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Eradication of biofilms formed by bacteria isolated from diabetic foot infections by potential antibiofilm agents alone and in combination with ciprofloxacin

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This study was performed to investigate the resistance of biofilm forming bacteria isolated from diabetic foot infection to different antibiotics and the eradicating activity of some potential antibiofilm agents alone and in combination with ciprofloxacin. Imipenem was the most active against biofilms formed by all tested strains, while tetracycline was the least active. For biofilms of Gram-positive bacteria, azithromycin and imipenem were the most potent, while tetracycline and vancomycin showed the lowest activity. Similarly, imipenem showed the highest activity against biofilms of Gram-negative bacteria, while ciprofloxacin, tetracycline and cefoperazone were the least active. Potential antibiofilm agents exerted antibacterial and biofilm eradicating activities. Apple and grape vinegars showed the highest activities, followed by estradiol, ambroxol and piroxicam. Dexamethasone, manuka and citrus honeys were less active. Ambroxol showed the highest synergistic activity with ciprofloxacin, followed by dexamethasone, manuka honey, piroxicam, estradiol and grape vinegar, while apple vinegar and citrus honey showed intermediate activity. In conclusion, this study recommends the use of antibiofilm agents in combination with antibiotics to combat the resistance of biofilms to antibiotics.

Key words: Diabetic foot infections, biofilm eradication, antibiofilm agents, ciprofloxacin, synergy.

INTRODUCTION

Diabetic foot infection (DFI) is a major problem in patients with diabetes. Reasons of this infection are peripheral neuropathy, reduced peripheral blood supply and lowered immunity. DFIs bear high risk for patients with diabetes because they may lead to gangrene and amputation (Abbott et al., 2002; CDC, 2005; Lauterbach et al., 2010). The microbial etiology of DFIs is complex. Resistance of bacteria causing DFIs to antibiotics is common and formation of biofilms complicates the problem (Roghmann et al., 2001). Biofilm is a community of sessile microbial cells attached to a surface and housed within a matrix of polysaccharides, proteins and nucleic acids (Hoiby et al., 2010).

Biofilms are remarkably resistant to antimicrobial agents. The mechanisms of biofilm resistance may include slow growth and metabolic rates, inactivation of

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Table 1. Bacterial strains used in this study.

Bacterial strains	Number
Proteus mirabilis	6
Proteus vulgaris	1
Pseudomonas aeruginosa	2
Pseudomonas mendocina	1
E. coli	3
Klebsiella ozaenae	1
Acinetobacter baumanii	1
Staphylococcus aureus	3
Staphylococcus epidermids	1
Entrococcus faecalis	1

antimicrobial agents by the extracellular matrix and the presence of an oxygen gradient that prevents the action of some antibiotics (Lynch and Robertson 2008; Hall-Stoodley and Stoodley, 2009). In addition, the biofilm matrix represents a diffusion barrier to antibiotics (Lynch and Robertson, 2008). Moreover, biofilms contain a large subpopulation of persister cells which are dormant cells that survive antimicrobial treatment (Lewis, 2010).

For these reasons, agents that can remove biofilms and act in synergism with antibiotics are urgently needed. This study investigated the *in vitro* activities of some potential antibiofilm agents alone and in combination with ciprofloxacin on the eradication of biofilms formed by bacterial isolates from diabetic foot infections.

MATERIALS AND METHODS

Media and chemicals

Tryptone soya broth, Tryptone soya agar and Mueller Hinton broth were the products of Oxoid (Hampshire, UK). Ambroxol hydrochloride, imipenem and Dimethyl sulphoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, USA). Antibiotics and chemicals used in this study were ciprofloxacin, amoxicillin-clavulinic acid and gentamicin (Egyptian Pharmaceutical Industries Company (EIPICO), 10th of Ramadan City, Egypt), Chloramphenicol (Alexandria Pharmaceutical and Chemical Industries Company, Alexandria, Egypt), tetracycline (Nile Pharmaceutical and Chemical Industries Company, Cairo, Egypt), cefoperazone, azithromycin and piroxicam (Pfizer, Cairo, Egypt), Manuka honey (Manuka health New Zealand Ltd., Te Awamutu, New Zealand), citrus honey (Isis Company, Egypt), estradiol and glutaraldehyde (El Nasr Pharmaceutical Chemicals Company, Cairo, Egypt), dexamethasone and cephalexin (Glaxo Smithkline, Cairo, Egypt), and vancomycin (Sigma Pharmaceutical Industries Company, Menoufia, Egypt). Apple and grape vinegar were purchased from the local market, Zagazig, Egypt. Other chemicals were of pharmaceutical grade.

Bacterial strains

Twenty isolates obtained from patients with diabetic foot infections admitted to the Surgery Department, Zagazig University Hospital were obtained from the stock culture collection of Department of Microbiology and Immunology, Faculty of Pharmacy, Zagazig University as shown in Table 1.

Quantitative assessment of biofilm by spectrophotometric method

The ability of tested strains to form biofilm was investigated according to Stepanovic et al. (2007). Overnight cultures of tested strains in Tryptone soya broth (TSB) were diluted with fresh TSB to a final inoculum of 1×10^6 CFU/ml. To the wells of 96-well sterile microtiter plates with rounded bottom, aliquots of 200 µl of the prepared suspensions were added and the plates were incubated for 24 h at 37°C. The contents of the microtiter plates were gently removed and the wells were washed 3 times with sterile phosphate buffered saline (PBS, pH 7.2). To fix adherent bacteria, aliquots of 200 µl of 99% methanol were added to the wells for 20 min. The wells were stained with 200 µl crystal violet (1%) for 20 min and the unbound dye was washed by distilled water. After air drying of the plates, the bound dye was eluted by aliquots of 160 µl of 95% ethanol. The optical densities of the stained adherent films were measured with a spectrofluorimeter (Biotek, USA) at 490 nm. Measurements were performed in triplicate and repeated 3 times. The cut-off optical density (ODc) was calculated as three times standard deviations above the mean OD of the negative control. The tested strains were classified according to the criteria of Stepanovic et al. (2007) into non-biofilm producer (OD ≤ ODc), weak biofilm producer (OD > ODc, but ≤ 2x ODc), moderate biofilm producer (OD>2x ODc, but ≤ 4x ODc), and strong biofilm producer (OD> 4x ODc).

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of tested antibiotics and potential antibiofilm agents was determined by the broth microdilution method according to Clinical Laboratory and Standards Institute Guidelines (CLSI, 2012).Standardized bacterial suspensions with a turbidity equivalent to that of 0.5 McFarland standard were prepared from overnight cultures in tryptone soya broth. The standardized bacterial suspensions were diluted to a cell density of 10^6 CFU/ml. Aliquots of 50 µl of the adjusted bacterial suspensions in Mueller-Hinton broth were added to the wells of a microtiter plate that contain aliquots of 50 µl of double the required dilutions of the tested agents in Mueller-Hinton broth. The plates were incubated at 37ºC for 20 h and the MIC was calculated as the lowest concentration of the tested agents that inhibited the visible growth in the wells.

Determination of minimum biofilm eradication concentration (MBEC)

The minimum biofilm eradication concentration was determined according to Ceri et al. (1999) with some modifications. Suspensions of the tested strains with a cell density of $1x10^8$ CFU/ml were prepared in Tryptone soya broth (TSB) and diluted in TSB to a cell density of $5x10^6$ CFU/ml. For biofilm formation, aliquots of 100 µl were inoculated into the wells of 96-well polystyrene microtiter plates and the plates were incubated for 24 h at 37ºC. The non-adherent cells were gently aspirated and the wells were washed three times with phosphate buffered saline (PBS). Aliquots of 100 µl of different dilutions of tested agents were transferred to the wells and the plates were again incubated for 24 h at 37ºC. The contents of the wells were removed and the wells were washed again. To resuspend the biofilms in the wells, aliquots of 100 µl of sterile phosphate buffered saline were added and the sides of the wells with a pipette tip were scrapped. To calculate

Table 2. Quantitative assessment of biofilm formation by bacterial isolates.

Isolate number	Optical density at 490 nm	Biofilm formation capacity
PM1	0.384	Strong
PM2	0.263	Strong
PM3	0.344	Strong
PM4	0.283	Strong
PM5	0.325	Strong
PM6	0.289	Strong
PV	0.289	Strong
PA1	0.281	Strong
PA2	0.346	Strong
K. ozaenae	0.308	Strong
SA1	0.333	Strong
SA2	0.351	Strong
SA3	0.338	Strong
SE	0.334	Strong
E. faecalis	0.316	Strong
AB	0.444	Strong
EC1	0.282	Strong
EC2	0.321	Strong
EC3	0.259	Strong
P. mendocina	0.346	Strong

PM, *Proteus mirabilis;* PV, *Proteus vulgaris*; PA, *Pseudomonas aeruginosa*; *K. ozaenae*, *Klebsiella ozaenae;* SA, *Staphylococcus aureus*; SE, *Staphylococcus epidermidis*; *E. faecalis, Enterococcus faecalis;* AB, *Acinetobacter baumanii*; EC, *E. coli*; *P. mendocina*, *Pseudomonas mendocina*

MBEC, 10 µl from each well was transferred onto Tryptone soya gar plates (TSA), incubated at 37°C for 24 h and MBECs were defined as the least concentrations that showed no growth on TSA.

Testing for synergy between potential antibiofilm agents and ciprofloxacin

For determination of the synergism of potential tested antibiofilm agents with ciprofloxacin, the same method of Ceri et al. (1999) was used, but instead of adding 100 µl of tested agent, aliquots of 50 µl of 1/2 MIC of antibiofilm agents were added to 50 µl aliquots of different dilutions of ciprofloxacin.

RESULTS

Assessment of biofilm formation

All tested strains were found to be strong biofilm forming (Table 2). The ODc was calculated as 0.064. According to the criteria of Stepanovic et al. (2007), the bacterial isolate is considered a strong biofilm-forming if the optical density is greater than 0.256.

Susceptibility of planktonic and biofilm cells to antimicrobial agents

Biofilm cells demonstrated higher resistance than planktonic cells to different antibiotics as demonstrated by the ratios of MBEC to MIC of antibiotics in Table 3. This ratio was lowest for imipenem (2-16) folds, followed by amoxicillin-clavulinic acid (2-32) folds, gentamicin (16-32), ciprofloxacin (8-64) folds, and was highest for tetracycline (4-256) folds. Considering biofilms formed by Gram-positive bacteria, highest resistance was found with vancomycin (1024) folds and tetracycline (32-256) folds, while low resistance was observed with azithromycin (4-8) folds and imipenem (4-16) folds, amoxicillin-clavulinic acid and cephalexin (8-32) folds each, gentamicin and ciprofloxacin (32 folds each), chloramphenicol (16-64) folds. Biofilm cells of Gram-negative bacteria were highly resistant to cefoperazone (4-512) folds and tetracycline (4-256) folds. Lower resistance was obtained with gentamicin (16-64) folds, ciprofloxacin (8-64) folds, chloramphenicol (16-32) folds, amoxicillin-clavulinic acid (2-32) folds, while imipenem showed the highest antibiofilm activity (2-8) folds.

Susceptibility of bacterial isolates to potential antibiofilm agents

Antibacterial and antibiofilm activities were found against planktonic bacteria (Table 4).Both apple and grape vinegars showed the highest activities, followed by

Table 3. Antimicrobial susceptibility of planktonic and biofilm cells.

Isolate number	Gentamicin			Ciprofloxacin			Chloramphenicol			Tetracycline			Amoxicillin/clavulinic acid		
	MIC	MBEC	MBEC/MIC	MIC	MBEC	MBEC/MIC	MIC	MBEC	MBEC/MIC	MIC	MBEC	MBEC/MIC	MIC	MBEC	MBEC/MIC
PM1	0.5	16	32	0.125	8	64	32	512	16	64	2048	32	1	32	32
PM2	1	32	32	0.25	16	64	64	1024	16	64	1024	16	64	128	2
PM3	16	512	32	2	128	64	64	1024	16	64	2048	32	32	256	8
PM4	16	512	32	2	128	64	64	2048	16	64	2048	32	512	2048	4
PM5	4	128	32	2	128	64	8	256	32	256	4096	16	8	64	8
PM6	1	32	32	1	128	64	2	32	16	1	256	256	1	32	32
PV	4	128	32	2	32	32	64	1024	16	16	2048	128	64	128	2
PA1	16	1024	32	2	128	64	256	4096	16	32	1024	32	1024	8192	8
PA2	8	256	32	32	256	8	256	4096	16	64	2048	32	1024	8192	8
K. ozaenae	32	1024	32	64	1024	16	256	4096	16	256	4096	16	32	256	8
SA1	0.25	8	32	1	32	32	8	256	32	1	256	256	0.5	16	32
SA2	0.5	16	32	1	32	32	4	128	32	2	512	256	0.5	16	32
SA3	0.5	16	32	1	32	32	8	256	32	2	512	256	0.5	16	32
SE	256	8192	32	1	32	32	64	1024	16	32	1024	32	0.5	16	32
E. faecalis	256	8192	32	1	32	32	4	256	64	32	1024	32	32	256	8
AB	256	8192	32	128	2048	16	128	2048	16	128	2048	16	1024	8192	8
EC1	16	1024	64	32	512	16	8	256	32	256	4096	16	32	256	8
EC2	1	32	32	1	32	32	2	32	16	256	4096	16	32	256	8
EC3	128	2048	16	128	4096	32	2	32	16	256	2048	8	128	1024	8
P. mendocina	1	32	32	1	32	32	4	128	32	64	256	4	16	128	8

PM, *Proteus mirabilis;* PV*, Proteus vulgaris*; PA, *Pseudomonas aeruginosa*; *K. ozaenae*, *Klebsiella ozaenae;* SA, *Staphylococcus aureus*; SE, *Staphylococcus epidermidis*; *E. faecalis, Enterococcus faecalis;* AB, *Acinetobacter baumanii*; EC, *E. coli*; *P. mendocina*, *Pseudomonas mendocina.*

oestradiol, ambroxol and piroxicam. Dexamethasone, manuka and citrus honeys were less active.

Synergy between ciprofloxacin and antibiofilm agents

Synergy was found between ciprofloxacin and different potential antibiofilm agents (Table 5). Ambroxol reduced MBEC of ciprofloxacin by 4-128 folds, grape vinegar by 2-64 folds, piroxicam by 2-32 folds, dexamethasone by 4-16 folds and apple vinegar and estradiol by 2-16 folds each.

DISCUSSION

In this study, the resistance of biofilm cells to antibiotics was higher than that of planktonic cells. The magnitude of biofilm resistance to individual antibiotics was measured by the ratio of MBEC/MIC expressed by ≥ 90% of the tested isolates. The resistance of biofilms formed by all tested strains was the least against imipenem (8 folds), followed by amoxicillin-clavulinic acid, gentamicin and chloramphenicol (32 folds each) and ciprofloxacin (64 folds). Resistance to tetracycline was the highest (256 folds) as shown in Figure 1. On the other hand the resistance of biofilms formed by Gram-positive strains was low against azithromycin (8 folds) and imipenem (16 folds) as shown in Figure 2. Intermediate resistance

Table 3. Contd.

Isolate number	Cefoperazone			Vancomycin			Imipenem			Azithromycin			Cephalexin		
	MIC	MBEC	MBEC/MIC	MIC	MBEC	MBEC/MIC	MIC	MBEC	MBEC/MIC	MIC	MBEC	MBEC/MIC	MIC	MBEC	MBEC/MIC
PM1	0.5	16	32	NT	NT	NT	2	8	4	NT	NT	NT	NT	NT	NT
PM2	256	1024	4	NT	NT	NT	4	8	2	NT	NT	NT	NT	NT	NT
PM3	64	1024	16	NT	NT	NT	2	8	4	NT	NT	NT	NT	NT	NT
PM4	32	1024	32	NT	NT	NT	4	8	2	NT	NT	NT	NT	NT	NT
PM5	16	32	2	NT	NT	NT	4	8	2	NT	NT	NT	NT	NT	NT
PM6	64	1024	16	NT	NT	NT	4	8	2	NT	NT	NT	NT	NT	NT
PV	2	1024	512	NT	NT	NT	2	8	4	NT	NT	NT	NT	NT	NT
PA1	8	512	64	NT	NT	NT	0.5	2	4	NT	NT	NT	NT	NT	NT
PA2	256	512	2	NT	NT	NT	64	256	4	NT	NT	NT	NT	NT	NT
K. ozaenae	256	4096	16	NT	NT	NT	1	8	8	NT	NT	NT	NT	NT	NT
SA1	NT	NT	NT	2	2048	1024	0.5	8	16	2	16	8	4	8	32
SA2	NT	NT	NT	1	1024	1024	1	8	8	2	16	8	32	512	16
SA3	NT	NT	NT	1	1024	1024	1	8	8	512	2048	4	4	128	32
SE	NT	NT	NT	1	1024	1024	2	8	4	256	1024	4	64	1024	16
E. faecalis	NT	NT	NT	0.5	512	1024	1	8	8	1024	8192	4	256	2048	8
AB	256	1024	4	NT	NT	NT	2	8	4	NT	NT	NT	NT	NT	NT
EC1	0.5	16	32	NT	NT	NT	0.5	2	4	NT	NT	NT	NT	NT	NT
EC2	0.5	16	32	NT	NT	NT	0.5	2	4	NT	NT	NT	NT	NT	NT
EC3	0.5	16	32	NT	NT	NT	0.5	2	4	NT	NT	NT	NT	NT	NT
P. mendocina	8	512	64	NT	NT	NT	0.5	2	4	NT	NT	NT	NT	NT	NT

Table 4. Antimicrobial and antibiofilm activities of potential antibiofilm agents.

Isolate number	Ambroxol (mg/ml)		Dexamethasone (mg/ml)		Piroxicam (mg/ml)		Estradiol (mg/ml)		Manuka honey (%)		Citrus honey (%)		Apple vinegar (%)		Grape vinegar (%)	
	MIC	MBEC	MIC	MBEC	MIC	MBEC	MIC	MBEC	MIC	MBEC	MIC	MBEC	MIC	MBEC	MIC	MBEC
M1	0.47	0.94	4	2	1.25	0.625	0.25	0.25	25	9.375	12.5	18.75	0.078	0.31	0.078	0.156
M2	0.47	0.94	4	2	1.25	0.625	0.5	1	12.5	9.375	25	18.75	0.078	0.31	0.039	0.156
M3	0.47	0.94	4	2	1.25	0.625	0.5	1	12.5	9.375	12.5	18.75	0.078	0.31	0.039	0.156
M4	0.47	0.94	4	2	1.25	0.625	0.5	1	12.5	9.375	25	18.75	0.078	0.31	0.078	0.156
M5	0.47	0.94	4	2	1.25	1.25	0.25	0.5	12.5	9.375	12.5	18.75	0.078	0.31	0.078	0.156
M6	0.47	0.94	4	2	1.25	1.25	0.5	0.5	12.5	9.375	12.5	18.75	0.078	0.31	0.078	0.156
PV	0.47	0.94	4	2	0.625	1.25	0.5	1	12.5	9.375	12.5	18.75	0.078	0.31	0.039	0.156

Table 4. Contd.

PA1	0.47	0.94	4	2	1.25	0.625	0.25	0.25	12.5	9.375	12.5	18.75	0.078	0.156	0.039	0.156
PA2	0.47	0.47	4	2	1.25	0.625	0.25	0.5	12.5	9.375	12.5	18.75	0.625	0.31	0.039	0.156
K. .ozaenae	0.47	0.47	4	2	1.25	0.625	0.25	0.25	25	9.375	25	18.75	0.195	0.31	0.078	0.156
SA1	0.47	0.47	1	2	0.625	1.25	0.125	0.25	12.5	9.375	25	18.75	0.078	0.31	0.039	0.156
SA2	0.47	0.47	1	2	0.625	0.625	0.25	0.25	12.5	9.375	12.5	18.75	0.078	0.31	0.078	0.156
SA3	0.47	0.47	1	2	0.625	0.625	0.125	0.25	12.5	9.375	25	18.75	0.078	0.31	0.078	0.156
SE	0.94	0.47	1	2	0.625	0.625	0.125	0.25	25	9.375	25	18.75	0.078	0.156	0.078	0.156
E. faecalis	0.94	0.47	1	2	0.625	0.625	0.5	0.25	25	9.375	25	18.75	0.078	0.156	0.078	0.156
AB	0.94	0.47	4	2	0.625	1.25	0.125	0.25	12.5	9.375	25	37.5	0.078	0.156	0.039	0.31
EC1	0.94	0.94	4	2	1.25	1.25	0.5	1	12.5	9.375	25	18.75	0.078	0.31	0.078	0.156
EC2	0.94	0.94	4	2	1.25	1.25	0.5	1	12.5	9.375	25	18.75	0.078	0.156	0.078	0.156
EC3	0.47	0.47	4	2	0.625	0.625	0.25	1	12.5	9.375	25	18.75	0.078	0.156	0.078	0.156
P. mendocina	0.47	0.94	4	2	0.625	0.625	0.25	0.5	12.5	9.375	25	18.75	0.078	0.31	0.0195	0.156

PM, *Proteus mirabilis;* PV, *Proteus vulgaris*; PA, *Pseudomonas aeruginosa*; *K. ozaenae*, *Klebsiella ozaenae;* SA, *Staphylococcus aureus*; SE, *Staphylococcus epidermidis*; *E. faecalis, Enterococcus faecalis;* AB, *Acinetobacter baumanii*; EC, *E. coli*; *P. mendocina*, *Pseudomonas mendocina.*

Table 5. Effect of potential antibiofilm agents on biofilm eradication by ciprofloxacin.

Isolate number	MBEC (µg/ml)								
	CIP	Apple vinegar /CIP	Grape vinegar /CIP	Citrus honey /CIP	Manuka honey / CIP	Ambroxol / CIP	Piroxicam / CIP	Dexamethasone / CIP	Estradiol/CIP
PM1	64	32	32	32	4	8	16	4	32
P. *mendocina*	256	128	16	128	128	8	64	32	32
SA2	256	32	16	128	64	16	128	64	32
AB	2048	128	32	64	64	16	64	32	128
EC2	512	128	16	128	64	32	64	128	32
K. ozaenae	1024	128	32	128	64	16	64	64	64
SE	32	32	16	8	4	8	16	4	8
E. faecalis	64	32	16	32	4	8	16	8	32

PM, *Proteus mirabilis;K. ozaenae*, *Klebsiella ozaenae;* SA, *Staphylococcus aureus*; SE, *Staphylococcus epidermidis*; *E. faecalis, Enterococcus faecalis;* AB, *Acinetobacter baumanii*; EC, *E. coli*; *P. mendocina*, *Pseudomonas mendocina;* CIP, ciprofloxacin.

was observed against cephalexin, amoxicillin-clavulinic acid, ciprofloxacin and gentamicin (32 folds each), while it was high against tetracycline (256 folds) and vancomycin (1024 folds). Imipenem was the least affected by biofilms formed by Gram-negative bacteria (4 folds) as

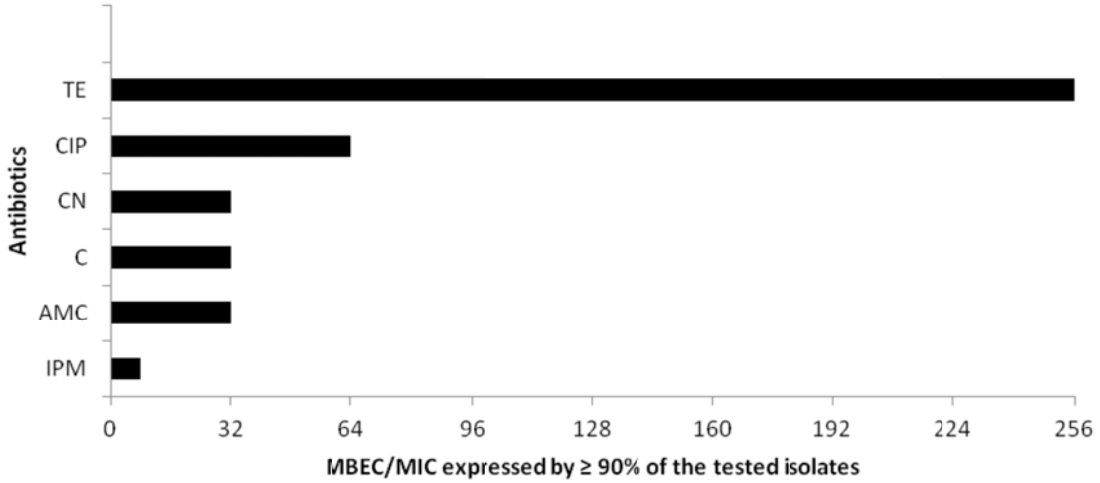

Figure 1. Biofilm eradicating activity of antibiotics against Gram-negative and Gram-positive bacteria IPM, imipenem; AMC, amoxicillin-clavulinic acid; C, chloramphenicol; CN, gentamicin; CIP, ciprofloxacin; TE, tetracycline

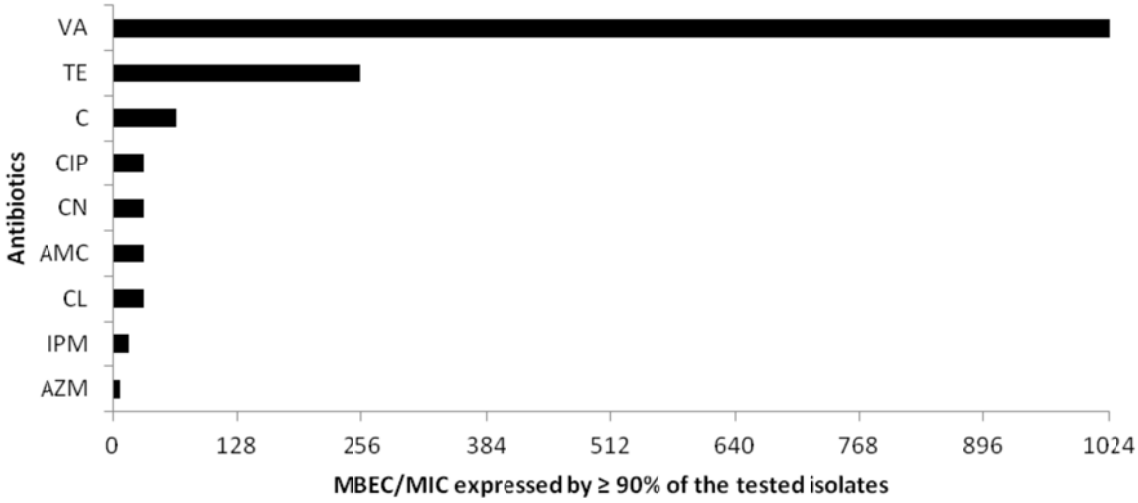

Figure 2. Biofilm eradicating activity of antibiotics against Gram-positive bacteria. AZM, azithromycin; IPM, imipenem; CL, cephalexin; AMC, amoxicillin-clavulinic acid; CN, gentamicin; CIP, ciprofloxacin; C, chloramphenicol; TE, tetracycline; VA, vancomycin.

shown in Figure 3. The biofilm resistance against chloramphenicol (16 folds), amoxicillin-clavulinic acid and gentamicin (32 folds each) was found to be intermediate, while the least active antibiotics against biofilm cells were ciprofloxacin and tetracycline (64 folds each) and cefoperazone (128 folds).

High resistance of biofilms to antimicrobial agents was reported by other studies. Thus Černohorská and Votava (2004) found that the susceptibility of biofilms formed by *E. coli*, *P. aeruginosa* and *Klebsiella pneumoniae* to cefoperazone and ciprofloxacin was much lower than that of planktonic cells. La Plante and Mermel (2009) reported that vancomycin was not effective for eradicating biofilms formed by *S. aureus* and *Enterococcus faecalis* as shown by MBEC/MIC ratios of ≥256 folds. Ceri et al. (1999) also reported high resistance of biofilms of *E. coli* to ciprofloxacin (MBEC/MIC >2048 folds), *P. aeruginosa* to ciprofloxacin (16 folds), gentamicin (64 folds) and imipenem (> 1024 folds), *S. aureus* to ciprofloxacin (1024 folds) and vancomycin (> 1024 folds).

As a result of the high resistance of biofilm cells to antibiotics, agents that can remove biofilms are necessary. A number of potential agents were tested. These agents include ambroxol, dexamethasone, piroxicam, manuka and citrus honeys, apple and grape vinegars and estradiol.

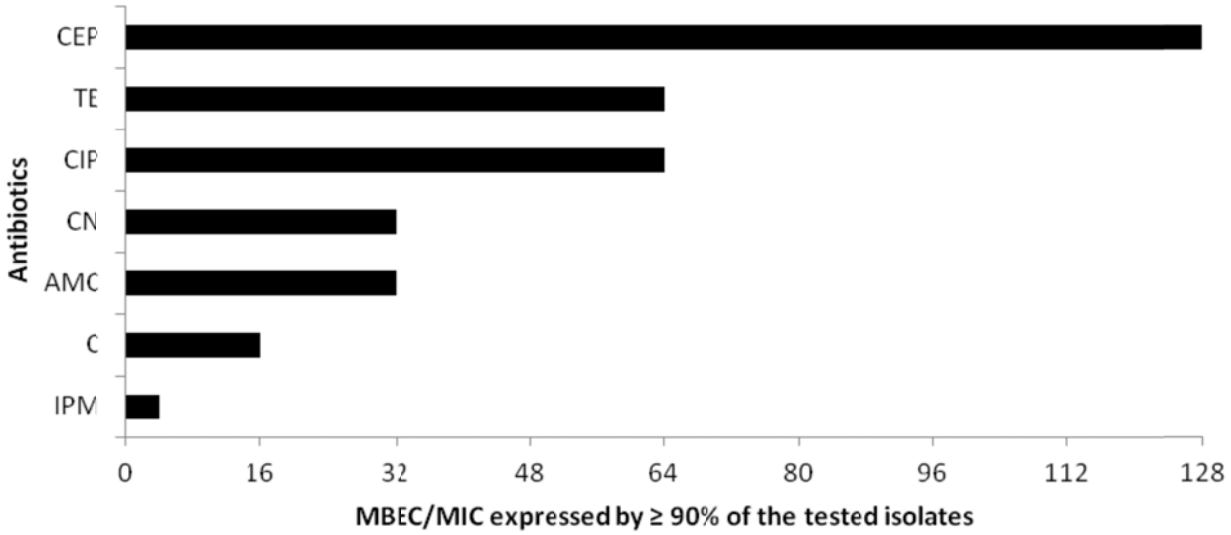

Figure 3. Biofilm eradicating activity of antibiotics against Gram-negative bacteria. IPM, imipenem; C, chloramphenicol; AMC, amoxicillin-clavulinic acid; CN, gentamicin; CIP, ciprofloxacin; TE, tetracycline; CEP, cefoperazone

Ambroxol was found to be a strong antiadhesion agent (Hafez et al., 2009). In addition to its antiahesive effects, ambroxol interferes with biofilm formation by interference with quorum sensing and decreasing the matrix production in *P. aeruginosa* biofilms (Li et al., 2008; Lu et al., 2010). Abbas (2013) also reported the ability of ambroxol to inhibit and eradicate biofilms formed by *Proteus mirabilis* isolated from diabetic foot infections. At 0.9 mg/ml, ambroxol caused 90.25-100% inhibition and 78.38-83.77% eradication of biofilm.

In this study, the MICs and MBECs of ambroxol against tested isolates were found to be 0.47-0.94 mg/ml. Lu et al. (2010) reported that at 1.875 and 3.75 mg/ml, ambroxol could inhibit quorum sensing, biofilm maturation and viability. Furthermore, Li et al. (2008) found that ambroxol at 3.75 mg/ml could disrupt the biofilms.

Honey has both a broad spectrum antibacterial and wound healing activities (Lusby et al., 2005). The antibacterial activity may be due to low water content, high osmolarity and low pH, hydrogen peroxide and non-peroxide phytochemical components of honey (Rhoads et al., 2008). Moreover, honey was reported to have antibiofilm activity (Saraf et al., 2009) that may be due to its quorum sensing inhibiting activity (Wang et al., 2012).

In this study, two types of honey were used; Manuka honey and citrus honey. Both showed comparable activity against planktonic growth, while Manuka honey was more active in biofilm eradication. Manuka honey is a broad spectrum antibacterial agent (Blair et al., 2009). In addition, it could detach established biofilms (Merckoll et al., 2009). On the other hand, citrus honey (20.3%) had a strong growth inhibiting activity against *S. aureus*, and intermediate activity against each of *P. aeruginosa*, *Klebsiella pneumoniae* and *E. coli* (Hegazy, 2011).

Vinegar is a sour liquid prepared by the fermentation of many fruits such as apples and grapes. Acetic acid is the main constituent of vinegar. Vinegar has bacteriostatic and bactericidal effect on microorganism (Entani et al., 1998; Nascimento et al., 2003). Its mechanism of action depends on penetration and disruption of the bacterial cell membrane (Parish et al., 2003; Yousef and Juneja 2003; Marriott and Gravani 2006). The high content of polyphenols contributes to the antimicrobial activity of apple and grape vinegars (Jafari et al., 2012). Vinegar could eradicate biofilm formed by *Candida albicans* on acrylic resin plates (Alberto et al., 2006). This may be due to polyphenols that were reported to inhibit streptococcal biofilm formation through inhibition of enzymes that produce exopolymers; a major component in biofilm (Sendamangalam, 2010).

In our study, grape vinegar produced slightly higher antibacterial and biofilm eradicating activities than apple vinegar. Apple vinegar could inhibit the planktonic growth at 0.078% except for *Klebsiella ozaenae* (0.195%) and one *P. aeruginosa* strain (0.625%), while grape vinegar produced similar effect at 0.039-0.078% except for *P. mendocina* (0.195%). Moreover, grape vinegar could eradicate biofilms of all tested strains at 0.156% except for *Acinetobacter baumanii* (0.31%), while apple vinegar MBECs ranged between 0.156-0.31%.

In this study, the non-steroidal anti-inflammatory agent piroxicam exerted slightly stronger antibacterial and antibiofilm effects than dexamethasone. The MICs of piroxicam and dexamethasone were 0.625-1.25 and 1-4 mg/ml, respectively. The biofilm eradication was achieved at 0.625-1.25 and 2 mg/ml for piroxicam and dexamethasone, respectively.

Non-steroidal anti-inflammatory drugs (NSAIDs) have

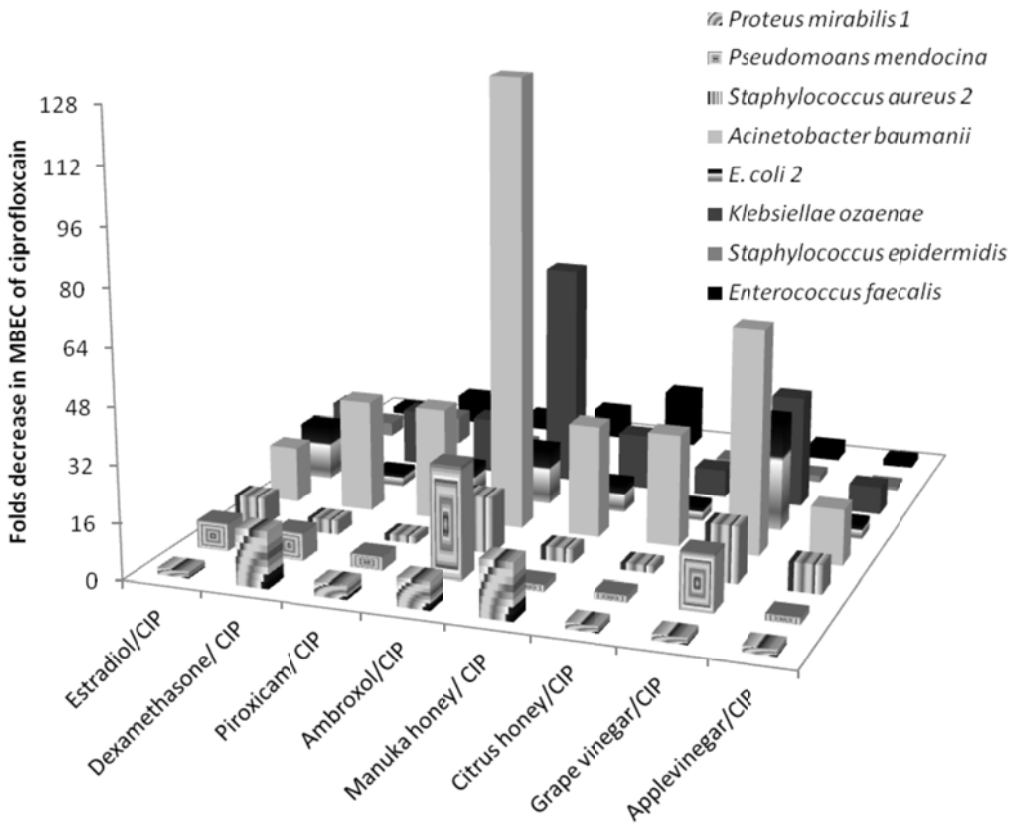

Figure 4. Effect of antibiofilm agents on biofilm eradication by ciprofloxacin.

good antimicrobial activities. This antimicrobial activity may be attributed to inhibition of bacterial DNA synthesis (Annadurai et al., 1998; Dastidar et al., 2000). Abbas et al. (2012a) found that piroxicam exerted antibiofilm activity against *P.aeruginosa* biofilms. Possible mechanisms of biofilm inhibition by NSAIDs are inhibition of bacterial adhesion, reduction of extracellular polysaccharide, modification of the surface properties of the bacterial cell (Farber and Wolff, 1992; Muller et al., 1998). Another possible mechanism is the inhibition of quorum sensing system. Piroxicam may inhibit biofilm formation by *P. aeruginosa* by decreasing the production of quorum sensing-dependent virulence factors (Ulusoy and Bosgelmez-Tinaz, 2013)

In our study, the steroidal hormone estradiol inhibited growth of free-living cells at 0.125-0.5 mg/ml and removed established biofilms at 0.25-1mg/ml. In a previous study, topical corticosteroids (fluticasone at 400 µg/200 µl, mometasone at 300 µg, 400 µg/200 µl and budesonide at 750 µg, 1,000 µg, and 2,000 µg/200 µl) were found to significantly reduce biofilms formed *in vitro* by *Staphylococcus aureus* isolated from chronic rhinosinusitis patients (Goggin et al., 2014).This activity may be due to the quorum sensing inhibiting activity of estradiol that was reported against *P. aeruginosa* (Beury-Cirou et al. 2013).

The synergistic effect of potential antibiofilm agents with ciprofloxacin was investigated. Ambroxol showed the highest synergistic activity (Figure 4). Ambroxol and dexamethasone showed synergistic effect against biofilms in all tested strains, but the magnitude of reduction in MBEC was higher in case of ambroxol. The synergistic effect was observed in 87.5% of tested strains with manuka honey, in 75% of isolates with each of piroxicam, estradiol and grape vinegar, but the magnitude of potentiation was greater with grape vinegar. Each of apple vinegar and citrus honey potentiated the biofilm eradicating activity of ciprofloxacin in 50% of isolates.

The biofilms formed by different strains were differently affected by antibiofilm agents-ciprofloxacin combinations. The most affected was *Acinetobacter baumanii* (all combinations showed synergism), followed by *Klebsiella ozaenae* and *E.coli* (all combinations showed synergism but with lower magnitude of MBEC decrease). Potentiation of the biofilm removal activity of ciprofloxacin was obtained by 6 combinations against *Staphylococcus aureus* biofilm, with 5 combinations against biofilms of each of *Pseudomonas mendocina*, *Enterococcus faecalis and Staphylococcus epidermidis*, but the magnitude of MBEC reduction was higher in *Pseudomonas mendocina* biofilm. The least affected was *Proteus mirabilis* biofilm; only 4 combinations showed synergism.

In accordance with our study, Li et al. (2008) found that ambroxol can increase the activity of ciprofloxacin against *P. aeruginosa* biofilms by increasing the permeability of ciprofloxacin. Abbas et al. (2012b) reported the potentiation of ciprofloxacin against established biofilms formed by 5 *P. aeruginosa* isolates by ambroxol. Synergistic activity of piroxicam with ciprofloxacin against pre-formed *P. aeruginosa* biofilms was also observed by Abbas et al. (2012c).

In summary, this study suggests that use of antibiofilm agents in combination with ciprofloxacin may be useful to overcome the high biofilm resistance to antibiotics, but further clinical trials should be done to test the clinical efficacy of such combinations.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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