

3-2-2012

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## Recommended Citation

Ayman M. Noreddin, Ghada Sawy, Walid Elkhatib, Ehab Noreddin and Atef Shibl (2012). Inhibition of Adhesion and Invasion of *Pseudomonas aeruginosa* to Lung Epithelial Cells: A Model of Cystic Fibrosis Infection, *Lung Diseases - Selected State of the Art Reviews*, Dr. Elvisegran Malcolm Irusen (Ed.), ISBN: 978-953-51-0180-2, InTech, Available from: <http://www.intechopen.com/books/lung-diseases-selected-state-of-the-artreviews/inhibition-of-adhesion-and-invasion-of-pseudomonas-aeruginosa-to-lung-epithelial-cells-a-model-of-cy>

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# Inhibition of Adhesion and Invasion of *Pseudomonas aeruginosa* to Lung Epithelial Cells: A Model of Cystic Fibrosis Infection

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## 1. Introduction

Cystic Fibrosis is a lethal autosomal recessive lung disorder resulting from a mutation in the transmembrane conductance regulator (CFTR) protein. The CFTR is a chloride channel encoded by a single gene on chromosome 7. More than 1200 mutations have been identified that result in defective or absent CFTR protein [1]. The most common mutation is  $\Delta F508$  (deletion of phenylalanine 508). This deletion leads to a non-functional, misfolded protein that is subsequently degraded [2]. While multiple organ systems are affected, CF is most often characterized by thickened mucus secretions and an inability to clear the lungs [1, 3-4]. In recent decades, improvements in life expectancy were achieved by new methods of antibiotic delivery as well as addition of new treatments to thin lung fluids. An example is the use of DNase to clear the buildup of extracellular DNA that occurs in the CF lung. This also relieves some of the exaggerated inflammatory response that is a characteristic of the CF lung [5].

Over their life time, CF patients experience multiple infections by various pneumonia-causing bacteria [6]. With more patients surviving to adulthood, chronic infections with *Pseudomonas aeruginosa* are coming to the forefront as a leading cause of death [7]. Problems presented by infected CF lung are multi-dimensional; the electrolyte balance and pH of the fluids are abnormal. The mucus is thick and of an alternative composition compared to normal lung and may contribute to colonization with *Pseudomonas aeruginosa* [2, 3, 5]. As such, research is multi-pronged and includes gene therapy to correct the defective protein, amelioration of inflammatory response and thinning of alveolar surface fluids [8, 9]. Significantly, *Pseudomonas* bacteria colonize the CF lung far easier than normal lung. Normal lung tissue has several naturally occurring defenses that work in concert with commonly prescribed antibiotics for recovery from lung infections [4, 10]. The CF patient appears to lack these natural defenses [1, 7].

*Pseudomonas aeruginosa* biofilms are difficult to treat due to multi-drug resistance. Many aspects of biofilms from physical structure to genetics and protein expression are under heavy investigation to elucidate the mechanisms by which biofilms develop antibiotic resistance. Within the biofilm matrix, bacteria are well protected from hostile environmental elements

and host defenses. Cells in different areas of the biofilm take on various patterns of gene expression, as such; biofilms are described as a community or as functioning similar to a multicellular organism [3]. Biofilms develop slowly, destroy surrounding tissue and evade even healthy immune systems. Standard dose antibiotic therapy can reduce biofilm as well as planktonic cells shed from the biofilm, but rarely eradicate the entire biofilm. This allows a cycle of subsequent regrowth and new shedding of planktonic cells which possess altered antibiotic resistance [11]. Some theories describe the heavy matrix, flow channels and oxygen gradients within the biofilm structure as contributing to problems with penetration of antibiotics into the biofilm. Others describe a counteraction of host defenses or alterations in gene and protein expression not only within the biofilm, but also in the CF host [11-13]. In this proposal we will discuss a novel experimental approach to study the pharmacodynamics of antibiotics used to treat *Pseudomonas aeruginosa* biofilms in CF lung versus normal lung.

*Pseudomonas aeruginosa* is an important human pathogen that can cause a wide range of infectious diseases that are associated with high morbidity and mortality rates [14]. Exposed tissue surfaces lined with epithelia are the most common targets of that pathogen e.g. epithelial surface of the eye and airways.

Adhesion of *Pseudomonas aeruginosa* to the airway cellular receptors is the initial event to establish respiratory colonization and infection [12, 15-16]. Interestingly, the affinity of *Pseudomonas aeruginosa* to bind to the inflamed or injured epithelial cells (CF or mechanically ventilated patients) is significantly higher than that to bind normal cell surfaces; thus preventing bacterial adherence may minimize lung pathology for high-risk patients [16].

The exploit of natural compounds, especially carbohydrates, to prevent infection has been considered in lung infectivity studies; since bacteria associated with pneumonia is known to bind the carbohydrate receptors on the pulmonary epithelium [12]. Dextran, a polymer of  $\alpha$  (1,6) linked D- glucose (branched at the 3 position), is a widely available polysaccharide that has been used in the *in vitro* and *in vivo* infection models to block the adherence of *Pseudomonas aeruginosa*, *Haemophilus influenzae* and *Staphylococcus aureus* [12, 15]. Berries of *Vaccinium* family (include cranberry) and their extracts that contain a saccharide effective in preventing the attachment of *Escherichia coli* to the uroepithelial receptors are well-known examples as well [12].

Recent studies suggested that many pathogenic organisms have the ability to invade and reside in the host cells during the early stage of infection. The discovery of antibiotic-resistant intracellular *Haemophilus influenzae* in the lung of individuals with Chronic Obstructive Pulmonary Disease (COPD) implicates intracellular bacteria as an important reservoir for the persistent infection [17]. *Pseudomonas aeruginosa* can also survive for up to 24 hours inside the lung epithelium without inducing cytotoxicity and is known to develop resistance to the treatments afterwards [17]. Accordingly, it is equally substantial to combat bacterial adhesion and invasion as a preventive step of the infection as well as the development of bacterial resistance.

It has been reported that some inexpensive natural extracts (e.g. Berries of *Vaccinium* family and dextran) have shown promising anti-adhesion properties with some micro-organisms (e.g. *Escherichia coli*) in the urinary tract infections, therefore, they might worth testing as topical therapeutics for prevention of *Pseudomonas aeruginosa* adhesion and invasion to lung epithelial cells [18-19].

In this study, the efficacy of some natural extracts with potential anti-adhesion properties and their combinations with ciprofloxacin was evaluated as blocking agents for the adhesion and the invasion of *Pseudomonas aeruginosa* PAO1 to A549 lung epithelial cells.

## 2. Building CF infection model

Ciprofloxacin and gentamicin sulfate were purchased from Sigma/Aldrich (St. Louis, MO, USA) and their stock solutions were prepared in 0.1N HCl (32 mg/ml) and water (32 mg/ml), respectively, and stored at -80°C. Before use; the antibiotic dilutions were made in F12-K cell culture medium (Cellgro, Mediatech, Inc, Manassas, VA, USA). Other Cell culture reagents were also obtained from Cellgro, Mediatech Inc. (Manassas, VA, USA). Dextran of molecular weight (MW) 40,000 was provided by Dr. John Brekke (University of Minnesota Duluth, MN, USA). Bacterial culture reagents were obtained from Difco Laboratories (Detroit, MI, USA).

The commercially available *Vaccinium macrocarpon* (cranberries) and *Glycine max* (soybean) were blended and extracted with distilled water (1:1 and 1:3.5, respectively) at room temperature.

Both crude mixtures were left to decant overnight at 4°C. Their supernatants were then subjected to two steps centrifugation at 4750 rpm for 20 min followed by 12,000 rpm for 10 min. The resultant supernatants were membrane filtered (0.22 µm, Millipore Corp. Billerica, MA). The sterile extracts were then stored at -80°C in 1ml aliquots till they were used in the adhesion or invasion assays.

The concentrations of the solid contents of such extracts were determined by evaporating the water content at 40°C and weighing the solid residues until constant weights were achieved.

*Pseudomonas aeruginosa* strain PAO1 was purchased from American Type Culture Collection (ATCC, Rockville, MD). *P. aeruginosa* PAO1 was grown until achieving the exponential growth phase over 16 hours at 37°C in Cation Adjusted Muller Hinton Broth (CAMHB) (Sigma-Aldrich, St. Louis, MO) from which 0.5 McFarland ( $1.5 \times 10^8$  CFU/ml) was prepared in F12-K tissue culture medium to initiate adhesion or invasion assays.

A549 lung epithelial cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD). The cells were passed in 75 cm<sup>2</sup> flasks (BD Falcon, San Jose, CA), and then  $1.5 \times 10^5$  cells were counted using the Hemocytometer and seeded into 12 well tissue culture plates (Becton Dickinson, NJ, USA). The cells were incubated in F12-K medium supplemented with 10% (vol/vol) Fetal Bovine Serum (FBS) at 37°C in the presence of 5% CO<sub>2</sub> to achieve confluence over 48 hours.

Minimum Inhibitory Concentration (MIC) and Minimum Cytotoxic Concentration (MCC) were two factors controlled the choice of the extract or ciprofloxacin concentrations selected in the adhesion and invasion assays. In order to prevent adhesion or invasion, the bacterial cells need to be alive but incapable of clinging to or intruding into the lung epithelial cells; therefore, sub-MIC had to be determined prior to the assays. On the other hand, the lung cells integrity was kept intact via using sub-MCC of the different tested agents. MICs were determined according to the Clinical and Laboratory Standards Institute (CLSI, M7-A7 and M100-S16, Criteria for *Pseudomonas aeruginosa*). Cytotoxicity of the tested agents against the A549 lung epithelial cell line was determined using crystal violet nuclei staining method as described by Gillies, *et al.* (1986) [10] with minor modifications. Briefly, confluent monolayer of A549 lung epithelial cell was incubated for 2 hrs with 200 µl of two-fold serial dilutions of different tested agents prepared in F12-K Medium, and then the cells were washed twice with phosphate-buffered saline (PBS). After rinsing, the cells were fixed with 1% (vol/vol) glutaraldehyde (Sigma/Aldrich, St. Louis, MO) and stained with 0.1% crystal violet (Fisher Scientific, Pittsburgh, PA) for 15 min. The dye was removed through multiples sterile water

rinsing and the absorbed crystal violet was then dissolved with 0.5% (vol/vol) Triton X-100 (Sigma/Aldrich, St. Louis, MO).

Absorbance was measured at  $\lambda$ 590 nm using Synergy™2 Microplate Reader (BioTech Instruments, Inc. Vermont, USA). Absorbance data were analyzed using Excel spread sheet and sub-MCCs were determined for different agents.

For SEM analysis, A549 lung epithelial cells were challenged with PAO1 cells for 1 hour, washed 3 times with F12-K medium and subsequently fixed with 5% (vol/vol) glutaraldehyde over 24 hours. The cells were then flushed with sterile deionized water to remove salts and dried before scanning with JSM-6490LV SEM (Peabody, Massachusetts) equipped with a tungsten filament, an accelerating voltages of 15-20 kV and a chamber pressure of 60-70 Pa according to the method described by Carterson *et al* with minor modifications [11]. All infectivity studies were carried out in a sterile class II biological safety cabinet (Sterilgard III Advance, Baker Company, Sanford, Maine, USA) according to the method described Plotkowski, *et al* [12] with some modifications. Initially, the confluent monolayer of A549 lung epithelial cells was incubated with 500 $\mu$ l fresh F12-K cell culture medium (control wells), 500 $\mu$ l of the natural extract or combination solutions (natural extract and ciprofloxacin) in F12-K medium at 37°C for 15 min followed by mixing with 500 $\mu$ l of 0.5 McFarland ( $1.5 \times 10^8$  CFU/ml) suspension of *P. aeruginosa* PAO1 prepared in F12-K medium for one minute to achieve homogenous bacterial distribution in Multiwell™ 12-well tissue culture plate (Becton Dickinson, NJ, USA). Ciprofloxacin (0.0625  $\mu$ g/ml), dextran (50.0 mg/ml), and the aqueous extracts of soybean (42.8 mg/ml), and cranberry (25.4 mg/ml) as well as the combinations of the three later agents with ciprofloxacin were applied as potential anti-adhesion agents in the aforementioned assay. The mixture was incubated at 37°C for 1 hour to establish bacterial adhesion. Each plate had a control well and all experiments were carried out in triplicates.

At the end of the incubation time, cells were gently washed thrice with FBS free F12-K medium to eliminate the non-adhered bacterial cells of *P. aeruginosa* PAO1 and then lysis of the mammalian cells was carried out with 1ml of 1% (vol/vol) tween-20 (Astoria-Pacific, Clackamas, Oregon, USA) at 37°C for 30 min.

After lysis, ten-fold serial dilutions for the *P. aeruginosa* PAO1 suspension in each well followed by plating onto Cation Adjusted Muller Hinton II (CAMHII) agar were carried out. All CAMHII agar plates were incubated at 37°C for 18 hours for determining CFU/ml through viable cell counting for different treatments as well as for the untreated control. Percentage adhesion for each treatment relative to the untreated control was calculated and the averages of triplicate experiments were expressed graphically  $\pm$  standard deviations (S.D). Data analysis was carried out using Graphpad prism 5 (GraphPad Software Inc. La Jolla, CA ) that utilized One Way Analysis of Variance (ANOVA) followed by Dunnett Multiple Comparison test to determine the significant treatment differences as compared to the untreated control.

Parallel experiments were carried out as described under adhesion assay except for an extra step performed to assess *P. aeruginosa* PAO1 invasion into A549 lung epithelial cells. After 15 min incubation with 500  $\mu$ L of the drug or the combination solutions in F12-K medium at 37°C, bacterial suspension (500  $\mu$ L of 0.5 McFarland) was added and mixed with the aforementioned solutions for 1min in Multiwell™ 12-well tissue culture plates. The plates were incubated at 37°C for 2 hours to allow for invasion. Gentamicin exclusion method as described by Fleiszig *et al* [13] was then used to kill the adherent bacterial cells. This step involved washing of the adherent cells with FBS free F12-K medium followed by incubation

of the infected cells with fresh F12-K medium containing 300µg/ml gentamicin for 1 hour at 37°C. After incubation, the dead bacteria were washed thrice with FBS free F12-K medium and the lung epithelial cells A549 were lysed with 1% (vol/vol) Tween-20 solution to determine the count of internalized *P. aeruginosa* PAO1 cells. The resultant cell suspensions were tenfold serially diluted, plated onto CAMHIII agar plates, and incubated as previously described under adhesion assay.

Percentage invasion for each treatment relative to the control was calculated and the averages of triplicate experiments were expressed graphically ± standard deviations (S.D). Data analyses were carried out using Graphpad Prism 5 (GraphPad Software Inc. La Jolla, CA) that utilized the one way ANOVA followed by Dunnett Multiple Comparison adjustment to determine the significance of treatment differences on the invasion of *P. aeruginosa* PAO1 as compared to the control.

Adhesion of *P. aeruginosa* PAO1 to lung epithelial cells A549 was visualized using Scanning Electron Microscopy (SEM). Imaging revealed that *P. aeruginosa* PAO1 could adhere to the lung epithelial cells without disrupting the lung cells morphology (Figure 1).

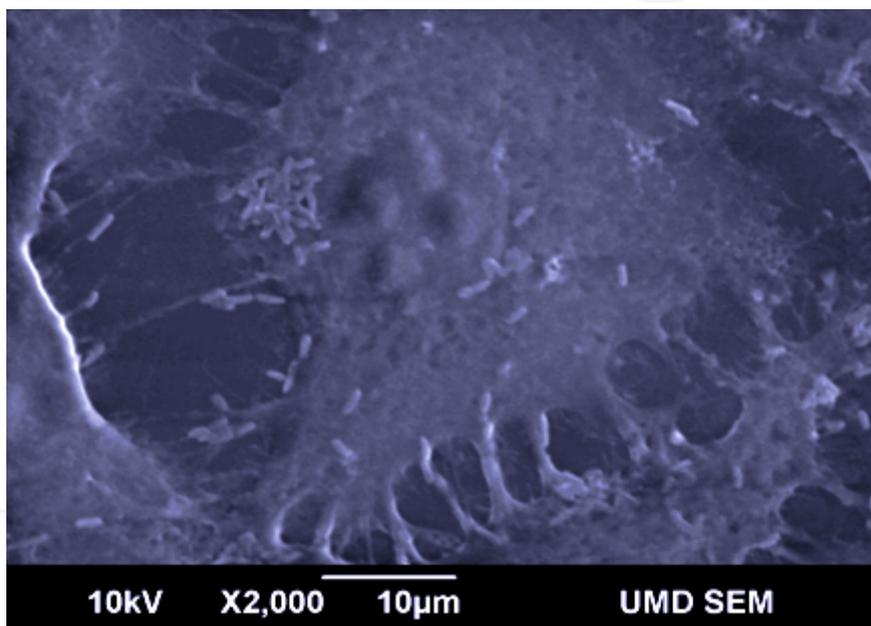


Fig. 1. Scanning Electron Micrograph illustrating the adherence of *Pseudomonas aeruginosa* PAO1 to A549 lung epithelial cells.

The soluble solid ingredients for the applied extracts were determined as described under Materials and Methods and their concentrations were 50.9 mg/ml and 85.7 mg/ml for the aqueous extracts of cranberry, and soybean respectively. Dextran was applied at a concentration level of 50.0 mg/ml.

MIC of ciprofloxacin and natural extracts against *P. aeruginosa* PAO1 and MCC of the same agents on A549 lung epithelial cells were determined according to CLSI criteria and the method described by Gillies *et al.* [10], respectively. The results showed that all the tested

natural extracts had relatively high concentrations of MICs against *P. aeruginosa* PAO1 and of MCC on A549 lung epithelial cells (Table 1). These results may indicate that these agents are relatively non-toxic to the A549 lung epithelial cells as well as they cannot inhibit the growth of *P. aeruginosa* PAO1 at the tested concentrations.

Treatment	MIC <sup>a</sup> (µg/ml)	MCC <sup>b</sup> (µg/ml)
Ciprofloxacin	0.125	> 64
Dextran	>10000	>10000
Cranberry Extract	>5090	>5090
Soybean Extract	>8570	>8570

<sup>a</sup>Minimum Inhibitory Concentration

<sup>b</sup>Minimum Cytotoxic Concentration

Table 1. MIC and MCC (µg/ml) values for ciprofloxacin and different natural extracts against *P. aeruginosa* PAO1 and A549 lung epithelial cells, respectively.

Based on the data shown in table (1), sub-MIC and sub-MCC concentrations of both ciprofloxacin and the natural extracts were selected in the adhesion and invasion assays to evaluate their effects, as single agents or in combination with ciprofloxacin, on *P. aeruginosa* PAO1 adhesion and invasion without interfering with the viability of the bacterial or mammalian cells. Accordingly, ciprofloxacin (0.0625 µg/ml), dextran (50.0 mg/ml), cranberry extract (25.5 mg/ml), and soybean extract (42.8 mg/ml) were selected at their subMIC/subMCC for testing the effects of the single treatments on *P. aeruginosa* PAO1 adhesion and invasion to the lung epithelial cells. In combination treatments, the effect of ciprofloxacin (0.0625 µg/ml) on the adhesion and invasion of *P. aeruginosa* PAO1 was assessed with halves of the aforementioned concentrations of the tested single agents.

### 2.1 Effect of natural extracts and their combinations with ciprofloxacin on the adhesion of *P. aeruginosa* PAO1 to A549 lung epithelial cells

Adhesion of the *P. aeruginosa* PAO1 to the lung epithelial cells was assessed through viable cell counting of the bound bacteria to the cell surface from each independent triplicate experiment and the untreated controls. Adhesion of *P. aeruginosa* PAO1 to A549 lung epithelial cells in the presence of different natural extracts, as single agents and in combination with ciprofloxacin, was expressed as the percentage of adhered bacterial cells to the epithelial cell surface and normalized to that of the untreated controls. In that regard, ciprofloxacin (0.0625 µg/ml), dextran (50.0 mg/ml), cranberry extract (25.5 mg/ml), soybean extract (42.8 mg/ml) could reduce *P. aeruginosa* PAO1 adhesion by 26.3%, 16.4%, 54.5%, and 45%, respectively compared to the untreated control. On the other hand, ciprofloxacin combination with dextran (25.0 mg/ml), cranberry extract (12.7 mg/ml), soybean extract (21.4 mg/ml) could reduce *P. aeruginosa* PAO1 adhesion by 87.5%, 100%, and 72%, respectively as compared to that of the control (Figure 2). Interestingly, combination of ciprofloxacin (0.0625 µg/ml) with cranberry extract (12.7 mg/ml) could completely inhibit the adhesion (0.0%) of *P. aeruginosa* PAO1 to A549 lung epithelial cells. Although dextran was relatively the least effective single anti-adhesion treatment (83.6%±12.1%), it achieved a significant higher effect upon combination with ciprofloxacin (12.5%±4.2%).

Soybean extract was an effective anti-adhesion agent (55.0%±6.4%) compared to ciprofloxacin (73.7%±2.08%) and their combination could synergistically (28.03%±0.65%) and significantly ( $P<0.0001$ ) reduce the ability of *P. aeruginosa* PAO1 to adhere to the A549 lung epithelial cells (Figure 2).

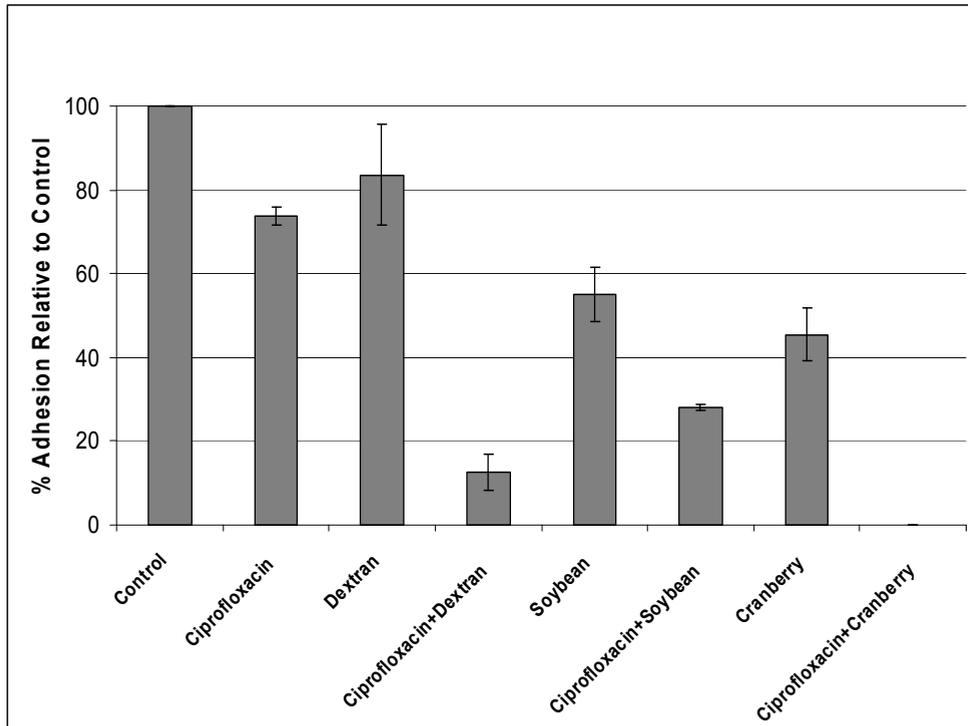


Fig. 2. The effect of different natural extracts alone and in combination with ciprofloxacin on the adhesion of *P. aeruginosa* PAO1 to A549 lung epithelial cells as compared to the untreated control.

### 2.2 Effect of natural extracts and their combinations with ciprofloxacin on the invasion of *P. aeruginosa* PAO1 to A549 lung epithelial cells

The ability of the adhered bacterial cells to internalize into the lung epithelial cells in the presence of different natural extracts, as single agents or in combination with ciprofloxacin, was assessed through calculation of percentage invasion of *P. aeruginosa* PAO1 from three independent experiments relative to the untreated controls. The results of gentamicin exclusion assay are believed to reflect the count of bacteria that has invaded cells [4, 13] with different treatments. Expectedly, both single cranberry and combination with ciprofloxacin were able to completely abrogate the invasion of *P. aeruginosa* PAO1 to the lung cells (0.0%). Although 45.5%±6.7% of the initial bacterial inoculum was able to bind to the epithelial cells after treatment in the presence of cranberry extract, none of this adhered population was able to penetrate the lung epithelial cells. Following the synergistic effect of cranberry extract, the combination of ciprofloxacin with both of soybean (17.6%±7.12%) and dextran

(11.8%±2.1%) achieved comparable and significant ( $P<0.0001$ ) anti-invasion effects compared to the control. Similar to the results for the adhesion assay, dextran and soybean were more effective in combination with ciprofloxacin in preventing invasion rather than the single agents (75.5%±2.1%) and (17.6%±11.8%) as shown in figure (3).

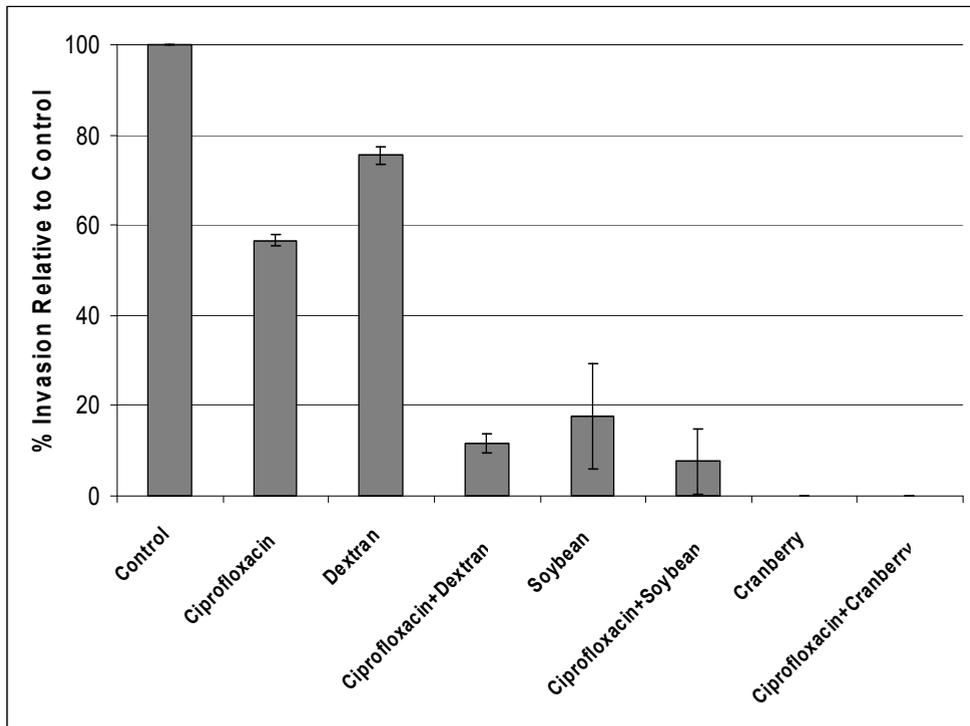


Fig. 3. The effect of different natural extracts alone and in combination with ciprofloxacin on the invasion of *P. aeruginosa* PAO1 to A549 lung epithelial cells as compared to the untreated control.

### 3. Discussion

*Pseudomonas aeruginosa* is a major cause for mortality seen in CF patients [6]. Adhesion and subsequent invasion of that organism to the lung epithelial cells are considered as the initial and substantial steps in the lung infection [5, 14-16]. Once the colonization of the organism is established, it is rarely eradicated. Several strategies have been developed to prevent *P. aeruginosa* infection in CF patients through different antibiotics and immunizations, but were not successful [8, 17-19]; therefore, development of other prophylactic measures as anti-adhesion and anti-invasion approaches is required. Ciprofloxacin, a fluoroquinolone, is considered as an antibiotic of choice for the treatment of the lung infections with *Pseudomonas aeruginosa* [7]. The current study aimed at introducing a new strategy to prevent *Pseudomonas aeruginosa* adhesion and invasion to the lung epithelial cells using different combinations of ciprofloxacin and aqueous extracts from widely available natural

products such as cranberry, and soybean. Dextran, which has been tested before in many occasions and found to be an effective anti-adhesion agent [2, 8, 11], was tested on that epithelial cell infection model as well to assess its anti-adhesion and anti-invasion activities compared to the other natural products, alone and in combination with ciprofloxacin.

The virulence factors of different *Pseudomonas aeruginosa* strains were attributed either to their direct host cell cytotoxicity or ability to adhere, invade, and survive within the epithelial cells. *P. aeruginosa* PAO1 virulence is categorized under the second types of isolates and invasion of that strain was suggested to contribute to biofilm formation and establishment of chronic lung infections [7]. Interestingly, the binding of *Pseudomonas aeruginosa* to the uninjured epithelial surfaces was found to be minimal. However, the binding ability of the organism increases dramatically in presence of epithelial surface inflammation or injury (such as in CF patients) [6, 20]. Scanning electron microscopy imaging revealed establishment of *P. aeruginosa* PAO1 binding to lung epithelial cells as the first step of *Pseudomonas aeruginosa* infection. Such binding did not affect the cells integrity or morphology (Figure1). Similarly, Fleiszig *et al.* [13] noted that integrity of the infected cells remains unaffected since the strain *P. aeruginosa* PAO1 does not use cytotoxicity as a virulence mechanism against the lung epithelial cells.

In order to exclude the inhibitory effect of ciprofloxacin and the applied natural extracts on *P. aeruginosa* PAO1 and A549 lung epithelial cells, the MICs of ciprofloxacin and the natural extracts against *P. aeruginosa* PAO1 and the MCCs of the same agents were determined to aid selection of the concentrations of different agents that will be tested on the adhesion and invasion models.

Unsurprisingly, the MICs and MCCs values could not be reached within the tested concentration levels for the different natural extracts, which were relatively high compared to ciprofloxacin, indicating that they are relatively non-toxic to the A549 lung epithelial cells as well as to *P. aeruginosa* PAO1. It has been reported that extracts of the natural sources are considered as endless reservoirs of safe and relatively inexpensive bioactive agents [9].

Dextran is a widely available polysaccharide that has been used in clinical settings as plasma expander [2]. It was previously tested as a carbohydrate treatment that blocks the epithelial glycoconjugates and impedes the bacterial legends from binding to pulmonary epithelial cell receptors [2]. Aerosolized dextran has been examined in a mouse infection model to prevent pneumonia caused by *Pseudomonas aeruginosa* and it could significantly reduce the development of pneumonia in the treated group relative to the untreated control animals, being an immuno-stimulant and a sputum rheology enhancer [2].

The combination treatment of dextran with ciprofloxacin disabled the internalization abilities of 85% of the bacterial population that adhered in the control experiments. Application of dextran resulted in an average of 14% reduction in both adhesion and invasion of *P. aeruginosa* PAO1 to the lung epithelia. This reduction is slightly less than that was found by Bryan *et al.* [2] who reported 35% reduction on *P. aeruginosa* PAO1 adhesion to nasal polyp primary culture cells when pretreated with dextran. Factors such as difference in the cell culture type or the experimental conditions may provide an explanation for the disparity in the activities. The same authors proposed that the likely mechanism through which dextran blocks bacterial adhesion was through the non-specific interaction with the epithelial cells; since pre-incubation of *P. aeruginosa* PAO1 with dextran before performing the infection abolished its anti-adhesion properties [2]. Consequently, the inhibitory action of dextran might not only involve *P. aeruginosa* PAO1 adhesion, but also other potential pathogens especially those targeting respiratory epithelia.

To our knowledge, there were no previous studies that reported the utilization of soybean extract as a potential anti-adhesion or anti-invasion treatment. The present study, however, proposes that the aqueous extract of soybean (*Glycine max*) is an additional promising treatment against adhesion and invasion of *P. aeruginosa* PAO1 to the lung epithelial cells. Compared to the control treatment, 28% and 17% of the applied bacterial inoculum were able to adhere and invade the lung epithelial cells, respectively, upon application of soybean extract combination with ciprofloxacin. These results suggest that on average half of the bacterial population that were able to adhere after treatment with soybean extract were not capable to invade the lung epithelial cells

Aqueous extract of cranberry (*Vaccinium macrocarpon*) was by far the most effective anti-adhesion and anti-invasion treatment when used in combination with ciprofloxacin. Cranberry belongs to *Vaccinium* family that has a wide spectrum of *in vitro* antimicrobial activity [21]. Many reports have demonstrated that proanthocyanidines and saccharides of cranberry were able to inhibit adhesion and invasion of some pathogenic microbes *in vitro*, and that cranberry juice clinically prevents urinary tract infection in women by inhibiting *Escherichia coli* adhesion to uroepithelial glycolipid receptors [3, 9]. The activity of the cranberry extract extended to provide protection against resistant strains of *Escherichia coli* by a mechanism that is unlikely to increase the selective pressure associated with antibiotics resistance [3]. Therefore, combining cranberry extract with ciprofloxacin sub-MIC may block the initial steps of infection, adhesion and invasion, as well as minimizing the development of bacterial resistance.

The results of this study may indicate that cranberry extract could turn the adhered bacteria unable to invade the lung epithelial cells since 45% of *P. aeruginosa* PAO1 population that were able to adhere to the lung cells under treatment with cranberry were, simultaneously, incapable of invading those cells and further molecular studies are required to elucidate this interesting point.

In published randomized clinical trial, cranberry juice was found to only affect harmful bacteria leaving normal bacterial flora unaffected; an observation that could suggest the therapeutic benefits of cranberry without having major unexpected side effects [21]. Cranberry juice, capsules and powder were also found to reduce urinary tract infection recurrences as well as the salivary counts of the bacteria causing dental carries [21-24].

The significance of proposing the anti-adhesion treatments emanates from the fact that the earlier the establishment of airway infection is prevented the better the expected therapeutic outcome; since the persistence of intracellular bacteria causes sloughing of epithelial cells containing these bacteria and may contribute to spreading of the infection, establishment of the biofilm, rendering the bacterial eradication by the antibiotics difficult and favoring the bacterial endurance in the host airway [7].

In light of these *in vitro* anti-adhesion and anti-invasion effects of the promising natural extracts, further *in vivo* studies are required to explore their putative applications as an alternative strategy to combat the respiratory infections and the potential reduction of the antibiotic resistance rates.

#### 4. Abbreviations

CF: Cystic Fibrosis

COPD: Chronic Obstructive Pulmonary Disease

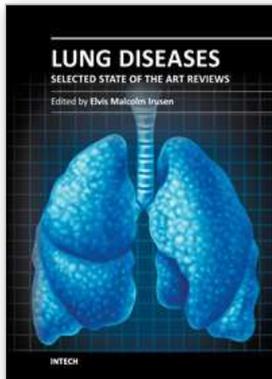
CAMHB: Cation Adjusted Muller Hinton Broth

MIC: Minimum Inhibitory Concentration  
MCC: Minimum Cytotoxic Concentration  
CLSI: Clinical and Laboratory Standards Institute  
SEM: Scanning Electron Microscopy  
ANOVA: Analysis of Variance

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## **Lung Diseases - Selected State of the Art Reviews**

Edited by Dr. Elvisegran Malcolm Irusen

ISBN 978-953-51-0180-2

Hard cover, 690 pages

**Publisher** InTech

**Published online** 02, March, 2012

**Published in print edition** March, 2012

The developments in molecular medicine are transforming respiratory medicine. Leading clinicians and scientists in the world have brought their knowledge and experience in their contributions to this book. Clinicians and researchers will learn about the most recent advances in a variety of lung diseases that will better enable them to understand respiratory disorders. This treatise presents state of the art essays on airways disease, neoplastic diseases, and pediatric respiratory conditions. Additionally, aspects of immune regulation, respiratory infections, acute lung injury/ARDS, pulmonary edema, functional evaluation in respiratory disorders, and a variety of other conditions are also discussed. The book will be invaluable to clinicians who keep up with the current concepts, improve their diagnostic skills, and understand potential new therapeutic applications in lung diseases, while scientists can contemplate a plethora of new research avenues for exploration.

### **How to reference**

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Ayman M. Noreddin, Ghada Sawy, Walid Elkhatib, Ehab Noreddin and Atef Shibl (2012). Inhibition of Adhesion and Invasion of *Pseudomonas aeruginosa* to Lung Epithelial Cells: A Model of Cystic Fibrosis Infection, Lung Diseases - Selected State of the Art Reviews, Dr. Elvisegran Malcolm Irusen (Ed.), ISBN: 978-953-51-0180-2, InTech, Available from: <http://www.intechopen.com/books/lung-diseases-selected-state-of-the-art-reviews/inhibition-of-adhesion-and-invasion-of-pseudomonas-aeruginosa-to-lung-epithelial-cells-a-model-of-cy>

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