

Novel Ferrocenyl Chalcone Compounds as Possible Antimicrobial Agents

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The increased presence of drug-resistant bacteria has quickly become a worldwide concern as infections spread from healthcare settings to the wider community. The swift spread of infections caused by bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) is influenced by factors such as misuse and abuse of traditional antimicrobial treatments and inferior drugs. Ferrocenyl chalcones, which are derivatives of plant-based flavonoids, have gained further attention from researchers because of their antimicrobial activity. Using 2-fold broth microdilution, results demonstrated that 5 of the 10 newly developed ferrocenyl chalcones, which contain increasing alkyl chains from 5-10 carbons on ring B, possessed greater antimicrobial activity against Gram-positive organisms than Gram-negative organisms. These novel compounds were active against 3 types of drug-resistant *S. aureus*, including a MRSA, and other non-resistant Gram-positive bacteria. The same compounds inhibited growth by potentially obstructing cellular respiration in Gram-positive bacteria. Images obtained through scanning electron microscopy revealed bacterial cells with severe external damage once exposed to a selected compound that showed activity. Findings indicate that these newly developed compounds could be important antimicrobial agents in the treatment of infections from clinically resistant bacteria.

Keywords Antimicrobial resistance; antimicrobial activity; ferrocenyl chalcones; lipophilicity; mechanism of action; cellular respiration; scanning electron microscopy

1. Introduction

Because of the dwindling supply of antibiotics, and the rise in multi-drug resistant (MDR) bacteria, there is a need for new antimicrobial agents (1). This drug resistance in microorganisms has resulted in the emergence of MDR bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Staphylococcus aureus* (VRSA) and carbapenemase-producing *Enterobacteriaceae* (CPE) leading to a greater prevalence in healthcare-acquired infections (HCAI) (2). Infections arising from these organisms have quickly become an issue of grave importance worldwide (3,4).

1.1 Emergence of colistin-resistant bacteria

Infections resulting from CPEs have driven greater use of colistin (polymixin E). This drug is a critical antimicrobial agent against some common MDR Gram-negative aerobic bacilli, including CPEs and has been considered as the last form of protection. The mode of action of colistin is to damage the integrity of the outer envelope of Gram-negative bacilli by causing instability of membrane-bound lipopolysaccharides (LPS) (5). This damage allows leakage of cellular matter, resulting in cell death, but the lethal effects on the human kidney prevented its use in routine antimicrobial therapy (6). Overuse of colistin has now resulted in infections caused by colistin-resistant CPEs. Initially, resistance was thought to result from chromosomal mutations (7) but recent studies have revealed that resistance can be facilitated by the transfer of plasmids containing the colistin-resistant gene known as MCR-1 (7). In a 2016 report by the European Centre for Disease Prevention and Control (ECDC), infections caused by this type of colistin resistance are described as a critical public health issue (ECDC, 2016).

1.2 Natural-based antimicrobial agents

Increasing prevalence of MDR bacteria has intensified research into natural-based compounds as a possible solution to this global concern (8,9). Potential sources of natural antimicrobial agents include soil and plants (10). In particular, researchers have discovered teixobactin, which is a compound derived from soil-based microbes (11). This chemical exhibits antimicrobial activity against *Mycobacterium tuberculosis* (12,13) and Gram-positive bacteria, including drug-resistant *S. aureus* (14) and *E. faecium* (15). Teixobactin inhibits bacterial cell wall synthesis by binding to lipids that are essential for cell wall integrity (16,17).

Another group of compounds that has gained interest are the chalcones, which are plant-based flavonoids (18). These biosynthetic intermediates are found widely in most plant material, including leaves and stems (19). These chemicals

are responsible for the colour of pollinating flowers and for protection against harmful ultra violet rays from the sun (20). Chalcones possess many biological activities such as anti-cancer, anti-parasitic, anti-fungal and antibacterial (21–26). From these chalcone compounds, medicinal chemists have synthesised derivatives that contain a ferrocene moiety (27,28).

Ferrocenyl chalcones have useful benefits including small size, increased lipophilicity for crossing cell membranes, they can be easily modified (29), and they show biological activities similar to those that were previously mentioned, especially their antimicrobial activity (30–33). In this study, ferrocenyl chalcone derivatives with increasing alkyl iodide chains were prepared [RS⁽²⁾] to determine their antimicrobial activity.

2. Antimicrobial activity of ferrocenyl chalcones derivatives

Due to their hydrophobic nature, ferrocenyl chalcones are usually dissolved in organic solvents such as ethanol, dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) (28). The ferrocenyl chalcone compounds that were provided by RS (UCLAN, UK) were highly water-insoluble because they contained alkyl iodide chains (methyl to decyl). Therefore, DMSO was the most favourable solvent used in this study because of its amphipathic feature, which allows for the delivery of lipophilic antibacterial agents, and because it is less inhibitive in terms of bacterial growth (34). For all assays, we prepared fresh stock solutions of each compound at 1 mg/ml in DMSO.

We assessed antimicrobial activity of each ferrocenyl chalcone solution in terms of their minimum inhibitory concentration (MIC) values against a panel of non-resistant and resistant bacterial clinical isolates as well as non-resistant laboratory-adapted organisms. *Staphylococcus aureus* NCIMB 8244, *Enterococcus faecalis* NCTC 12697, *Kocuria kristinae* NCIMB 8884, *Escherichia coli* NCIMB 9483, *Klebsiella pneumoniae* and *Salmonella serotype Manchester* NCTC 7832 were prepared by suspending at least 3–4 colonies of each organism in individual sterile 10 ml aliquots of sterile Oxoid Mueller-Hinton (MH) broth (Fisher Scientific, Loughborough, UK) and incubated for 15–20 minutes at 37°C in air while stirring. Each inocula was compared to 0.5 MacFarland standard. Suspensions were diluted 1:100 in sterile MH broth to gain starting inocula of 10⁵ per BSAC standards. Clinical isolates of non-resistant *E. coli*, fully sensitive *S. aureus*, resistant *S. aureus* (penicillin; erythromycin/penicillin/clindamycin) and a highly resistant MRSA were prepared as previously described. *K. kristinae* NCIMB 8884 was prepared 1:10 also according to British Society of Antimicrobial Chemotherapy standards (35).

2.1 Minimum inhibitory concentration assay

Antimicrobial activity of newly developed compounds is commonly determined using 2-fold serial broth microdilution (36–38). This method is used to measure MIC values, the lowest concentration of antimicrobial agent that inhibits growth of organism (39). Each ferrocenyl chalcone compound was diluted with sterile MH broth. 75 µl of each prepared inocula was added to an equal value of diluted ferrocenyl chalcone solution in Nunc 0.2 ml flat bottom 96-well 12-column microtitre plates (Fisher Scientific, Loughborough, UK). Column 11 was treated with either penicillin or oxytetracycline and column 12 was left untreated. Plates were then incubated at 37°C for 18–24 hours. Absorbance values were measured using a Rosys Anthos 2010 microplate reader (Salzberg, Austria) at 620 nm adapted from Medu (2013). Results from this assay indicated that the ferrocenyl chalcone compounds with shorter alkyl chains (from methyl to pentyl) were less effective than those compounds with longer alkyl chains (from hexyl to decyl). The mean (± SD) MIC values of the methyl to pentyl ferrocenyl chalcones were determined to be 0.125 mg/ml (± SD), while the mean (± SD) MIC values of the hexyl to decyl ferrocenyl chalcones ranged from 0.008 mg/ml (± SD) to 0.063 mg/ml (± SD) (Table 1). The MIC values of the ferrocenyl chalcone compounds were within the reported values for penicillin (0.00015–0.128 mg/ml) against *S. aureus* and *E. faecalis*, and for oxytetracycline, an analogue antibiotic of tetracycline, against *Enterobacteriaceae* (0.00025–0.128 mg/ml) (35). However, growth inhibition of Gram-negative bacteria may also have resulted from exposure of the organisms to DMSO. DMSO has been shown to have an inhibitory effect at percentages equal to or above 12.5% v/v (34). The latter group of compounds also showed greater activity against Gram-negative bacteria than Gram-positive bacteria (Table 1). The MIC values of the hexyl to decyl ferrocenyl chalcone compounds are also equal to the minimum bactericidal concentration (MBC) of the same group of compounds for non-resistant *S. aureus* NCIMB 8244, *E. faecalis* NCTC 12697 and *K. kristinae* NCIMB 8884 (Table 1). MBC is defined as the lowest concentration at which an antibiotic either completely inhibits bacterial growth or facilitates the reduction of the bacterial population to ≤ 99.9% (40).

Table 1 Mean (\pm SD) MIC values of 10 ferrocenyl chalcone compounds against non-resistant and resistant laboratory-adapted bacteria and clinically isolated bacteria. CRH = Chesterfield Royal Hospital. *Minimum Bactericidal Concentration of the compounds.

Organism	Mean (\pm 0.000, n=6) MIC (mg/ml)									
	Methyl	Ethyl	Propyl	Butyl	Pentyl	Hexyl	Heptyl	Octyl	Nonyl	Decyl
<i>S. aureus</i> NCIMB 8244	0.125	0.125	0.125	0.125	0.125	0.063*	0.063*	0.063*	0.063*	0.031*
<i>K. kristinae</i> NCIMB 8884	0.125	0.125	0.125	0.125	0.125	0.031*	0.008*	0.016*	0.016*	0.016*
<i>E. faecalis</i> NCTC 12697	0.125	0.125	0.125	0.125	0.125	0.063*	0.063*	0.063*	0.063*	0.063*
<i>S. aureus</i> Fully Sens. (CRH)	-	-	-	-	-	0.125	0.063	0.063	0.063	0.063
<i>PEN-resistant S. aureus</i> (CRH)	-	-	-	-	-	0.063	0.031	0.063	0.063	0.063
<i>PEN/ ERY/ CLI-resistant</i> (CRH)	-	-	-	-	-	0.063	0.031	0.063	0.063	0.063
<i>MRSA</i> (CRH)	-	-	-	-	-	0.063	0.063	0.063	0.063	0.063
<i>E. coli</i> NCIMB 9483	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125
<i>K. pneumoniae</i> (IH)	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125
<i>Salmonella "Manchester"</i> NCTC 7372	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125
<i>E. coli</i> Fully Sens. (CRH)	-	-	-	-	-	0.125	0.125	0.125	0.125	0.125

2.2 Inhibitory activity of ferrocenyl chalcone chemicals

The difference in MIC values with respect to the Gram-negative and Gram-positive organisms may be because of increasing alkyl chain length of the ferrocenyl chalcone chemicals. One possible reason that contributed to the difference in activity is that the compounds may pass across the thick hydrophilic peptidoglycan layer of the Gram-positive bacteria because of the amphipathic DMSO. The long chains may become trapped in the cell membrane allowing the attached ferrocenyl groups, which are relatively smaller than the alkyl chains, to enter the cytoplasm. Since Gram-negative bacteria have outer envelopes with membrane transporter proteins such as porins, followed by thin peptidoglycan layers and cell membranes in their cellular envelopes, entry into these cells may be more difficult (41).

These porins allow hydrophilic compounds to enter, while hydrophobic compounds may diffuse across the lipid bi-layer of the outer envelope (42). However, because of the fluidity of the outer lipid bi-layer (43), the long alkyl chains of the ferrocenyl chalcones may become trapped in the outer envelope and would be unable to cross the peptidoglycan layer and cell membrane into the cells. Another possible reason is that the organisms such as *E. coli* have become used to living in enriched media, which promotes vigorous growth (44). The ferrocenyl chalcone compounds exhibited antimicrobial activity against bacteria that were resistant to penicillin (PEN), clindamycin (CLI), erythromycin (ERY), and a MRSA that was resistant to penicillin, flucoxacillin, trimethoprim, ciprofloxacin and a cephalosporin. The modes of action of these antibiotics include cell wall synthesis inhibition (45), protein synthesis inhibition (46), blockage of essential bacterial reductases (47) and the inhibition of DNA replication (48). The possible antimicrobial mechanism of the ferrocenyl chalcone compounds was further explored using a bacterial cell viability assay.

3. Bacterial cell viability assay

In order to determine a potential mode of action of the ferrocenyl chalcones, cell viability, in terms of respiration, can be used. The assay involved using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), a positively charged compound that can easily diffuse into cells (49). The yellow MTT compound is reported to be reduced to the purple formazan product by a dehydrogenase system of enzymes within the cell (50). Once formed, the formazan product, which is insoluble in water, can be dissolved in an organic solvent such as dimethyl sulfoxide and then measured by spectrophotometry at 570 nm (50). Although the exact mechanism is not known, the process occurs in actively respiring cells (51). The amount of formazan present is proportional to cell viability. Bacterial cell viability of resistant and non-resistant bacteria at MIC was determined by inoculating 96-well microplates as described above, followed by the addition of 10 µl of MTT solution (5 mg/ml) (Sigma-Aldrich, Dorset, UK). Plates were incubated at room temperature for 3 hours followed by the addition of 50 µl of DMSO. Absorbance values were measured at 570 nm.

The results of the assay of non-resistant Gram-positive laboratory organisms demonstrated that the percentage of actively respiring cells, in terms of formazan product observed (Figure 3-1) decreased after exposure to ferrocenyl chalcone compounds at the MIC value. A mean percentage of 0% was observed for *S. aureus* NCIMB 8244 when exposed to hexyl, octyl and nonyl. Similar percentage were seen for *E. faecalis* NCTC 12697 when exposed to hexyl, heptyl and octyl. For *K. kristinae* NCIMB 8884, mean percentage of 0% were seen for hexyl and heptyl. The highest percentage was measured for *S. aureus* NCIMB 8244 after incubation with decyl (4.241%). In the MTT assay of resistant and non-resistant Gram-positive clinical isolates the percentage of actively respiring cells, in terms of formazan product observed (Figure 3-2) also decreased after exposure to chalcones at the MIC value. Mean percentage of 0% were seen for fully sensitive *S. aureus* (CRH) when exposed to hexyl, heptyl and octyl, for PEN-resistant *S. aureus* (CRH) when exposed to heptyl, octyl, nonyl and decyl, for PEN/ERY/CLI-resistant *S. aureus* (CRH) and MRSA when exposed to hexyl, heptyl, nonyl and decyl ferrocenyl chalcones. The highest percentage was determined for fully sensitive *S. aureus* (CRH) after incubation with nonyl (2.242%).

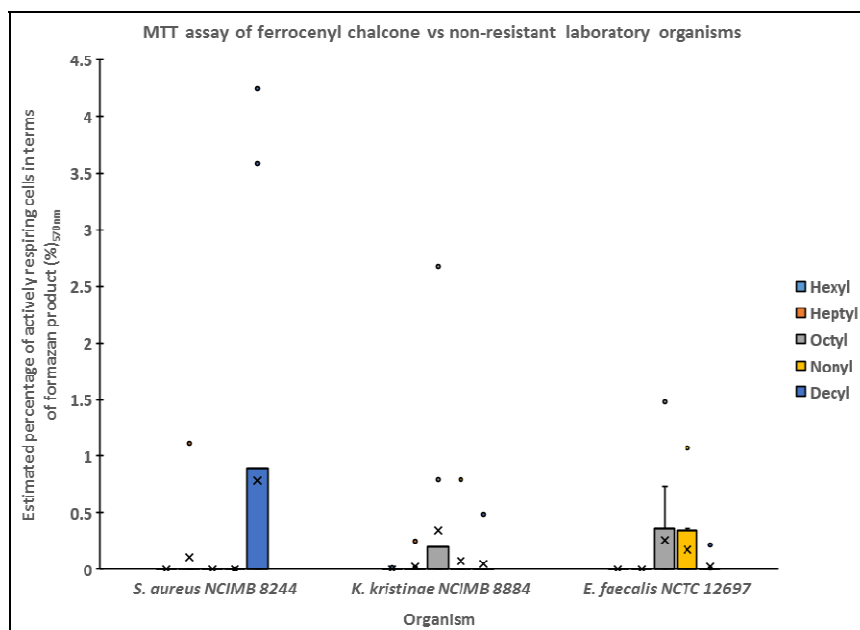


Figure 3-1 Estimated percentage of actively respiring non-resistant laboratory bacterial cells when treated with ferrocenyl chalcone at MIC. Box plots represent the lower and upper quartiles with the medians shown as black lines. Whiskers represent the minimum and maximum percentages and each X represents the mean values. Dots represent outlier values.

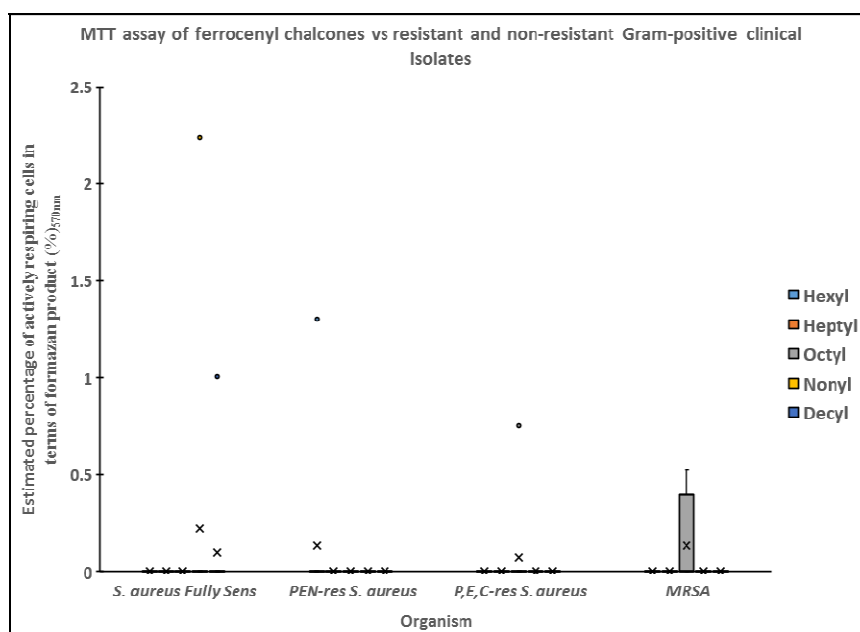


Figure 3-2 Estimated percentage of actively respiring resistant and non-resistant clinically isolated bacterial cells when treated with ferrocenyl chalcone at MIC. Box plots represent the lower and upper quartiles with the medians shown as black lines. Whiskers represent the minimum and maximum percentages and each X represents the mean values. Dots represent outlier values.

3.1 Statistical analysis

Statistical analysis of the MTT assay data in the study was performed using a One-Way ANOVA to determine if the mean percentage of actively respiring cells differed between the hexyl to decyl ferrocenyl chalcones treatments. The Kolmogorov-Smirnoff test was used to determine data normality of the MTT assay data.

In the microplate assays, the overall trend demonstrated that the chalcones had a greater inhibitory effect on Gram-positive organisms than on Gram-negative organisms. The trend also indicated that the differences between hexyl to decyl chalcones in terms of mean percentage (\pm SD) of actively respiring cells present were not significant for *S. aureus* NCIMB 8244 ($p=0.107$), *K. kristinae* NCIMB 8884 ($p=0.326$) and *E. faecalis* NCTC 12697 ($p=0.118$). Thus, the compounds were equally effective at inhibiting respiration in these bacterial cells. Similarly, the hexyl to decyl

ferrocenyl chalcones were equally effective at respiration inhibition for fully sensitive *S. aureus* ($p=0.523$), penicillin-resistant *S. aureus* ($p=0.418$), PEN/ERY/CLI-resistant *S. aureus* ($p=0.418$) and a MRSA ($p=0.418$).

3.2 Possible mechanism of action

When compared to MTT screening of ferrocenyl chalcone antimicrobial activity against *Mycobacterium tuberculosis*, the MIC values in this study lay within the reported range (0.016-0.128 mg/ml) (49), except for *K. kristinae* NCIMB 8884 where a lower MIC (heptyl chalcone) was used. Increased chain length may allow the ferrocene group to enter the cytoplasm of Gram-positive organisms. Ferrocene groups have been theorised to be inhibitors of cellular respiration, in which the ferrocene groups act as uncouplers (29). Since Gram-negative organisms have outer envelopes, thin peptidoglycan layers with increased periplasmic space and cell membranes in their cell envelopes, entry into these cells may be more difficult. Another possibility (SB) is that the cell membrane of Gram-positive bacteria is compromised such that the electron transport chain cannot function (52). Cell viability, as indicated by MTT metabolism to formazan, decreased in Gram-positive organisms when compared to Gram-negative organisms. Therefore, a possible mechanism of action of the chalcones with longer alkyl chain lengths may be inhibition of cellular respiration. This effect causes physical damage to the bacterial cells and was observed in images obtained from scanning electron microscopy.

4. Bacterial Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy is an imaging technique used to investigate the surface topography of biological specimens at the nanometre level (53). These samples usually possess a higher water content, which decreases their conductivity under high vacuum conditions in the electron microscope and results in distortion and destruction of the material (54). Biological samples, such as bacterial cells, must be chemically fixed, dehydrated, dried and sputter coated with a metal such as gold or using carbon under vacuum (55). Fixed bacterial samples must also be washed with an appropriate buffer such as phosphate buffered saline (PBS). For the purpose of SEM, bacterial samples should be captured on membrane filter with a diameter 13 mm and with a pore size that ranges between 0.2 μm to 1 μm (56).

SEM has been used to examine the external morphological effects of sugar fatty acid esters on selected Gram-positive and Gram-negative bacteria (57), and to observe the morphological changes to *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 after exposure to cinnamon essential oil (58).

4.1 SEM study of treated and untreated bacteria

In the current study, treated and untreated non-resistant bacteria were assessed. *S. aureus* NCIMB 8244, *K. kristinae* NCIMB 8884 and *E. faecalis* NCTC 12697 cells, which were examined in the SEM. The treated organisms were exposed to decyl ferrocenyl chalcone solution at MIC values and incubated for 18-24 hours at 37^oC, whilst untreated cells were incubated under the same conditions in the absence of chalcone. Treated and untreated cells were incubated with 2% glutaraldehyde (prepared with sterile deionised water) for 1 hour then washed with sterile phosphate buffer saline (PBS) (59). The cells were then dehydrated with a graded series of sterile ethanol and re-suspended in sterile deionised water. At the end of each step, the solutions were centrifuged. 10 μl of re-suspended cells were pipetted on to 0.2 μm Cyclopore Track Etch polycarbonate membrane filter discs (Whatman International Limited, Maidstone, UK) and sputter-coated with gold. Secondary electron images were taken using the JEOL JSM 6610V SEM (Herts, UK).

4.2 Morphological damage to bacterial cells

The SEM images revealed that exposure to decyl ferrocenyl chalcone resulted in severe external damage to bacterial cells at MIC and supra-MIC. The affected cells were shrivelled and wrinkled in appearance as if their cell wall structural integrity had been affected (Figure 3-3).

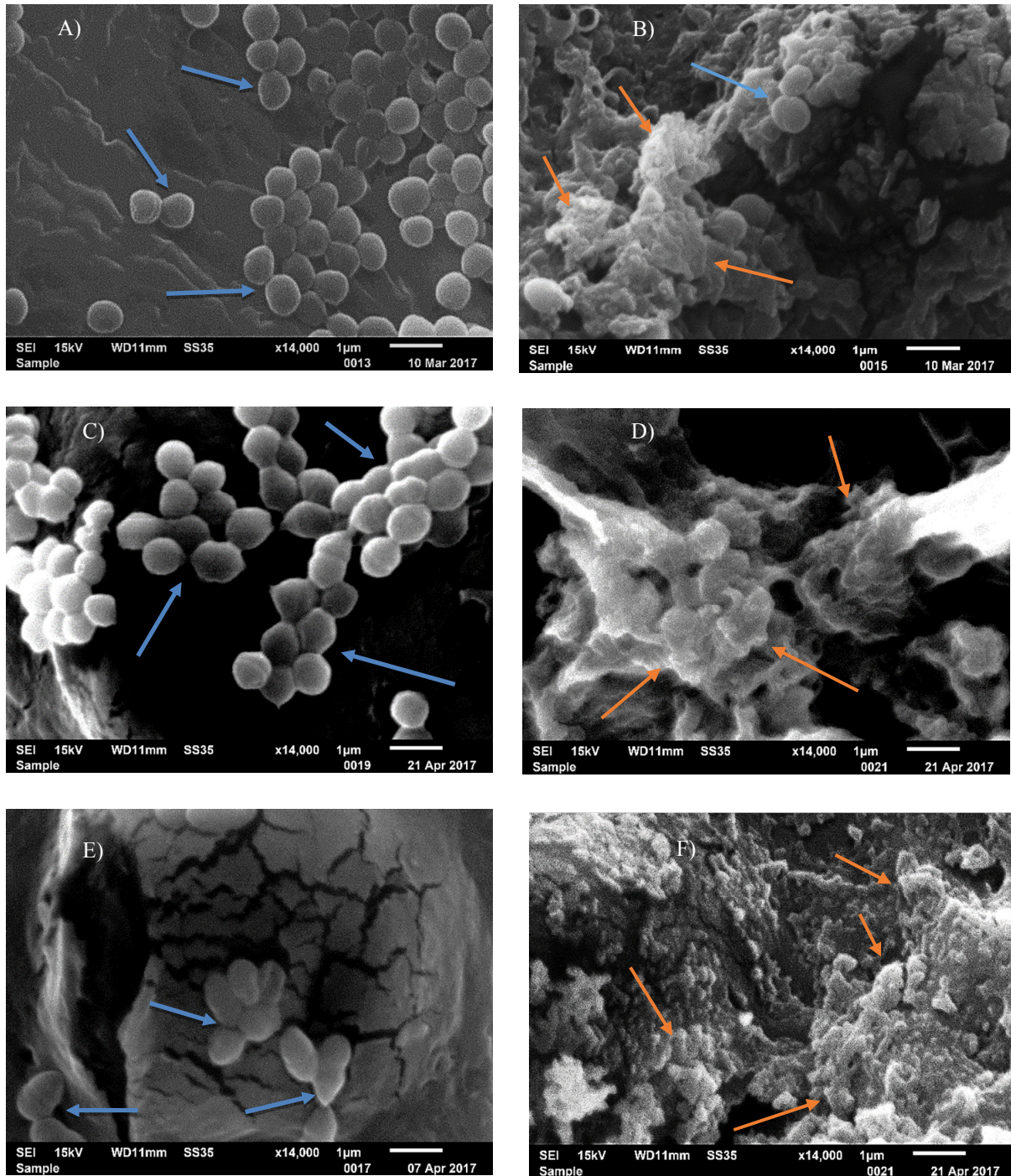


Figure 3-3 SEM images of untreated bacterial cells and cells that were treated with decyl ferrocenyl chalcone compound where blue arrow indicates unaffected cell and orange arrows indicate severely damaged cells. A) Untreated *S. aureus* NCIMB 8244. B) Treated *S. aureus* NCIMB 8244. C) Untreated *K. kristinae* NCIMB 8884. D) Treated *K. kristinae* NCIMB 8884. E) Untreated *E. faecalis* NCTC 12697. F) Treated *E. faecalis* NCTC 12697.

5. Conclusion

Infections caused by the spread of drug-resistant bacteria must be addressed as quickly as possible. This spread is in part caused by the misuse of antibiotics and the unavailability of new antimicrobial agents. Key discoveries of the

research are that ferrocenyl chalcone compounds with longer alkyl chains (hexyl to decyl) exhibit greater antimicrobial activity than those with shorter alkyl chains (methyl to pentyl) and that compounds with antimicrobial activity are more effective against Gram-positive bacteria than against Gram-negative bacteria. This is the first report to also demonstrate that ferrocenyl chalcones, which can be structurally altered by synthetic methods, possess significant antimicrobial activity against non-resistant laboratory organisms and resistant and non-resistant clinical isolates. Another important result of this study is that chalcone activity may be characterised by blocking bacterial respiration. Findings from this study reveal that these novel ferrocenyl chalcone compounds are potential antimicrobial agents against clinical bacterial isolates requiring further investigation involving efficacy of the compounds against biofilms and cytotoxicity against mammalian cells.

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