

Poster Session 3 – Microbiology

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Antibacterial activity of total extracts and essential oil of *Nigella sativa* seed on mice

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Due to the increased resistance of bacterial pathogens to commercially used antibiotics and the growing interest in herbal medicine, more scientists are investigating the antibacterial activity of plant extracts and fractions. *Nigella sativa* is one of the native plants of Iran. There are in-vitro reports on the antibacterial activity of the total extract of this seed (Hanafy & Hatem 1991; Morsi 2000).

The aim of this study was to evaluate the antibacterial activity of total extracts (TE) and essential oil (EO) of *N. sativa* seed in mice infected with *Staphylococcus aureus* and *Escherichia coli*.

Different extracts of seed (methanol, chloroform and aqueous) were prepared. The proper dilutions were made by sterile normal saline.

Male mice, 25 ± 3 g, were infected with 0.1 mL (10^8 colony-forming units mL^{-1}) of suspension of each microorganism intraperitoneally. After 24 h, the infected mice were subjected to different doses of TEs and EO in groups of 8. Also, 8 infected mice received 33 mg kg^{-1} gentamicin (positive control group) and 8 mice received 0.1 mL of normal saline (negative control group). After 24 h , aspirated specimens from intraperitoneal fluid were cultured on soybean casein digest agar plate surface.

Results showed that the inhibitory effect of the methanol extract at a dose of 2.14 g kg^{-1} in mice infected with *Staph. aureus* was 87.5% ($P < 0.01$, Fischer test) and at dose of 1.2 and 2.14 g kg^{-1} in mice infected with *E. coli* was 100% ($P < 0.01$) compared with the normal saline group. While the aqueous extract did not show any inhibitory effect, for both microorganisms, the effect of chloroform extract at dose of 2.6 g kg^{-1} was equal to 33 mg kg^{-1} gentamicin. However EO at dose of 0.3 g kg^{-1} in mice infected with *Staph. aureus* and *E. coli* showed 100% ($P < 0.01$) inhibitory effect compared with normal saline. In conclusion, TE of *Nigella sativa* seed in methanol and chloroform and its EO has antimicrobial activity on Gram-positive and Gram-negative organisms tested, and the effect is dose dependent in mice.

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Morsi, N. M. (2000) *Acta Microbiol. Pol.* **99**: 63–74

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The antimicrobial effect of buffered benzoic acid in-vitro

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Controlled Therapeutics Scotland Ltd are developing a controlled-release formulation which delivers a triple antimicrobial therapy to combat mucositis, a severe side effect of radiotherapy in patients with head and neck cancer. The formulation delivers two antibiotics (colistin and tobramycin) and one antifungal (amphotericin B) into the buccal cavity by means of a muco-adhesive hydrogel polymer system. The colistin and tobramycin are contained within the hydrogel matrix and the amphotericin is spray-coated onto the surface of the dosage form. Benzoic acid is used in the loading of colistin and tobramycin to facilitate the uptake of the colistin and tobramycin actives into the hydrogel matrix. Benzoic acid is a preservative used in many oral and topical pharmaceutical preparations and is stated only to be active at a pH of 5 or less (Kibbe 2001). Although not classed as an active in the buccal hydrogel unit, the effect of benzoic acid from an antimicrobial perspective was investigated in-vitro.

The pH of saliva after insertion of the antimicrobial buccal insert was measured to determine the pH of solution to be used in testing. The average pH of saliva after 60 min was approximately 6.5. A suitable biological buffer (Buffered Peptone Water; Oxoid) was then used to prepare the benzoic acid dilutions. Buffering eliminates the antimicrobial effect of low pH.

The Minimum Inhibitory Concentration range for benzoic acid at pH 5 or below is stated to be 100–1600 μg mL^{-1} (Kibbe 2001). A range of benzoic acid concentrations was chosen to encompass this. The microorganisms chosen were QC type-strains representative of the species reported to be problematic during mucositis (Symonds et al 1996; Wijers et al 2001).

Tryptic Soya Agar (Oxoid) spread plates were prepared for each microorganism at a level conducive to confluent or near-confluent growth. Each microorganism was challenged with the full range of benzoic acid concentrations buffered to approximately pH 6.5 (from approximately 3.5).

Table 1 Microbial recovery on TSA spread plates after exposure to varying concentrations of benzoic acid

	Benzoic acid concn (μg mL^{-1})					
	0	200	500	1000	1600	2000
B. cepacia	++	++	++	++	++	++
C. albicans	++	++	++	++	++	++
E. cloacae	+++	+++	+++	+++	+++	++
E. coli	++	++	++	++	++	++
K. pneumonia	+++	+++	+++	+++	+++	+++
S. aureus	++	++	++	++	++	++

+ + + = confluent growth; + + = near confluent growth

It was found that the benzoic acid had no effect on the growth of the micro-organisms chosen (Table 1). This confirmed the theory that even at concentrations outside the normal MIC range the benzoic acid was inactive at pH 6.5, the pH of the saliva 60 min after insertion of a buccal unit.

Kibbe (2001) *Handbook of pharmaceutical excipients*. Pharmaceutical Press, London
Symonds, R. P., et al (1996) *Br. J. Cancer* **74**: 312–317
Wijers, O. B., et al (2001) *Int. J. Radiation Oncol. Biol. Phys.* **50**: 343–352

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A sensitivity test to evaluate tobramycin and ceftazidime in combination against clinical isolates of *Pseudomonas aeruginosa*

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Due to the rise in resistance in *Pseudomonas aeruginosa* in the clinical setting, the use of antibiotic combinations is increasingly necessary. The choice of which antibiotics to combine is generally based on clinical experience rather than laboratory evidence of efficacy. One of the limitations of testing antibiotic combinations is the labour-intensive nature of traditional tests such as the checkerboard method. Break-point sensitivity testing is a truncated version of the standard sensitivity test for individual antibiotics (MacGowan & Wise 2001). Fixed concentrations of the antibiotic are employed that conform to agreed cut-off points of sensitivity or resistance. The aim of this study was to investigate the activity of tobramycin and ceftazidime combinations based on breakpoint concentrations as an alternative method for combination testing.

The minimum inhibitory concentration (MIC) of tobramycin and ceftazidime for clinical isolates of *Ps. aeruginosa* was determined in Iso-sensitest broth based on the BSAC recommended broth macrodilution method (Andrews 2001). For combination tests, antibiotics were prepared to give final breakpoint concentrations (tobramycin 8 and 2 mg L^{-1}, ceftazidime 4 and 2 mg L^{-1}) in the following four combinations: high concentrations of both, alternate high and low concentrations of each, and low concentrations of both antibiotics.

Results of individual MICs were determined from observation of turbidity after incubation for 48h at 37°C. With individual agents, isolates were categorised as sensitive (S), intermediate (I) or resistant (R). Interpretation of the combination test was as follows: no growth in any tube, sensitive combination (SC); growth in all tubes, resistant combination (RC); growth in $\leqslant 3$ tubes, intermediate combination (IC). Results are shown in Table 1.

Table 1 Sensitivity of *Ps. aeruginosa* isolates to tobramycin and ceftazidime, alone and in combination

Sensitivity of isolates to individual antibiots		No. isolates	Results for combinations
Tobramycin	Ceftazimie		
S	R	4	4(SC)
I	I	1	1(SC)
I	R	6	4(SC) I(IC) 1(RC)
R	I	1	1(SC)
R	R	5	3(IC) 2(RC)

S, sensitive; I, intermediate; R, resistant; SC, sensitive, IC, intermediate; RC, resistant

Fifteen of the 17 isolates tested were resistant to ceftazidime and only four were sensitive to tobramycin. These four isolates were also sensitive to the combination. The benefits of the combination were seen where isolates were intermediate or resistant to either individual antibiotic. For these 13 isolates, 6 (46%) were classed as sensitive in combination, suggesting that it would be of clinical benefit to administer the two antibiotics together. This method employs only four tubes, making it much simpler to carry out than the microtitre combination method. Furthermore, since it employs critical concentrations of antibiotics established for break-point sensitivity testing, the outcomes are more meaningful to the clinical situation.

Andrews, J. M. (2001) *J. Antimicrob. Chemother.* **48** Suppl. S1: 5–16

MacGowan, A. P., Wise, R. (2001) *J. Antimicrob. Chemother.* **48** (Suppl. S1): 17–28

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An investigation into the effect of aluminium and iron on activity of tetracyline

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Tetracycline (Tc) is a broad-spectrum antibiotic with activity against both Gram-negative and Gram-positive bacteria. It works by binding to bacterial ribosome and prevents the formation of bacterial proteins. It is a commonly prescribed drug for treatment of infections. The efficacy of Tc is affected by the presence of metal ions particularly the di and tri-valent cations. Aluminium (Al^{3+}) and iron (Fe^{3+}) can act as hard acids and can theoretically interact with hard bases such as the oxygen atoms present in the Tc structure.

Numerous in-vivo studies have been carried out to look at interactions between Tc and Fe^{3+}/Al^{3+} identifying a reduced bioavailability of the antibiotic when co-administered with metal ion containing medicaments such as antacids (Gupta et al 1979). However, to date no work has been done on investigating the antimicrobial activity of a complex of the tetracycline molecule with either Al^{3+} or Fe^{3+}.

The effect of these ions on *E. coli* (W3110) was investigated in the presence of Tc. The strain used is known not to produce aerobactin and enterochelin siderophores which may adversely affect the results. Siderophores are iron-scavenging compounds produced by *E. coli* in situations of low iron levels to obtain iron from the bacterial surroundings.

Preliminary studies were carried out to determine stable sub-inhibitory concentrations of Al, Fe and Tc. These were chosen based on growth over 24 h; no precipitation was observed and no significant change in pH was seen over a 24-h period. Complexes, 1:1, 1:1/2 and 1:1/3 were formed using the sub-inhibitory concentrations of Al^{3+}, Fe^{3+} and Tc. The effects of these complexes on growth of *E. coli* were determined and compared with the effect of sub-inhibitory concentration ($4\,\mu g\,mL^{-1}$) of Tc.

Statistical analysis (anaylsis of variance) was carried out to determine the significance of these results.

Fe:Tc complexes had a significant inhibitory effect on *E. coli*. In contrast, Al:Tc complexes produced little significant effect on *E. coli* growth.

These studies therefore suggest that Fe^{3+} exhibits synergistic antimicrobial effects with Tc while Al^{3+} does not influence the activity of Tc. It is hypothesised that *E. coli* could take up the Fe:Tc complexes but was unable to take up Tc when it was complexed with Al^{3+}. This may be due to the increased selectivity of membrane protein receptors involved in iron uptake or increased antibacterial activity of the complexes compared with the Tc.

Gupta, et al (1979) *Inorganica Chemica Acta* **32**: L95–L96.

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An investigation into the influence of aluminium (III) and iron (III) on the antibacterial effects of oxytetracycline

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Tetracyclines are broad range antibiotic that have been used for over 50 years to treat Gram-positive and -negative infections as well as being used as growth promoters in animal feeds. The pharmacokinetic interaction between medicaments containing metal ions, such as antacids, and tetracyclines is well documented as forming unabsorbable chelates, which consequently reduces antimicrobial properties (Gugler & Allgayer 1990).

Many studies (Jogun & Stezowski 1975) have investigated the binding site of multivalent metal ions to oxytetracycline (including other tetracyclines). Surprisingly little work has been concentrated on determining the effect multivalent metal ions have on the potency of oxytetracycline as well as other tetracyclines.

In this study the influence of both aluminium (Al^{3+}) and ferric (Fe^{3+}) ions on the antimicrobial action of oxytetracycline using turbidometry was investigated. A strain of *Escherichia coli* (W3110) unable to produce the siderophores enterochelin and aerobactin was utilised to measure changes in oxytetracycline activity.

Following preliminary work, a concentration of $0.8\,\mu g\,mL^{-1}$ oxytetracycline was found to be sub-inhibitory and retarded the growth of W3110. Preliminary studies showed that all metal ion concentrations used were sub-inhibitory. This concentration was utilised throughout the study and complexed with various molar ratios of both Al and Fe (1:1, 2:1, 3:1, 1:10), (oxytetracycline: metal). Two controls were used, an antibiotic standard containing only oxytetraycline and an *E. coli* control.

Results obtained following 7 h growth curves suggested that a molar ratio of 1:1 (and 1:10) for both Al and Fe significantly inhibited the action of oxytetracycline ($P < 0.05$), whereas molar ratios of 2:1 and 3:1 were found to have no significant inhibitory effect on oxytetracycline. This was shown, by an increased cell number when a molar ratio of 1:1 of previously complexed oxytetracycline–Al^{3+}, resulted in $10.8 \times 10^6 \pm 0.051$ cells, compared with the oxytetracycline control ($8.1 \times 10^6 \pm 0.99$). A similar inhibition was observed when oxytetracycline was complexed with a 1:1 molar ratio of Fe^{3+}. Cell numbers were found to be $10.9 \times 10^6 \pm 0.072$ at 420 min compared with the oxytetracycline control ($8.1 \times 10^6 \pm 0.99$).

Oxytetracycline reversibly blocks the aminoacyl-tRNA A site at the bacterial ribosome, so oxytetracycline must cross the outer membrane (if present) and cytoplasmic membrane to gain access to the ribosome. It is possible that both Al and Fe, when complexed to oxytetracycline, alter its affinity for proton motive force coupled transport or phosphate bond hydrolysis coupled transport thus reducing cytoplasmic concentrations and impairing activity.

The authors would like to thank Dr Phil Rowe for his statistical input.

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Jogun, H. K., Stezowski, J. J. (1975) *J. Am. Chem Soc.* **98**: 6018–6026

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In-vitro development of gentamicin adaptive resistance in *Pseudomonas aeruginosa*

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Aminoglycosides are widely used in the prevention and treatment of serious infections. However, their efficacy in the treatment of *Pseudomonas aeruginosa* infection may be affected by adaptive resistance, a phenomenon that describes a reversible refractoriness by bacteria to the bactericidal action of an antibiotic following first exposure (Barclay & Begg 2001). Adaptive resistance is induced rapidly in *P. aeruginosa* following a dose of an aminoglycoside, developing within 2 h of first exposure, but is reversible over a number of hours, unlike true resistance if no further doses of aminoglycoside are administered. Knowledge of the time-course of adaptive resistance displayed by clinical bacterial strains would be useful when deciding the most appropriate dosage interval for aminoglycoside treatment, as a dosage interval longer than the period of adaptive resistance should ensure that adaptive resistance has resolved before second and subsequent drug exposure. The aim of this study was, therefore, to determine whether adaptive resistance was induced by gentamicin in clinical strains of *P. aeruginosa* and, if so, to determine the time-course of the adaptive resistance following a single exposure of gentamicin.

The minimum inhibitory concentration (MIC) of gentamicin for a control *P. aeruginosa* strain (NCTC 12934) and for clinical *P. aeruginosa* strains was determined by the broth microdilution method (Andrews 2001). Adaptive resistance in each strain was initiated with a 1-h gentamicin exposure at 8 × MIC. Following exposure, the gentamicin was removed by centrifugation and the test culture was resuspended in drug-free broth to yield a culture containing approximately 10^4 to 10^5 viable postexposure cfu mL^{-1}. A growth control was processed in an identical manner, and its final bacterial concentration was adjusted to approximate that of the test culture. Samples were taken from both test and control cultures immediately and at suitable intervals thereafter, and exposed to gentamicin at the MIC for 1 h. Colony count were performed before and after exposure, and the difference was expressed as bacterial killing (log decrease cfu mL^{-1}) after 1 h. Adaptive resistance was observed when the bactericidal effect in the test culture was less than that noted at the same time point in the control culture.

All of the *P. aeruginosa* strains tested developed adaptive resistance following exposure to gentamicin (Table 1). However, adaptive resistance was strain dependent with differences apparent in both the extent and the duration of the adaptive resistance displayed by different clinical strains.

Table 1 Bacterial killing of *P. aeruginosa* strain NCTC 12934 by gentamicin at the MIC

	Log decrease in cfu mL^{-1} at time (h):						
	0	1	2	3	4	5	6
Test	−2.48	−3.00	−1.60	−1.94	−0.39	−0.42	−0.26
Control	−5.00	−5.70	−5.36	−5.89	−2.96	−2.08	−1.37

This study has shown that clinical strains of *P. aeruginosa* develop adaptive resistance on exposure to gentamicin and highlights the importance of carefully selecting the dosage and dosage interval when using aminoglycosides to treat infection caused by these bacteria.

Andrews, J. (2001) *J. Antimicrob. Chemother.* **48** (Suppl. S1): 5–16

Barclay, M. L., Begg, E. J. (2001) *Drugs* **61**: 713–721

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Combination antibiotic therapy for treatment of cystic fibrosis lung infection

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Respiratory disease as a result of recurrent bacterial infections is the main cause of mortality and morbidity in patients with cystic fibrosis (CF), with more than 95% of deaths due to respiratory failure. Eradication of infecting bacteria such as *Pseudomonas aeruginosa* and *Burkholderia cepacia* in CF airways is virtually impossible and treatment of acute exacerbations usually necessitates the use of a combination of two or more antibiotics with different mechanisms of action in an attempt to prevent the development of resistance and provide synergistic activity. These antibiotic combinations are frequently selected on the basis of the susceptibility pattern of the organism against each individual antibiotic, and the assumption that certain combinations are synergistic. However, empirically chosen antibiotic combinations may not be synergistic and, therefore, not effective against multiresistant bacteria resulting in a less than optimal treatment outcome for the patient and the development of antibiotic resistance. The aim of this study, therefore, was to determine, using synergy testing, whether the empiric antibiotic combinations used to treat lung infection in individual patients demonstrated synergy in-vitro and, if not, to determine whether other antibiotic combinations would have been more effective.

The susceptibility to tobramycin, ceftazidime, meropenem, piperacillin and chloramphenicol of 6 *P. aeruginosa* and 3 *B. cepacia* strains isolated from CF patients admitted to the Belfast City Hospital with acute exacerbations of lung infection were determined using the broth microdilution method (Andrews 2001). Various paired combinations of the antibiotics were cross-titrated using the microdilution chequerboard technique as described previously (Scott et al 1995) and the effect of the selected antibiotic combination was interpreted as synergistic, additive, indifferent or antagonistic following calculation of the fractional inhibitory index (FIX) (Berenbaum 1978).

Seven of the nine isolates tested were resistant to tobramycin with 3/9, 4/9, 2/9 and 3/3 isolates tested resistant to ceftazidime, meropenem, piperacillin and chloramphenicol, respectively. Lung infection in 8 of the 9 patients was treated with a combination of tobramycin and ceftazidime and synergy testing of the tobramycin-ceftazidime combination against the bacteria isolated from these patients revealed an indifferent effect for 2 strains, an additive effect for 2 strains and an antagonistic effect for 4 strains. Infection with *B. cepacia* in the other patient was treated with a combination of tobramycin, meropenem and chloramphenicol and synergy testing revealed that the tobramycin-meropenem combination had an indifferent effect and that the tobramycin-chloramphenicol combination had an antagonistic effect. Tobramycin in combination with meropenem was synergistic against only 1 of the 9 strains tested whereas a tobramycin-piperacillin combination was not synergistic against any of the 6 strains tested. In addition, a tobramycin-chloramphenicol combination was antagonisitc against the 3 *B. cepacia* strains tested.

The results of this study highlight the importance of using synergy testing to determine which antibiotic combinations are synergistic prior to commencing dual or triple antibiotic therapy for the treatment of CF lung infection.

Andrews, J. (2001) *J. Antimicrob. Chemother.* **48** (Suppl. S1): 5–16

Berenbaum, M. C. (1978) *J. Antimicrob. Chemother.* **137**: 122–130

Scott, E. M., et al (1995) *Antimicrob. Agents Chemother.* **39**: 2610–2614

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In-vitro activity of anti-pseudomonal antibiotics against *Pseudomonas aeruginosa* and *Burkholderia cepacia* using standard, high and mixed inocula

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In-vitro antibiotic susceptibility tests are routinely performed to provide information that is used when choosing the most appropriate antibiotic therapy for the treatment of infection. The tests are highly standardised in terms of the composition of media, culture conditions and inoculum size used with a standard inoculum size of 5×10^5 cfu mL^{-1} usually employed. These standardised conditions may not reflect the in-vivo situation at the site of infection. For example, in cystic fibrosis patients with lung infection, the number of bacteria at the site of infection has been shown to be significantly higher (8×10^8 cfu mL^{-1} sputum) than the standard inoculum size used (Bilton et al 1995). In addition, there may be more than one infecting bacteria present at the site of infection and the killing of bacteria in mixed culture may not necessarily be identical to killing in pure culture. The aim of this study was, therefore, to determine the effect of both an increased inoculum size and the presence of mixed culture infection on the in-vitro susceptibility of *Pseudomonas aeruginosa* and *Burkholderia cepacia* to antipseudomonal antibiotics.

The minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of gentamicin (GENT), piperacillin (PIP), aztreonam (AZT) and ceftazidime (CAZ), for *P. aeruginosa* and *B. cepacia* strains were determined using the broth microdilution method (Andrews 2001) at both a standard (5×10^5 cfu mL^{-1}) and a higher (5×10^7 cfu mL^{-1}) inoculum size. The susceptibility to the antibiotics of selected *P. aeruginosa* and *B. cepacia* strains either in pure culture or in combination as a mixed culture was also determined using the broth microdilution method.

Of the antibiotics tested, piperacillin displayed the greatest inoculum effect with its inhibitory effect at the higher inocula between 8 and > 64 fold lower than that at the standard inocula for all of the strains tested (Table 1). The inoculum effect was not as apparent with the other antibiotics, with either less significant or no decreases in the inhibitory effect apparent at the higher inocula. When *P. aeruginosa* and *B. cepacia* strains were combined as a mixed culture, both strain dependent and antibiotic dependent increases in both the MIC and the MBC were apparent.

Table 1 Susceptibility of *P. aeruginosa* strains to antibiotics at standard and high inocula

		MIC (μg mL^{-1})			
Strain	Inocula	GENT	PIP	AZT	CAZ
6009	Standard	8	32	16	4
	High	32	> 256	16	16
6014	Standard	8	16	16	4
	High	32	256	16	4
NCTC					
12934	Standard	4	4	< 4	< 2
	High	8	> 256	8	128

This study has shown that when determining MICs and MBCs using in-vitro tests it is important that conditions reflect the in-vivo situation. In certain circumstances, it may, therefore, be appropriate to use a higher inoculum and when infection is caused by more than one strain of bacteria it may be necessary to test the efficacy of the antibiotics against the infecting bacteria in mixed culture.

Andrews, J. (2001) *J. Antimicrob. Chemother.* **48** (Suppl. S1): 5–16
Bilton, D., et al (1995) *Europ. Resp. J.* **8**: 948–953

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An investigation into the effects of aluminium and ferric ions on the activity of a sub-inhibitory concentration of ciprofloxacin

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Ciprofloxacin is one of the most potent of the fluoroquinolone antibiotics demonstrating a broad spectrum of activity against Gram-positive and Gram-negative bacteria. Cruz et al (1999) and others have demonstrated the ability of ciprofloxacin to chelate polyvalent cations in-vivo, impairing the absorption from the gastro-intestinal tract.

The aim of this study was to investigate the effect of two polyvalent ions (Al^{3+} and Fe^{3+}) on the antimicrobial action of ciprofloxacin. *E. coli* W3110 was used as this strain is known not to produce the two potent iron chelating siderophores, enterochelin and aerobactin, which may otherwise adversely affect the uptake of the ions under investigation.

Preliminary studies indicated that, at concentrations in the range 0.01–1 mM Al^{3+} and Fe^{3+}, there was no significant effect on the growth of *E.coli*, which remained stable over at least a 24-h period. Aqueous solutions of ciprofloxacin and Al^{3+} and Fe^{3+} (1:1, 2:1, 3:1 and 10:1 of ciprofloxacin–metal ion) were prepared 24 h before use to ensure sufficient time for equilibrium. *E. coli* growth was measured turbidometrically.

No statistically significant difference was observed between *E. coli* growth over 5.5 h for ciprofloxacin–aluminium ($P = 0.068$) and ciprofloxacin–iron ($P = 0.094$) over all concentration and ratio ranges of both Fe^{3+} and Al^{3+}. However, over 24 h, growth of *E. coli* with ciprofloxacin (0.0125 μg mL^{-1}) demonstrated a significant difference ($P \leqslant 0.002$ and $P \leqslant 0.001$ for ciprofloxacin combined with Al^{3+} and Fe^{3+}, respectively). The results suggest possible synergism between Al^{3+} and ciprofloxacin, and iron may be inhibitory to the action of ciprofloxacin.

Ciprofloxacin acts by inhibiting DNA-gyrase and thereby preventing negative supercoiling of bacterial chromosome. This complex is thought to pass through the bacterial porins present in *E. coli* more favourably than ciprofloxacin alone, possibly due to the change in the hydrophobicity of the molecule, (Piddock et al 1999), thereby enabling greater intracellular accumulation of the complex. It is possible that this complex may dissociate within the cell, releasing ciprofloxacin and metal ion.

While Fe^{3+} serves many important biological functions in *E. coli*, Al^{3+} is known to be toxic to the cell, and therefore the observed synergism may be a result of the combined bactericidal action of both Al^{3+} and ciprofloxacin. Al^{3+} toxicity may result from interactions with donor hard atoms present on base pairs of bacterial DNA, or via membrane damage (Suwalsky et al 2002). In contrast, Fe^{3+}may, on entering the cell, promote cell growth, or facilitate enzyme activity within the cell (Park & Gunsalus 1995).

Cruz, S. R, et al (1999) *Pharm. Acta Helv.* **73:** 237
Park, S. J., Gunsalus, R. P (1995) *J. Bacteriol.* **177**: 6255
Piddock, L. J. V., et al 1999) *J. Atimicrob. Chemother.* **43**: 61–70
Suwalski, M., et al (2002) *Coordination Chem. Rev.* In press

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Investigation into the influence of aluminium (III) and iron (III) on the antibacterial effects of herb oils

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The effect of alkali metal ions such as Al^{3+} and Fe^{3+} on the absorption of antibiotics has always been of great interest amongst researchers (Cruz 1999), with very little work done on possible synergism in terms of antibacterial activity. Tooray et al (2001) have demonstrated potentiation of activity with Al^{3+} and nalidixic acid.

In this study, cinnamon, clove, garlic and rosemary oils were selected to investigate their antimicrobial activity, with and without Al^{3+} and Fe^{3+}, on the growth of *E. coli*. The strain of *E. coli* (W3110) used was known not to produce the potent iron-chelating siderophores enterochelin and aerobactin, which may adversely affect iron effects. Preliminary data, using agar diffusion assays (Kumar et al 1998), showed both garlic and rosemary oils to have no inhibitory activity and these were not used in further investigation. For cinnamon and clove, MICs of 0.5% and 5% were determined, respectively. Al^{3+} and Fe^{3+} concentrations of 0.1 mM and 0.01 mM, respectively, were found to be sub-inhibitory and were consequently used in further studies.

Interaction studies in nutrient broth suggest statistically significant effects between 5% clove and metal ions after 24 h incubation (but with no effect for 0.5% cinnamon), giving the following viable counts in the presence of 5% clove oil with and without Al^{3+}: $1.93 \times 10^7 \pm 0.142$ (without), $1.049 \times 10^7 \pm 0.023$ (0.1 mM) and $1.3 \times 10^7 \pm 0.119$ (0.01 mM). Similarly, *E. coli* counts without/with Fe^{3+} were shown to be $1.56 \times 10^7 \pm 0.112$ (without), $0.83 \times 10^7 \pm 0.017$ (1 mM), $1.13 \times 10^7 \pm 0.023$ (0.1 mM) and $1.36 \times 10^7 \pm 0.30$ (0.01 mM).

Eugenol (the active antibacterial component of clove) produces its antibacterial effects by interacting at the *E. coli* membrane. Kim et al (1998) demonstrated an increase in the uptake of metal ions in the presence of eugenol resulting in a eugenol–metal ion complex accumulating in the membrane. This complex may dissociate and both entities produce their respective effects, eugenol disrupting membrane integrity and metal ions upsetting the ion concentration gradients resulting in destruction of cellular oxidation processes. Bacterial uptake of ferric complexes is likely to be through outer membrane proteins (OMP). Perhaps up-regulation of OMP in the presence of low metal ion concentrations is responsible for the increase in potentiation effects. Alternatively, specific ratios of metal ion to eugenol may be required to achieve high effect.

Cruz, M. S. R., et al (1999) *Pharm. Acta Helv.* **73**: 237
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Kumar, M., et al (1998) *J. Appl. Microbiol.* **84**: 213
Tooray, V., et al (2001) *British Pharmaceutical Conference* Abstract: 103

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Triclosan and tetracycline cross-resistance in *Escherichia coli*

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Triclosan is a broad-spectrum antimicrobial agent. It has found extensive use in beauty, healthcare and household products, particularly for the control of Gram-positive organisms (Bhargava & Leonard 1996). For many years it has been accepted that the primary target for triclosan is the bacterial cytoplasmic membrane; this non-specific mode of action has been considered to militate against the development of resistance. However, in 1998 an alternative mechanism of action was proposed by McMurry et al (1998a), identifying the membrane-bound enzyme enoyl reductase as a primary target. Mutations in this target, and multidrug efflux pumps, have been found to be responsible for low-level triclosan resistance.

Growth of *E. coli* K12 under triclosan selective pressure was used to develop and characterise resistant strains by reference to their minimum growth inhibitory concentrations (MIC). Reserpine addition (10–100 $\mu g\,mL^{-1}$) was employed to inhibit energy-dependent efflux pumps in an attempt to locate the mechanism of resistance. Resistant isolates were tested for cross-resistance to 25 and 50 μg tetracycline by an agar diffusion method.

Wild-type *E. coli* K12 exhibited a triclosan MIC of 0.25 $\mu g\,mL^{-1}$. Selective pressure increased the growth inhibitory concentration at least 20 fold, giving an MIC in excess of 5 $\mu g\,mL^{-1}$. Reserpine (100 $\mu g\,mL^{-1}$) reduced this value to 1 $\mu g\,mL^{-1}$. Zones of inhibition against tetracycline demonstrated an increased resistance in triclosan-mutants (Table 1).

Table 1 Tetracycline-induced zones of growth inhibition

E. coli strain	Presence of triclosan (1 $\mu g\,mL^{-1}$)	Zone of growth inhibition (mm)	
		25 μg Tet.	50 μg Tet.
Wild type	−	2	10
Tr resistant mutant	−	0	0
Tr resistant mutant	+	2	7

Tr, triclosan; −, absent; +, present; Tet., tetracycline

Wild-type *E. coli* can be trained to grow in elevated concentrations of triclosan. This appears to arise through a mutation selecting for the operation of a reserpine-sensitive efflux pump, an observation consistent with the studies of McMurry et al (1998b), who recognised increased expression of the AcrAB multidrug efflux pump. Low-level tetracycline resistance emerged as triclosan resistance developed. Interestingly, tetracycline sensitivity was restored in the presence of sub-MIC levels of triclosan, suggesting that these two agents share competitively the same efflux mechanism.

Triclosan, a widely used biocide, has the capacity to induce resistance in *E. coli* consistent with the appearance of a multidrug efflux pump. This mechanism may cause antibiotic cross-resistance to arise, raising concerns over the widespread use of triclosan.

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Minimum inhibitory concentrations of four herbal extracts for potential use in contact lens solutions

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With the increasing popularity of natural products, it is important that any beneficial claims product manufacturers make can be backed up with scientific data. *Calendula* has previously been shown to have antiviral activity (De Tommasi et al 1990) and antifungal activity (Kasiram et al 2000). *Echinacea* has been used therapeutically by native Americans as a remedy for eye conditions. *Achillea millefolium* has been found to have antifungal (Fiori et al 2000) and anti-inflammatory (Zitterl-Eglseer et al 1991) effects. *Euphrasia* has been used by ~5% of patients to supplement their glaucoma treatment, though there is no evidence that it has any beneficial effect (Rhee et al 2001). This communication describes an investigation of whether these natural products have any useful antimicrobial properties, with a view to their potential use in contact lens care solutions.

Three methods were used to determine the antimicrobial activity of the herbal extracts, using 10-fold serial dilutions of 1–1 $\times 10^{-7}$% w/v. For diffusion zone analysis, solutions of extract were added to wells in an agar plate inoculated with appropriate bacteria and left to incubate over night, after which the zones of inhibition were measured. For agar dilution, extract dilutions were mixed into nutrient agar, allowed to set and then surface inoculated with selected bacteria. The plates were incubated overnight to determine the lowest concentration of herbal extract that completely inhibited the growth of the bacteria (minimum inhibitory concentration — MIC). For broth dilution, MIC determinations were carried out by inoculating selected bacteria into tubes of nutrient broth containing increasing concentrations of extracts. After overnight incubation the tubes were examined for evidence of growth.

None of the methods of analysis revealed any indication of antimicrobial activity in any of the extracts. No MIC values could be determined. Solutions of higher concentrations were not checked as these were brown in colour and did not seem viable for addition to a contact lens solution.

There are many problems associated with natural extracts, especially when considering using them in contact lens solutions. These studies indicate that there would, however, be no antimicrobial advantage of including these products in contact lens care solutions.

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An investigation into the antibacterial mechanism of action of triclosan against *Escherichia coli* NCIMB 8879

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Triclosan, a broad-spectrum antimicrobial agent, has been used extensively for over 30 years. It is active against a range of Gram-negative and Gram-positive bacteria and is used regularly as a hand-wash for homes and hospitals. It is incorporated into numerous household products including toothpaste, deodorant and more recently chopping boards and children's toys.

Recent studies indicate that bacteria resistant to low levels of triclosan have mis-sense mutations in the gene, which encodes for the enzyme enoyl-acyl carrier protein reductase (ENR) (Mc Donnell et al 1998). ENR catalyses the final step in the type II fatty acid synthetase cycle, and is a major component of the membrane lipid biosynthetic pathway. If this enzyme is a specific cellular target for the action of triclosan, it might account for observed apparently non-specific perturbations to cytoplasmic membrane structure and function (Regos & Hitz 1974). This would have serious implications for the emergence of future resistance.

In this study, the single-target theory was tested by exposing non-growing *E. coli* cells to varying concentrations of triclosan. Under these conditions a pre-formed membrane structure will not be influenced by ENR inhibition. Experiments were performed at 37°C and employed aqueous solutions of triclosan prepared in HEPES buffer (pH 7.4).

Cells were exposed to a range of concentrations of triclosan and survivors determined at set intervals. Triclosan was neutralised by dilution in Letheen broth. Triclosan-induced potassium leakage was investigated by exposing cells to a range of concentrations of triclosan. At set time intervals samples were removed, filtered through a 0.4-μm filter and the amount of extra-cellular potassium determined by atomic absorption spectrophotometry. Triclosan's ability to modify proton flux across the bacterial membrane was investigated using a method based on that of Mitchell & Moyle (1967). Cells pre-treated with triclosan were exposed to a pulse of 10 mM HCl, and the subsequent rate of increase in pH measured.

At both bacteriostatic and bactericidal triclosan concentrations, potassium leakage was manifest, up to 40% of intracellular potassium lost at MIC levels giving a clear indication of membrane damage. Proton translocation was observed at sub-MIC and to a greater extent at bactericidal concentrations. This is an indication that triclosan acts as a lipid-soluble proton carrier, catalysing movement of protons across the membrane and uncoupling respiration and ATP synthesis, critically impairing cellular metabolic activity.

These studies indicate that triclosan can cause significant damage to the cell under conditions that limit the significance of the ENR system. Whilst ENR may be a target for the growth inhibitory properties of triclosan, it is likely that the disinfectant potential of the agent derives from additional target damage.

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Assessment of the activity of an electrochemically activated water against a glutaraldehyde-resistant clinical isolate of *Mycobacterium chelonae*

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Mycobacterium chelonae is a non-tuberculous, rapidly growing mycobacterium with a high level of resistance to antimicrobial agents. Although it exhibits a low level of pathogenicity, mainly nosocomial infection following accidental trauma or surgery, it is important that this strain is eradicated from washer disinfectors as it has been implicated in the formation of biofilms (Hall-Stoodley et al 1999) and in the misdiagnosis of TB (Heifets 2001). The purpose of this study was to investigate the efficacy of an electrochemically activated water (EAW) in its neat form and in a diluted form, against a washer disinfector isolate of this bacterium found to be resistant to glutaraldehyde. EAW is a liquid biocide consisting of a mixture of active species derived from salt by electrolysis thorough a proprietary electro-chemical cell; the main active agent is hypochlorous acid, (HOCl). EAW is a broad spectrum, non-toxic alternative to glutaraldehyde, used in conjunction with a washer-disinfector for the reprocessing of heat sensitive medical devices.

M. chelonae was grown for 3 days in Middlebrook 7H9 broth, washed and resuspended in deionised water (plus 5% calf serum), at a final stock concentration of 10^9 cfu mL^{-1}. EAW was generated using a Sterilox 2501 generator, (Sterilox Technologies, Stoke-on-Trent, UK). The cells were then added to neat EAW (1:9) and following a 5-min test period (equivalent to the contact time in a washer disinfector), the reaction mixture was added to neutraliser containing lecithin, Tween 80 and sodium thiosulphate (1:9). Serial dilutions were prepared with sterile deionised water and aliquots plated in triplicate onto Middlebrook 7H11 agar. Following incubation, for one week at 30°C, the colonies were counted and the concentration of surviving cells calculated. Dilutions of the EAW were prepared using sterile deionised water and a washed cell suspension was added to the various dilutions (1:99) to a final concentration of 10^4 cfu mL^{-1}. Following a 2-min test period (equivalent to the rinse cycle in a washer-disinfector), the reaction mixture was filtered through a 0.22-μm sterile filter which was washed with neutraliser, placed on a fresh Middlebrook agar plate and incubated as before. The resulting colonies were counted and the Log reduction calculated (Table 1).

Table 1 Activity of EAW against glutaraldehyde-resistant *M. chelonae*

T = 0 count (cfu mL^{-1})	Post-disinfection count (cfu mL^{-1})	Sterilox concn (% of neat)	Log_{10} reduction (cfu mL^{-1})	
			2 min	5 min
7.2×10^7	0	100	–	>7
7.2×10^7	0	100	–	>7
7.2×10^4	0	20	>4	–
7.2×10^4	0	15	>4	–
7.2×10^4	0	10	>4	–
7.2×10^4	0	5	>4	–
7.2×10^4	1.97	2	>3	–
7.2×10^4	TNTC	0	N/$	–

The EAW was very effective within 5 min in destroying 7.2×10^7 cfu mL^{-1} of the glutaraldehyde-resistant *M. chelonae* in the presence of a 0.5% organic challenge. These findings emphasise its efficacy in the disinfection/sterilisation of endoscopes and other heat sensitive medical devices. It was also very effective against 7.2×10^4 cfu mL^{-1} *M. chelonae* at dilutions as low as 5%.

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