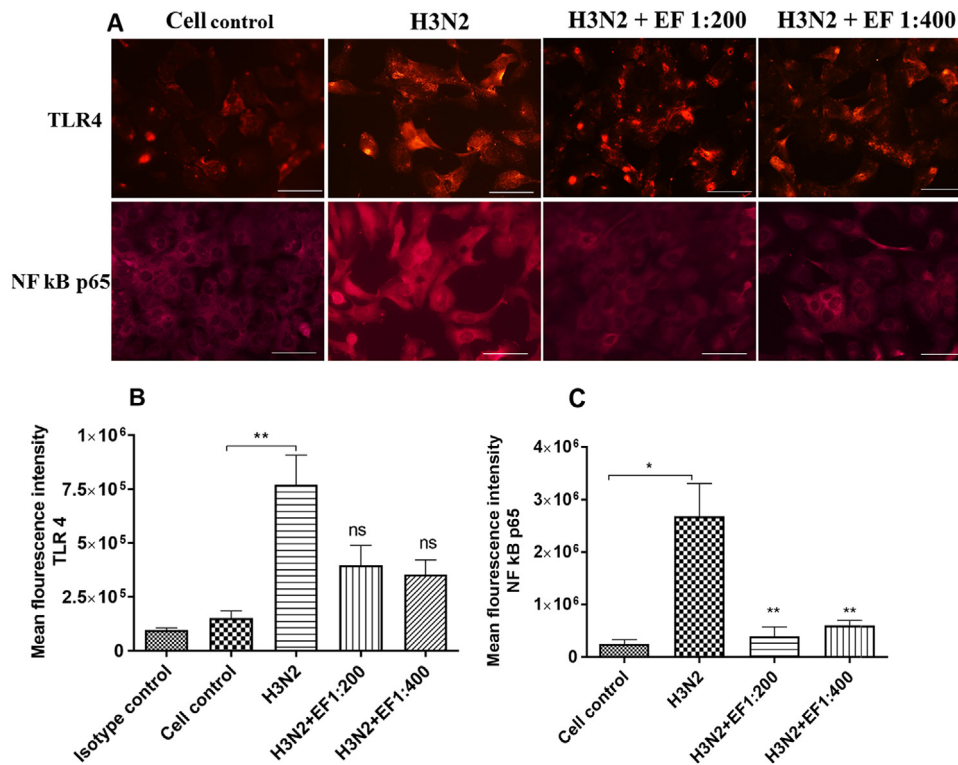
Fig. 7. The effect of Echinaforce® extract (EF) on influenza virus-induced TLR4 and NFκB p65 expression by BEAS-2B cells. (A) Surface TLR4 (upper panel) and intracellular NFKB p65 (lower panel) expressions were measured by fluorescence microscopy. The results are shown for one representative experiment (of three independent experiments), magnification 20×. (B) (TLR4) and (C) (NFκB p65). Graphs show mean fluorescence values ± SD of three independent experiments done in triplicates. Intensities were calculated using ImageJ; *$p < 0.05$, **$p < 0.01$. Scale bar = 100 μm.

extracts like EF, it is reasonable to assume that many points of activities are influenced, maybe to different extent. In our experiments we focused on the clinically relevant net effect, i.e. bacterial adherence and found receptors to play a central role. Further research is warranted to explain how EF extract could regulate bacterial adherence even in absence of viral infection, which might also be accomplished by receptor regulation (e.g. ICAM-1 and NFκB).

A meta-analysis has shown that standardized extracts from *E. purpurea* prevent recurrent infections and RTI complications. Schapowal et al. (2015) found that *E. purpurea* reduced the overall risk for respiratory complications, including sinusitis, bronchitis, otitis media, tonsillitis, and pneumonia by approximately 50% ($p < 0.0001$). That analysis was based on a sample of 2546 patients (Schapowal et al., 2015). The therapeutic effect was most prominent for pneumonia (68% reduction, $p < 0.0001$), which is often associated with *H. influenzae*. The observed pharmaceutical effects therefore largely correspond with observations in patients.

Interestingly, the meta-analysis also reported that *E. purpurea* extracts reduced recurrent viral infections, which could be explained by reduced ICAM-1 induction as well. Not only does the expression of ICAM-1 by bronchial cells increase the risk for bacterial colonization, this receptor is also the docking structure for rhinoviruses. Rhinovirus infections following influenza infections have been observed *in vivo* (Schapowal et al., 2015).

Our results, together with the published clinical data, indicate that *E. purpurea* may reduce the risk of complications from infections by preventing virus-induced bacterial adhesion and by cytokine storm inhibition. These results are highly important given the current view that there is massive abuse of antibiotics for respiratory illnesses, leading to drug resistance and newly-emerging bacterial strains, such as NTHi, for which there is currently no vaccine (Cerquetti and Giufrè, 2016). We showed that EF acts by regulating bacterial adhesion receptors and inflammation, so the

use of EF might represent a nonspecific and broadly effective strategy for preventing bacterial superinfections including pneumonia, bronchitis, and sinusitis.

5. Conclusion

We showed that the standardized antiviral herb *Echinacea purpurea* (Echinaforce) prevents adherence of clinically important pathogens *Haemophilus influenzae* and *Staphylococcus aureus* to influenza virus infected bronchial epithelial cells. The upregulation of bacterial ligands like ICAM-1, PAFr and fibronectin is inhibited by Echinacea together with the overexpression of proinflammatory cytokines. Further studies are needed to determine the exact molecular role of NFκB. Our results are in support of clinical data and suggest a beneficial use of *E. purpurea* for the prevention of secondary complications including pneumonia, sinusitis or bronchitis.

Conflict of interest statement

The authors Dr. Selvarani Vimalanathan and Dr. James Hudson have no conflicting financial interests.

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