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Bactericidal and anti-inflammatory properties of a standardized *Echinacea* extract (Echinaforce®): Dual actions against respiratory bacteria

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ABSTRACT

Common symptoms of upper respiratory infections, such as sore throat, cough, and inflammation, are often caused by bacteria, sometimes as a complication of virus infection. Extracts of *Echinacea purpurea* (Asteraceae) have been advocated traditionally for use by individuals suffering from these symptoms, although the underlying basis for the beneficial effects of *Echinacea* is not known. We hypothesized that *Echinacea* could inactivate certain respiratory bacteria and could also reverse inflammatory effects caused by these bacteria in epithelial cells. In order to test this we used a commercial standardized extract of *Echinacea purpurea* (Echinaforce®), and a novel cytokine array system designed to measure simultaneously the levels of 20 different cytokines secreted by bronchial epithelial cell cultures in response to infection. *Streptococcus pyogenes* (Group A Strep), which is often associated with sore throat and more severe pulmonary infections, was readily inactivated by *Echinacea*, which also completely reversed the cellular pro-inflammatory response. *Hemophilus influenzae* and *Legionella pneumophila* were also readily inactivated, and their pro-inflammatory responses reversed. *Staphylococcus aureus* (methicillin-resistant and sensitive strains) and *Mycobacterium smegmatis* were less sensitive to the bactericidal effects of *Echinacea* however, but their pro-inflammatory responses were still completely reversed. In contrast some other pathogens tested, including *Candida albicans*, were relatively resistant. Thus Echinaforce® exerts a dual action against several important respiratory bacteria, a killing effect and an anti-inflammatory effect. These results support the concept of using a standardized *Echinacea* preparation to control symptoms associated with bacterial respiratory infections.

Introduction

"Common colds and flu" affect millions of people every year and, although they are not usually life-threatening in healthy individuals, they are responsible for significant morbidity and the risk of respiratory complications, particularly in children and in immune compromised people (Roxas and Jurenka, 2007; Augustin et al. 2008). In addition the acute episode of a cold or 'flu is often accompanied by, and may even enhance, a significant bacterial infection, which may lead to more severe pulmonary and other diseases, as well as inflammatory activity (Wang et al. 2008; Beisswenger et al. 2009; Wang et al. 2009). The commonest bacterial isolates from people with cold syndromes include normal naso-pharyngeal flora, such as *Streptococcus pyogenes* (a group A Streptococcus, GAS), responsible for pharyngitis or "strep throat" (Diep and Otto, 2008); *Staphylococcus aureus*, which may be highly antibiotic resistant, eg MRSA (Rubinstein et al. 2008; Niki et al. 2008); as well as *Hemophilus influenzae* (Erwin and Smith, 2007), and other opportunistic organisms such as *Legionella pneumophila*, the agent of "Legionnaires disease" (Diederen, 2008; Isberg et al. 2009). Any of these bacteria can lead to serious complications (Augustin et al. 2008; Niki et al. 2008). In addition *Candida* yeasts are often present and may colonize respiratory tissues.

Different species and parts of *Echinacea* (Asteraceae) have been used traditionally in North America for the treatment of various symptoms of "colds and flu", as well as the treatment of candidiasis, respiratory diseases, and wound healing (Bauer, 1998; Barrett, 2003; Barnes et al. 2005). Many clinical trials have been conducted with *Echinacea* preparations, although with variable results; not surprising since the preparations had seldom been standardized or chemically characterized (Schoop et al. 2006; Vohra et al. 2009). In addition these trials were usually designed to evaluate the anti-rhinovirus potential of *Echinacea*, and did not address the issue of bacterial infections. Nevertheless previous studies suggested the potential of *Echinacea* in controlling bacterial infections (Sharma et al. 2008a). Furthermore, since certain *Echinacea* preparations can reverse the pro-inflammatory

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effects of some viruses in cultures of human bronchial cells (Sharma et al. 2009), we also hypothesized that respiratory bacteria might induce pro-inflammatory responses in these cells, and that *Echinacea* could also reverse this property.

In order to test this hypothesis we decided to evaluate the capacity of a standardized commercial preparation of *Echinacea purpurea* (Echinaforce®), with demonstrable potent antiviral activity (Sharma et al. 2009), for bactericidal activity against organisms associated with naso-pharyngeal and pulmonary infections, and in addition we examined anti-inflammatory activity, by means of cytokine antibody arrays, in a line of bronchial epithelial cells in which these bacteria induced the secretion of pro-inflammatory cytokines.

Materials & methods

Echinacea

The test preparation was Echinaforce® (A. Vogel Bioforce AG, Switzerland), a 65% ethanol extract of freshly harvested aerial parts of *Echinacea purpurea* (L.) Moench supplemented with 5% *E. purpurea* roots. HPLC analysis documented the presence of caftaric acid, 264.4 μg/ml; chlorogenic acid, 40.2 μg/ml; cichoric acid, 313.8 μg/ml; echinacoside, 6.9 μg/ml; PID 8/9 (alkylamide) 36.3 μg/ml; but no detectable caffeic acid, cynarin, or polysaccharides (Sharma et al. 2009). However it should be noted that biologically significant amounts of immuno-stimulating polysaccharides could be present (Bauer 1998). The recommended oral dose is approximately 1.0 ml Echinaforce in 10.0 mL water, corresponding to 1.6 mg/ml dry mass per volume and 6.5% ethanol v/v. No endotoxin was detected (<0.1 unit per ml), according to the Lonza endotoxin test kit (Lonza, Walkersville, MD).

Bacteria

The bacteria were standard ATCC (American Type Culture Collection) strains, purchased from PML Microbiologicals (Wilsonville Oregon). Most of the aerobes were propagated and assayed on sheep blood agar plates, at 35 °C. Anaerobes were cultivated in anaerobic chambers at 35 °C. *Legionella pneumophila* was cultivated on charcoal agar plates, *Mycobacterium smegmatis* on Middlebrook agar plates with a 5% CO_2 atmosphere, and *Hemophilus influenzae* on chocolate agar with a 5% CO_2 atmosphere.

In addition specimens of *Candida albicans*, a wild strain and an ATCC strain, and a wild strain of *Trichoderma viride* (courtesy Eduardo Jovel, University of British Columbia), were grown and assayed on Sabouraud-dextrose agar. All culture plates and related reagents were obtained from PML Microbiologicals (Oregon, USA), unless specified otherwise.

Bactericidal Assay

The standard assay method was as follows. Several isolated colonies of each bacterium were removed and dispensed into PBS (phosphate buffered saline) by vortex mixing, to give a homogeneous suspension of approximately 1×10^8 cfu (colony forming units) per ml. Aliquots of suspension were mixed 1:1 with the diluted extract to give final concentrations Echinaforce 1:100 (in PBS), equivalent to 160 μg/ml dry mass per vol plus 0.65% ethanol, or PBS alone, or 0.65% ethanol in PBS, in transparent sterile plastic tubes in ambient light or covered in aluminum foil (for dark exposure), for a period of 60 min. with incubation at room temperature (20 °C) on a rocker platform. The rationale for comparing light and dark exposure was based on evidence indicating the existence of photoactive ingredients in *Echinacea* extracts (Merali et al. 2003; Hudson et al. 2005).

Following the treatment, each mixture was serially diluted ($10\times$ dilutions) in PBS, and replicate 2.5 μl aliquots were spotted onto appropriate agar plates, divided into sectors for each dilution, and spread uniformly with sterile plastic loops, allowed to dry, and the plates incubated under the conditions described above. After incubation of the plates for 24–72 hours, depending on the bacteria, colonies were counted manually and compared with untreated samples.

Cytokine Analysis

BEAS-2B cells, a tracheo-bronchial epithelial cell line, and A-549 cells, a lung epithelial cell line, were obtained from ATCC. Human skin fibroblasts (courtesy Dr. Aziz Ghahary) were used in their sixth passage They were all propagated in Dulbecco MEM (Invitrogen, Ontario), supplemented by 5-10% fetal bovine serum (Invitrogen), without antibiotics or anti-mycotic agents. For experimental purposes, they were grown in complete medium, in 6-well trays, to produce freshly confluent monolayers. Bacteria were added to the cells at 70 cfu/cell for one hour, followed by aspiration and replacement by a 1:100 dilution of *Echinacea* in DMEM without serum, corresponding to a final concentration of 160 μg/ml (dry mass/vol). Cell free culture supernatants were harvested at 48 hours after infection (Sharma et al. 2008b) for ELISA tests and Quantibody cytokine array analysis. In additional experiments we showed that medium alone, with or without an equivalent volume of ethanol, and cell free supernatant derived from control uninfected BEAS-2B cells, did not induce the secretion of cytokines.

ELISA Assays

These were carried out according to the instructions supplied by the companies (either R&D for IL-8 and TNFα, or e-Bioscience, USA, for IL-6).

Cytokine Antibody Arrays

The Raybiotech fluorescent antibody array system was used. The array format (#QAH-CYT-1 from Raybiotech Inc. Norcross GA) consisted of quadruplicate antibody spots for 20 cytokines and inflammation-related mediators. The array slides were incubated with cell free supernatants, and treated and processed according to Manufacturer instructions. Data Acquisition was performed via a Perkin Elmer ScanArray Express laser microarray scanner (courtesy Prostate Research Centre, Vancouver) and subsequent quantification using ImaGene 8.0 software from BioDiscovery. Signal intensity medians were background corrected and the means of the replicates calculated. Some of the slide wells were treated with pure antigens (as part of the Raybiotech fluorescent antibody array system kit) in order to calculate a standard curve. The standard curve was used to convert the calculated mean intensities to concentrations (pg/ml).

Results

We evaluated the anti-bacterial (bactericidal) effects of Echinaforce against a range of common human pathogenic bacteria associated with respiratory infections. The data, summarized in Fig. 1, show considerable selectivity in anti-bacterial activities. Three of the pathogens, *Streptococcus pyogenes*,

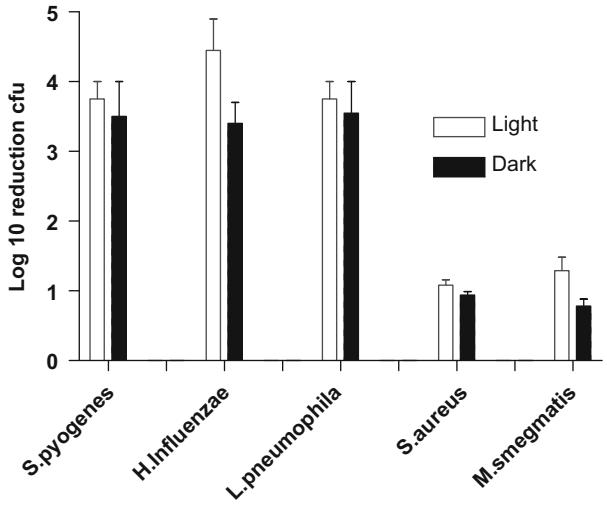


Fig. 1. Anti-bacterial Activity of Echinaforce. Equal volumes of the different bacteria, at concentrations of $\sim 6 \times 10^8$ cfu/ml in PBS, in duplicate, were separately incubated with 1:100 dilutions of Echinaforce in PBS, at 20 °C for 30 min, with or without exposure to light (white and black bars respectively), as described in Methods. Control cultures received no Echinaforce. Serial 10-fold dilutions were made and these were plated onto appropriate agar plates for enumeration of surviving cfu.

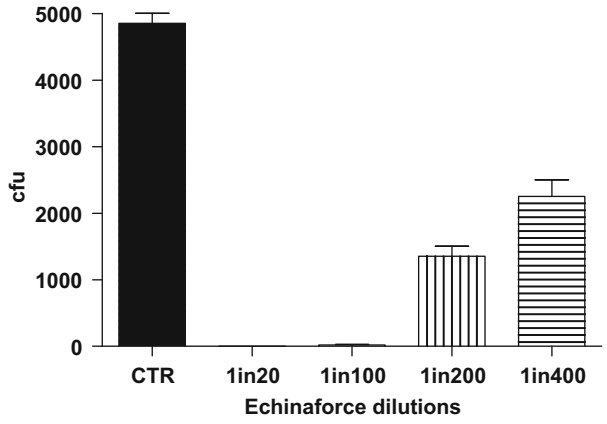


Fig. 2. Effect of Echinaforce Concentration on Anti-bacterial Activity. Different dilutions of Echinaforce were made in PBS, and incubated as described in Fig. 1 legend with aliquots of *S. pyogenes,* in duplicate, in ambient light. Serial 10-fold dilutions were made for enumeration of surviving cfu on blood agar plates. These were compared with untreated control bacteria (CTR). No colonies were observed with 1:20 and 1:100 dilutions. The recommended oral dose of Echinaforce is equivalent to 1:10 dilution in water.

Hemophilus influenzae, and *Legionella pneumophila*, were very sensitive to Echinaforce at a dilution of 1:100 or less (equivalent to < 160 μg dry mass/ml). The tests were run in the presence and absence of light exposure, in case photosensitizers were involved (Merali et al. 2003; Sharma et al. 2008a), although we observed few effects of light in this study. For each of these bacteria, ≥ 4 $\log_{10}$ inactivation was consistently obtained.

Fig. 2 shows a dose response graph for *S. pyogenes.* This illustrates the anticipated potency of the preparation in conditions of normal consumption. The higher concentrations completely inactivated the bacteria, while a dilution of 1:400 (40 μg dry mass/ml of extract) still showed substantial anti-bacterial activity.

The other bacteria tested were much less vulnerable to Echinaforce. *Staphylococcus aureus* (MRSA and MSSA) and *Mycobacterium smegmatis* showed approximately one $\log_{10}$ inactivation (Fig. 1), although such effects could still be significant since Echinaforce is routinely consumed in much greater concentration than that tested here. Other organisms found to be relatively resistant (< 1 $\log_{10}$ inactivation) included *Acinetobacter baumanii, Bacillus cereus*, *Bacillus subtilis, Enterococcus fecalis* (vancomycin resistant), *Escherichia coli, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa.* The two fungal organisms *Candida albicans* and *Trichoderma viride* were essentially resistant to Echinaforce.

Cytokines

Fig. 3 shows a typical set of results of a cytokine antibody array slide for *S. pyogenes*. The quadruplicate fluorescent spots are shown, and the corresponding cytokine concentrations in pg/mL are shown, for the main responders, below the slides. The colors indicate the relative degree of fluorescence, with red being the largest amount, and blue the least. The untreated cells secreted little in the way of cytokines, except for MCP-1, which was consistently secreted in small amounts by the BEAS-2B cells (Fig. 3 control slide). Echinaforce by itself did not have any effect, but infection by *S. pyogenes* induced substantial amounts of the pro-inflammatory cytokines IL-6, IL-8 (CXCL8), MCP-1, GMCSF, GROα, and smaller but significant amounts of IL-4 and MIP-1α. Values are also shown for VEGF, which was not significantly affected, for comparison. Echinaforce treatment resulted in complete abrogation of all the bacterially induced cytokines, such that the arrays for *S. pyogenes* plus Echinaforce were indistinguishable from control arrays (Fig. 3). In the case of MCP-1, even the low control value was no longer detected.

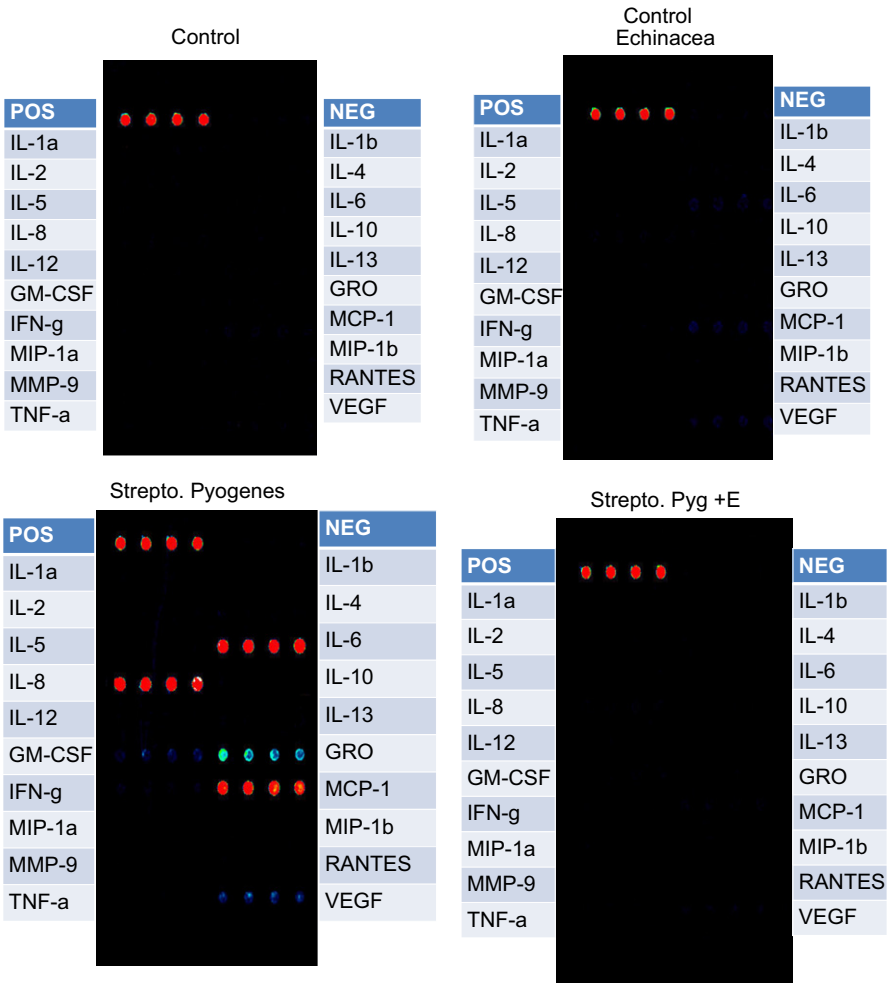
The pattern of cytokine induction was a little different for each bacterium tested, but in all cases *Echinacea* treatment reversed this induction. Array results for *S. aureus* (MRSA) are shown in Fig. 4 for the bacterial-induced cytokines and MRSA plus *Echinacea*, the latter being indistinguishable from control array (not shown, similar to Fig. 3). Concentrations of each cytokine are again displayed beneath the array figures. Thus even though *S. aureus* was relatively resistant to the bactericidal effects of *Echinacea*, the induction of cytokines (in this case IL-4, IL-6, IL-8, MCP-1, VEGF, and GROα) was still strongly inhibited.

ELISA assays for IL-6 and IL-8 (CXCL8) were carried out on the cell free supernatants for several of the bacterial infections, and these are summarized in Table 1 for BEAS-2B and A-549 cells. For all the organisms tested, *S. pyogenes, S. aureus, H. influenzae*, and *L. pneumophila, Echinacea* completely inhibited the secretion of IL-6 and IL-8. Similar results were obtained for ELISA's on supernatants of A549 cells. TNFα assays were also carried out, but none was detected in any of the cultures.

Discussion

Each of the potentially pathogenic bacteria examined in this study has the capacity to cause a variety of upper respiratory symptoms, resulting from initial interactions with epithelial cells of the oral and nasal mucosa, and various parts of the lung. General features of infection by these organisms include proliferation and spread of the bacteria with resultant cellular damage, often aided by products of bacterial virulence genes, and the induction of excessive pro-inflammatory cytokines, which can lead to migration of various types of leukocyte to the site of infection. A standardized herbal medicine that can reverse this inflammatory process, in addition to possessing bactericidal properties, could provide benefits to individuals suffering from the symptoms.

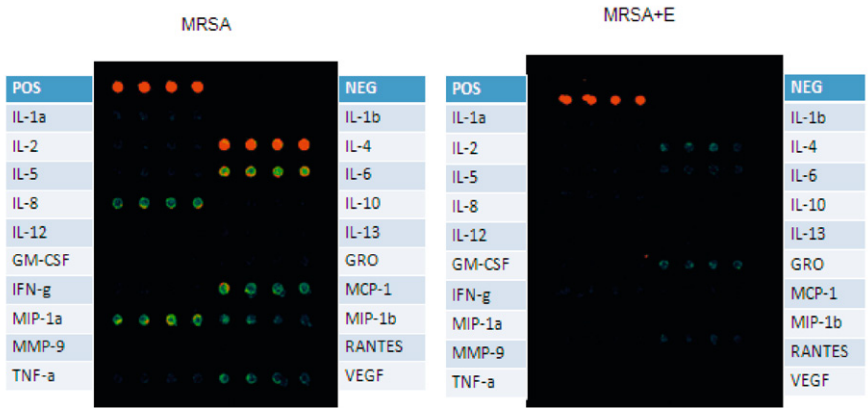
Streptococcus pyogenes (Goup A streptococcus, or GAS) is responsible for widespread infections, ranging from hundreds of



	IL-4	IL-6	IL-8	MIP-1α
C	92.82±11.8	13.51±15.7	7.88±8.9	67.68±22.32
C+E (Echinaforce)	80.23±24	10.67±8.77	9.2±10.2	54±14
S.pyogenes	130.24±11.9	6711.99±45	3615.59±14	141.47±28.9
S.p + E	81.4±34.9	10.67±47	6.87±12.9	56.2±11

	GRO-α	GM-CSF	MCP-1	VEGF
C	106.73±15.31	5.32±12.4	369.08±31.96	160.15±14.12
C+E	100.3±12.8	6.3±5.6	290±13.8	110.5±32
S.pyogenes	3002.31±56	87.27±10.7	2743.38±16	105.13±34.1
S.py + E	97.53±29	11.38±7.8	58.03±9.8	74.67±16.98

Fig. 3. Cytokine Antibody Arrays of Supernatants from Untreated and*S. pyogenes*infected cells, +/− Echinaforce. Replicate BEAS-2B monolayer cultures were infected with *Streptococcus pyogenes* (70 cfu/cell) for 1 hour, followed by Echinaforce at 1:100 dilution. Control uninfected cells were also treated, or not, with Echinaforce. At 48 hours after infection, cell free supernatants were harvested for analysis by cytokine antibody arrays (as described in Materials and Methods). The fluorescent quadruplicate spots of each cytokine are shown. Pos and Neg spots represent internal positive and negative controls. The relative intensities of the reactions are indicated by colour, with red being the highest reaction and blue the least. Intensity values were converted to cytokine concentrations, in pg/ml, by means of built-in standard curves (not shown) and these values are displayed below the slides for the most significant responders.



	IL-4	IL-6	IL-8	MIP-1α
MRSA	3402.7±35	169±27.2	27.29±5.8	1597±56
MRSA+E	663±25	40±7.9	4.76±2.7	331±22

	GRO-α	MCP-1	VEGF
MRSA	197.2±11.9	133.3±23	131±16.7
MRSA+E	53.8±9.8	73.94±15.8	80±12.9

Fig. 4. Cytokine Antibody Arrays of Supernatants from MRSA (methicillin reistant*S. aureus*) infected cells, +/− Echinaforce. Replicate BEAS-2B monolayer cultures were infected with MRSA (70 cfu/cell) for 1 hour, followed by Echinaforce at 1:100 dilution. Control uninfected cells were also treated, or not, with Echinaforce. At 48 hours after infection, cell free supernatants were harvested for analysis by cytokine antibody arrays (as described in Materials and Methods). The fluorescent quadruplicate spots of each cytokine are shown. Pos and Neg spots represent internal positive and negative controls. The relative intensities of the reactions are indicated by colour, with red being the highest reaction and blue the least. Only the MRSA +/− Echinaforce are shown. The uninfected +/− Echinaforce samples were indistinguishable from the controls (as in Fig. 3). Intensity values were converted to cytokine concentrations, in pg/mL, by means of built-in standard curves (not shown) and these values are displayed below the slides for the most significant responders.

Table 1
Effect of *Echinacea* (Echinaforce) on Bacterially-Induced IL-6 Secretion (ELISA assays).

	BEAS-2B cells pg/ml	Fibroblast cells pg/ml
Control	48 ± 0.09	94 ± 0.42
Control +*Echinacea*	53.6 ± 0.05	64 ± 0.89
H. influenzae	490.3 ± 0.2	890 ± 0.53
H. influenzae + Echinacea	194.6 ± 0.8	223.2 ± 0.89
L. pneumophila	297.6 ± 0.08	760 ± 0.79
L. pneumophila + Echinacea	83 ± 0.31	56.5 ± 0.65
MSSA	70.6 ± 0.07	245 ± 0.78
MSSA +*Echinacea*	37.3 ± 0.41	56 ± 0.86
MRSA	198 ± 0.076	543 ± 0.05
MRSA +*Echinacea*	66 ± 0.9	76 ± 0.68
S. pyogenes	600 ± 0.67	945.6 ± 0.56
S. pyogenes + Echinacea	64 ± 0.9	49.2 ± 0.49

millions of relatively mild cases of pharyngitis globally, to more severe toxic shock syndromes (STSS) and flesh-eating disease (necrotizing fasciitis, NF), the more severe symptoms being ascribed to inflammatory cytokines (“cytokine storms”). In addition several *Streptococcal* gene products or virulence factors have been described which aid the bacteria in persistence in oral epithelia and saliva and dissemination to other tissues (Tsai et al. 2006; Tart et al. 2007; Wang et al. 2008; Beisswenger et al. 2009). Consequently the dual actions of Echinaforce in killing the bacteria and reversing their pro-inflammatory activities could be a significant aid in combating such infections.

Staphylococcus aureus has long been recognized as part of the normal skin flora, but Methicillin resistant (MRSA) strains have been associated in recent years with increased frequency of hospital acquired infections, resulting in severe pneumonia (Diep and Otto. 2008; Rubinstein et al. 2008). In this study Echinaforce had relatively little effect on growth of MRSA or MSSA (methicillin sensitive *S. aureus*), but it was very effective in inhibiting the pro-inflammatory response to the bacteria, indicating at least partial benefits in counteracting the detrimental effects of MRSA infection.

Hemophilus influenzae is part of the normal naso-pharyngeal flora. Recently additional pathogenic strains have been associated with otitis media, chronic bronchitis, and pneumonia. Initial interaction with epithelial cells can result in pro-inflammatory cytokine secretion, via toll receptors and other mediators (Erwin and Smith 2007; Beisswenger et al. 2009). However the present study has shown that Echinaforce kills this organism readily and also inhibits the cytokine induction in bronchial epithelial cells.

Legionella pneumophila, associated with Legionnaires’ disease and sometimes more severe cases of pneumonia, is ubiquitously distributed in water and soil, from which the organism can be inhaled as an aerosol, and once inside alveolar macrophages localizes in a relatively resistant vacuole, in which it replicates (Diederen, 2008; Isberg et al. 2009). The organism is however very sensitive to Echinaforce and its induction of pro-inflammatory cytokines is inhibited by Echinaforce treatment.

In view of the widespread anti-inflammatory (cytokine inhibiting) properties of *Echinacea* (Sharma et al. 2008b, 2009), it is possible that Echinaforce could be beneficial in any respiratory bacterial infection, even in the absence of bacterial killing, and this benefit could extend to the secondary infections that often accompany viral “colds and flu”.

Therefore *Echinacea* has potentially multiple targets and mechanisms that could lead to alleviation of symptoms and disease associated with colds, 'flu and related infections. However at present we do not know which components of *Echinacea* are responsible for these benefits, although there are recognized chemical markers, such as those referred to in Materials and Methods for Echinaforce, which can be used to standardize the preparation and assure consistency. Thus *Echinacea* polysaccharides and alkylamides have been shown to possess immune-modulating activity in different model systems, although contributions from other constituents, such as caffeic acids and other phenolics, are feasible (Bauer, 1998; Barnes et al. 2005).

In conclusion, the standardized preparation Echinaforce® has two modes of anti-bacterial actions, a bactericidal effect against some of the bacteria incriminated in upper respiratory infections, and an anti-inflammatory effect which could reverse the inflammation caused by these bacteria.

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