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Combination antibiotic-phytochemical effects on resistance adaptation in *Staphylococcus aureus*

Shuchi Arora[1*], Bhone Myint Kyaw[2] and Chu Sing Lim[2]

[1]School of Chemical and Biomedical Engineering, Nanyang Technological University, 62 Nanyang Drive, 637459, Singapore.
[2]School of Mechanical and Aerospace Engineering, Nanyang Technological University, 50 Nanyang Avenue, 639798, Singapore.

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Emergence and rapid spread of antibiotic resistance has become one of the leading causes of treatment failures in case of bacterial infections. Antibiotic combinations are generally used to enhance the overall efficacy of therapy with the aim to generate synergistic outcomes. It further helps in reduction of total antibiotic dosage. Phytochemicals are known to have multiple bacterial targets that modulate or modify resistance in bacteria. In the present study, a microchannel-based device and monitoring system was used to demonstrate and investigate short and long term effects of antibiotic-phytochemical combinations in different proportions on *Staphylococcus aureus* as test organism. Novel and unconventional combinations of antibiotic ciprofloxacin with the phytochemicals, quercetin, rutin, protocatechuic acid and ethyl gallate, were tested. Based on the experimental results, the strains exposed the antibiotic, generated resistant strains in four days, with 8 to 64 fold increase in their minimum inhibitory concentration (MIC) from the parent strain. The strains exposed to antibiotic-phytochemical combinations, however, showed no resistance causing mutations. The results were verified by standard laboratory practices such as disk-diffusion, mutation frequency, population profiling and molecular studies on the exposed strains. The phytochemicals were able to potentiate antibiotic activity; thereby, increasing the antibacterial efficacy and time span of the treatment with a common antibiotic.

Key words: Antibiotic combination therapy, antibiotic resistance, ciprofloxacin, microfluidic-device, phytochemical, potentiation, *Staphylococcus aureus*.

INTRODUCTION

Traditionally, phytochemicals and plant extracts have been used in medicine for centuries to treat a number of diseases and disorders that include high blood pressure, pain, asthma, depression, viral infection, cancer, diabetes,

*Corresponding author. E-mail: arora.shuchi@gmail.com. Tel: +65-9349-3212. Fax: +65-6792-4062.

etc (Cragg and Newman, 2005; Fiore et al., 2008; Sarris, 2007). Their intrinsic antibacterial property has been harnessed in a number of occasions to treat bacterial infections (Cushnie and Lamb, 2005; Gibbons, 2004; Raos and Recio, 2005). However, with discovery of antibiotics and other modern medicines, the use of herbal remedies and phytochemicals as therapeutic agents has generally reduced. On the other hand, antibiotic discovery rate has drastically reduced in recent years (Barrett, 2005; Bax et al., 2000; Norrby et al., 2005).

Phytochemicals have been under close and careful scrutiny by many researchers for their antibacterial properties (Alviano and Alviano, 2009; Cowan, 1999; Lewis and Ausubel, 2006; Raos and Recio, 2005; Shibata et al., 2005). These have been used in combination with antibiotics to treat multi-drug resistant or extensively drug resistant (MDR and XDR, respectively) bacterial species. Increasing resistance in bacteria to old and existing antibiotics (Grundmann et al., 2006; Levy and Marshall, 2004), forms the basis for such investigations. The pathogen is either reported to become totally resistant to the drug or is susceptible at a higher dose (8 folds or higher). Particularly, *Staphylococcus aureus* has been reported as one of the superbugs due to its resistance to many classes of antibiotics (Lindsay and Holden, 2004; Livermore, 2000). Alternative and newer antibacterial solutions are hence, constantly required to check the ever growing resistance in bacterial populations (Bax et al., 2000).

To overcome the fast pace of adaptive resistance in pathogenic bacteria, combination therapy is of significant interest (Cottarel and Wierzbowski, 2007; Moellering Jr, 1983). Screening of drugs in combination with other drugs, and more recently, with herbal-based extracts has thus, become increasingly popular (Cottarel and Wierzbowski, 2007; Sibanda and Okoh, 2007). The main interest behind these studies is to find beneficial combinations that typically occur at sub-minimum inhibitory concentration (MIC) levels of the antibiotic (Sakharkar et al., 2009). Combination of an antibiotic with an appropriate phytochemical may help to reduce the antibiotic dosage as well as harness the benefits of plant-derived antibacterials (Kyaw et al., 2011). They are believed to modify and/or modulate bacterial internal resistance and potentiate the effect of antibiotics when used in combination (Sibanda and Okoh, 2007). Generally, these may thus, increase the efficacy of the treatment by inhibiting the phenomenon of resistance causing elements, such as mutation of genes encoding target enzymes, proteins and efflux pumps (Marquez et al., 2005; Sibanda and Okoh, 2007; Stapleton et al., 2004a; Stapleton et al., 2004b; Tegos et al., 2002).

In this study, selected phytochemicals were shown to delay the antibiotic resistance when used in combination with ciprofloxacin as test antibiotic on *S. aureus*. A previously validated and tested microchannel-based system (Arora et al., 2009), was used to perform the long term screening of drug combinations on the pathogen.

METHODS AND MATERIALS

The current study was performed on a microchannel-based, microfluidic-device and monitoring system. The design, fabrication and validation of the system for drug combination studies have been reported by the authors previously (Arora et al., 2009, 2011). A brief description of the setup and the microchannel device has been explained in the following section. Novel combinations of ciprofloxacin and purified phytochemicals were investigated against two strains of *S. aureus*. Four phytochemicals were chosen based on their previous antibacterial reports and positive interaction with ciprofloxacin (tests not shown in this study), quercetin and rutin-flavanoids, ethyl gallate - a major tea catechin, and protocatechuic acid - a phenolic antioxidant.

Experimental system and microchip

The setup consisted of a microfluidic-device (micro-device), designed to simultaneously separate and mix two injected fluids into six varying mixtures including their original concentrations. The same device was also used as a platform for cell culturing (Arora et al., 2009). An incubation chamber was designed to provide a controlled optimum growth environment for the cells and manipulate the micro-device for monitoring. Temperature control and heating was maintained by a programmed microcontroller circuitry (ATmega16 8-bit microcontroller). The wells were monitored using a spectrometer detector at 600 nm wavelength to measure optical density (OD) of the bacteria growing under influence of drug combination at specific time intervals. The OD was translated using a conversion equation to measure bacterial growth into cell count and when compared with the laboratory-based gold standards of microbial plating and counting. The schematic diagram of the incubation chamber, nutrient supply and monitoring system is illustrated in Figure 1. The fluids were injected using pre-programmed syringe pumps into two inlets marked A and B via silicone tubings. The same inlets could be used to extract fluid, as necessary.

Bacterial strains and media

S. aureus ATCC 29213 and ATCC 43300 for methecillin sensitive and resistant species (MSSA and MRSA), respectively, were used as test organisms. Both strains were kept suspended in Luria-Bertani (LB) broth containing 40% glycerol (v/v) and stored at -80°C. Iso-sensitist (IS) broth and agar powders were used as liquid and solid media, respectively, procured from Oxoid, Biomedia Bloxwich, Singapore. The bacterial stocks were prepared in IS agar plates and stored at 4°C. Bacteria from the stock was further sub-cultured onto IS agar plates one day before each experiment.

Antibiotics and phytochemicals

Purified powders of ciprofloxacin, quercetin (quer), rutin (rut), protocatechuic acid (PCA or 3,4-dihydroxybenzoic acid) and ethyl gallate (EG) were procured from Sigma-Aldrich, Singapore. Stock solutions were prepared in DI water and the respective diluent to aid dissolution. Ciprofloxacin was dissolved in 40% (v/v) 1 M NaOH to a concentration of 10 mg/mL. Quercetin and rutin were dissolved

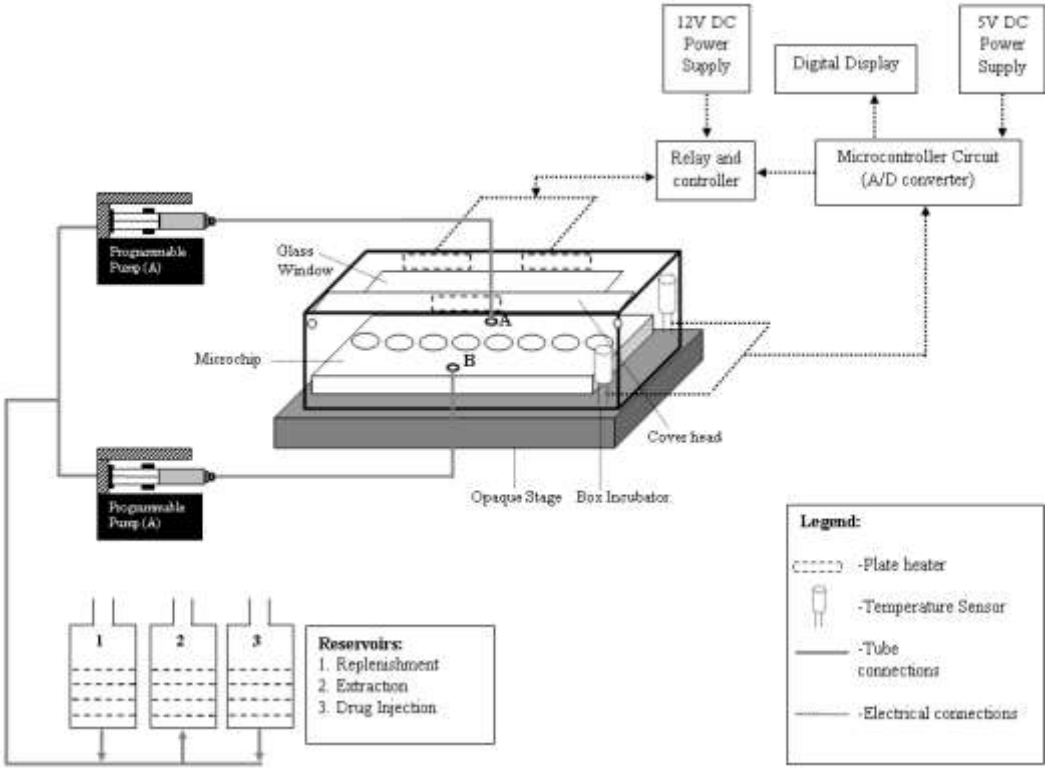

Figure 1. Schematic illustration of the monitoring system. The incubation chamber was connected to a microcontroller circuitry, pre-programmed to maintain the temperature inside the chamber at 37°C (± 0.2°C). The tubing from each syringe was connected on one side to the respective inlet (A or B) of the micro-device and on the other side connected to its respective reservoir.

in 60 and 50% (v/v) 0.1 M NaOH, respectively to a concentration of 102.4 mg/mL. PCA and EG were dissolved in 50 and 100% (v/v) ethanol (99.9%) to a concentration of 102.4 mg/mL. The stock solutions were filtered, aliquoted and stored at -20°C as per recommendations by CLSI (Standards, 2000).

MIC and FIC determination

The MICs of the drugs were determined in triplicates by broth microdilution method in IS broth as explained by Andrews (2001). The antibiotic concentration ranged from 0.0125 to 128 µg/mL and 8 to 8152 µg/mL for the phytochemicals. The titer plate was inoculated by bacteria of 0.5 Macfarland standards (Standards, 1992) and incubated at 37°C for 24 h aerobically. The MIC endpoint was determined as absence of turbidity in the wells followed by spectrometer analysis at 600 nm wavelength. The fractional inhibitory concentration (FIC) was established to interpret the combination effect of the drug-phytochemical pair under investigation. This was determined by checkerboard broth microdilution method as explained elsewhere (Pillai et al., 2005). The FIC index (FICI) for the drug combination was calculated as the sum of FIC of the two drugs.

Antibiotic-phytochemical combination tests

The tests were performed separately on MSSA and MRSA with ciprofloxacin in combination with one of each of the phytochemicals: quercetin, rutin, PCA and EG. A total of four combinations were tested on both strains, viz. ciprofloxacin with quercetin at starting concentrations equal to the MICs determined earlier (abbreviated as Cip1-Quer512, numbers indicating the MIC in µg/mL), ciprofloxacin with rutin (Cip1-Rut4096), ciprofloxacin with PCA (Cip1-PCA4096) and ciprofloxacin with EG (Cip1-EG1024). The combinations studies were performed on the experimental system explained earlier. Growth and sterility controls of the same volumes were also prepared to obtain comparative results. All experiments were performed in triplicates. A translation curve for OD versus bacterial cell number was established using exponential regression to determine the equation relating the two entities. The combinations indicating maximum bacterial growth reduction, with optical density < 0.055 and/or with cell count < 2.5×10^7 CFU/ml, after 24 h (represented as T_{24}) were then tested with the standard methods to obtain the time-kill plots to determine bactericidal activity of the selected antibiotic-phytochemical combination pair (Lorian, 2005). Bactericidal activity was established at T_{24} from the time-kill assays as greater than 3 Log_{10}-fold decreases from the starting concentration (at T_0) (Schwalbe et al., 2007). The procedures for combination screening and time-kill assays are briefly explained in the following paragraphs.

Overnight bacterial culture in IS broth was injected from the two inlets of the microchip to fill the wells to 45 µl. Subsequently, ciprofloxacin and one of the four phytochemicals were simultaneously injected from the microchip inlets. 5 µl of total solution at the required concentration were fed into each well

containing the bacterial suspensions. The end solution in the wells hence, consisted of six concoctions of the two drugs, including the original drug concentration. The resulting volume after drug injection in each well was 50 µl. The wells contained decreasing concentrations of ciprofloxacin in steps of 20% below its MIC and vice-versa for the phytochemicals. The following combinations were obtained, 20% ciprofloxacin with 80% phytochemical, denoted as Cip20-Phy80 (Phy = quer, rut, PCA or EG), similarly, Cip40-Phy60, Cip60-Phy40, Cip80-Phy20 and 100% (=MIC), Cip100 or Phy100. The microchip was incubated at 37°C in the incubation chamber (Figure 1) for 24 h. Bacterial growth was monitored by directly reading the OD of the wells at 600 nm wavelength and later translating to bacterial number using the regression equation, after 2, 4, 6, 8 and 24 h. An identical protocol was used for MRSA.

As a control experiment for comparison of combination efficacy, another experiment was performed on both strains with ciprofloxacin at one inlet and sterile DI water at the other, to check the sole effects of ciprofloxacin diluted at concentrations less than its MIC at 20% step reduction.

After analyzing the results of the above experiments for the combination of drugs and ciprofloxacin at sub-MIC concentrations, a time-kill assay was performed on the combination that suggested maximum growth inhibition indicated by the T_{24} readings from the micro-device (for cell counts < 2.5×10^7 CFU/mL). The colony counts as CFU/mL (colony forming units) were plotted against time for statistical analysis. The time-kill assay procedure is explained elsewhere (Pillai et al., 2005).

Long term behavioral study and mutant selection under drug combination effect

After the selection of useful combination mixtures of ciprofloxacin and phytochemicals, a 15-day long study was conducted. Five sterile micro-devices were inoculated with MSSA suspensions (~10^7-10^8 CFU/ml). Ciprofloxacin from inlet A and phytochemical or DI water from inlet B at concentrations equal to their respective MICs were then injected into the device. The zero hour reading or T_0 was taken at the start of Day 1. The inoculated devices incubated at 37°C for 24 h in the incubation chamber of the system. At the following day at T_{24}, 80% of the fluid in the wells (~40 µl) was extracted directly from the wells for analysis in order for fresh drug combinations and media to be added from the inlets. The extracted fluid was used to conduct disk diffusion test with 5 µg ciprofloxacin disks (Becton Dickinson, Singapore) following the procedure as previously reported (Schwalbe et al., 2007). Each sample was analyzed for ciprofloxacin susceptibility using standard manufacturer guidelines regarding the zone diameters in millimeters. This process was repeated every 24 h.

A part of the T_{72}, T_{144}, T_{216} and T_{360} samples was diluted in sterile phosphate buffer saline and plated onto agar plates for storage. These samples were sub-cultured seven times on drug free agar, followed by population analysis profiling (PAP), to study the heterogeneity of the bacterial population after continuous drug exposure (Schwalbe et al., 2007). The PAP was carried out using standard protocol on IS agar plates drugged with ciprofloxacin at concentrations ranging from 0 to 2 µg/mL (0, 0.06, 0.125, 0.25, 0.5, 1 and 2 µg/mL). Together with the disk diffusion and PAP tests, the susceptibility of ciprofloxacin was established for each sample after continuous exposure to ciprofloxacin alone (at MIC and sub-MIC levels) and to ciprofloxacin-phytochemical combinations.

The T_{360} samples (15^{th} day samples) were stored and sub-cultured at least seven times on drug free IS agar medium and used to determine the mutation frequency and mutation prevention concentration (MPC) of ciprofloxacin after continuous drug exposure. These would reveal information on whether the addition of phytochemicals delays the emergence of resistance in *S. aureus* with continuous antibiotic external pressure. The MPC window of mutation selection (window: minimum-MIC and maximum-MPC) for ciprofloxacin on *S. aureus* would also elucidate the antibiotic potentiation by the addition of phytochemicals. Calculation and determination of mutation frequency was carried out as explained elsewhere (Schwalbe et al., 2007).

For MPC calculation, an inoculum containing 10^9 CFU/ml was spread on agar plates (100 µl each plate) drugged with 2, 4, 8, 16 times MIC of the sample for ciprofloxacin. They were allowed to incubate for 72-120 h and mutation selection window was determined. The mutants selected at 2× and 4× respective MICs were also assessed further for their MICs to determine the stability of resistance. Five to ten random colonies were taken and streaked on drug free agar at least seven times before their MICs were determined. The MIC microdilution assay was carried out in triplicates to get consistent MIC values. The strains with 8-fold or higher increase in MIC from the parent MSSA ATCC 29213 strain were scrutinized further by molecular analysis to confirm occurrence of genetic mutations, if any.

Ciprofloxacin resistance was confirmed by carrying out molecular studies. Amplification of quinolone resistance-determining regions (QRDR) of *gyrA*, *gyrB*, *grlA* and *grlB* genes and *norA* promoter were performed using polymerase chain reaction (PCR), as explained by Sutandar et al. (2008).

RESULTS

The results of the antimicrobial susceptibility tests performed on MSSA and MRSA strains are summarized in Table 1. It can be noted from the preliminary micro-dilution studies and checker board analysis that the FICI for the combination of ciprofloxacin with the phyto-chemicals is > 0.5 and ≤ 1.0 that suggest synergistic/ additive outcome of the selected phytochemical with the antibiotic.

The preliminary data from the micro-device experiments provided useful combinations for further tests. In the observations from the control tests that were performed with ciprofloxacin and its dilution with DI water for MIC and sub-MIC levels (0 to 100 % ciprofloxacin), the wells showed significant bacterial growth (CFU/ml > 7×10^{10}) for concentrations less than MIC up to 20% (0.2, 0.4, 0.6, 0.8 µg/mL) and 0% as the control (data not shown) for both MSSA and MRSA. The addition of phytochemicals in place of DI water showed enhanced bacterial reduction at T_{24}. Quercetin and EG addition had a potentiating effect on ciprofloxacin dose and were able to suppress bacterial growth for 24 h for both MSSA and MRSA. All combinations of ciprofloxacin with quercetin and EG, on the micro-device gave CFU/ml < 2.5×10^7 and hence, were further tested with ciprofloxacin to determine long term usage and bactericidal activity, if present. However, for PCA and rutin on MSSA, only two combinations each were selected for further testing, Cip20-PCA/Rut80 and Cip20-PCA/Rut80. The combinations selected for PCA and rutin on MRSA were Cip20-PCA80, Cip40-PCA60, Cip80-PCA20, Cip20-Rut80 (results not shown in this study).

Table 1. MIC, FIC and FICI of Ciprofloxacin (cipro) with quercetin (quer), PCA, rutin and EG.

Organism	MIC when single drug was used (µg/ml)		MIC when drug used in combination (µg/ml)		FICI
	Cipro	Phytochemical	Cipro	Phytochemical	
MSSA	1	Quer - 512	0.25	Quer - 256	0.75
	1	Quer - 512	0.5	Quer - 128	0.75
	1	PCA - 4096	0.25	PCA - 2048	0.75
	1	PCA - 4096	0.5	PCA - 1024	0.75
	1	Rutin - 4096	0.5	Rutin - 2048	1.00
	1	EG - 1024	0.25	EG - 512	0.75
	1	EG - 1024	0.5	EG - 256	0.75
MRSA	1	Quer - 512	0.25	Quer - 256	0.75
	1	Quer - 512	0.5	Quer - 128	0.75
	1	PCA - 4096	0.25	PCA - 2048	0.75
	1	Rutin - 4096	0.5	Rutin - 2048	1.00
	1	EG - 1024	0.25	EG - 512	0.75
	1	EG - 1024	0.5	EG - 256	0.75

The bactericidal activity of the selected combinations was determined by time-kill assays. The time-kill plots are shown in Figure 2 for combinations against MSSA. From the graphs in Figure 2, it can be observed that for quercetin and EG, three out of four combinations showed total bactericidal activity, and one of the combinations showed bacterial growth suppression or inhibition only. For PCA, both the selected combination percentages showed bactericidal effects. For rutin, out of the two selected combinations from the previous experiments, one of the combinations showed bactericidal effect while the other showed bacteristatic effects.

After the 15-days experiment, the PAP studies of the samples collected at T_{72}, T_{144}, T_{216} and T_{360}, revealed the presence of subpopulations capable of growing on plates with ≥ 1 µg/ml (MIC) of ciprofloxacin. These sub-populations were not seen in the T_0 (or parent strain) PAP. Several samples from the ciprofloxacin combination pairs, including the ciprofloxacin-rutin combination (Cip20-Rut80 and Cip80-Rut20), the ciprofloxacin-quercetin combination (Cip80-Quer20) and the ciprofloxacin-EG combination (Cip20-EG80 and Cip80-EG20) showed growth on plates with 1 µg/mL ciprofloxacin in the T_{72} PAP, but no growth was observed on 2 µg/mL at T_{360} (PAP data not shown). The averaged MICs of the T_{360} samples and their respective mutation frequencies are tabulated in Table 2.

Based on the MICs and mutation frequency calculations, the ciprofloxacin exposed strains of *S. aureus* obtained at the end of 15 days (T_{360} sample) showed higher selection of mutants as compared to those exposed to combinations. Selection of mutants was also observed at 4× MIC for the respective ciprofloxacin exposed strain. Whereas, the MIC of the final strains increased up to 64 folds as compared to the starting MIC, and also compared to the T_{360} sample of the control strain. The mutation prevention concentration or MPC window for the control strain was determined as 1-4 µg/mL of ciprofloxacin. However, as seen from the table, there were mutants selected from significantly higher concentration of ciprofloxacin (32-64 µg/mL), indicating the development of ciprofloxacin-resistant *S. aureus*. For the T_{360} samples exposed to combination, the mutation frequency remained lower than the corresponding ciprofloxacin exposed strains (≤ 10^{-6}). In some cases, there were no mutants selected at two and four times MIC. The mutation frequency was hence less than 10^{-9}. The MIC of these strains was also maintained at ≤ 2 µg/mL, with Cip80-Quer/EG20 being the exceptions. The MICs of the isolates obtained from plates with respective MIC or twice MIC of ciprofloxacin is summarized in Table 3. Strains exposed to ciprofloxacin at MIC and sub-MIC (strains - Cip100-20) showed resistance to ciprofloxacin with MIC ≥ 32 µg/ml. This resistance was further confirmed by genetic analysis.

For isolates with MIC ≥ 8 µg/ml, genetic evaluation of the QRD region and *norA* promoter sections of the bacterial genome was performed. For all the ciprofloxacin exposed strains, the *gyrA*, *gyrB*, *grlA* and *norA* genes sections remained unaltered. However, a point mutation in *grlB* gene at codon 470 was found, the original asparagine amino acid was substituted by isoleucine. The resultant strain was similar to the one found by Sutandar et al. (2008) where they had exposed the parent *S. aureus* strain (ATCC strain) to 50% MIC (0.5 µg/mL) of ciprofloxacin for seven days. Interestingly,

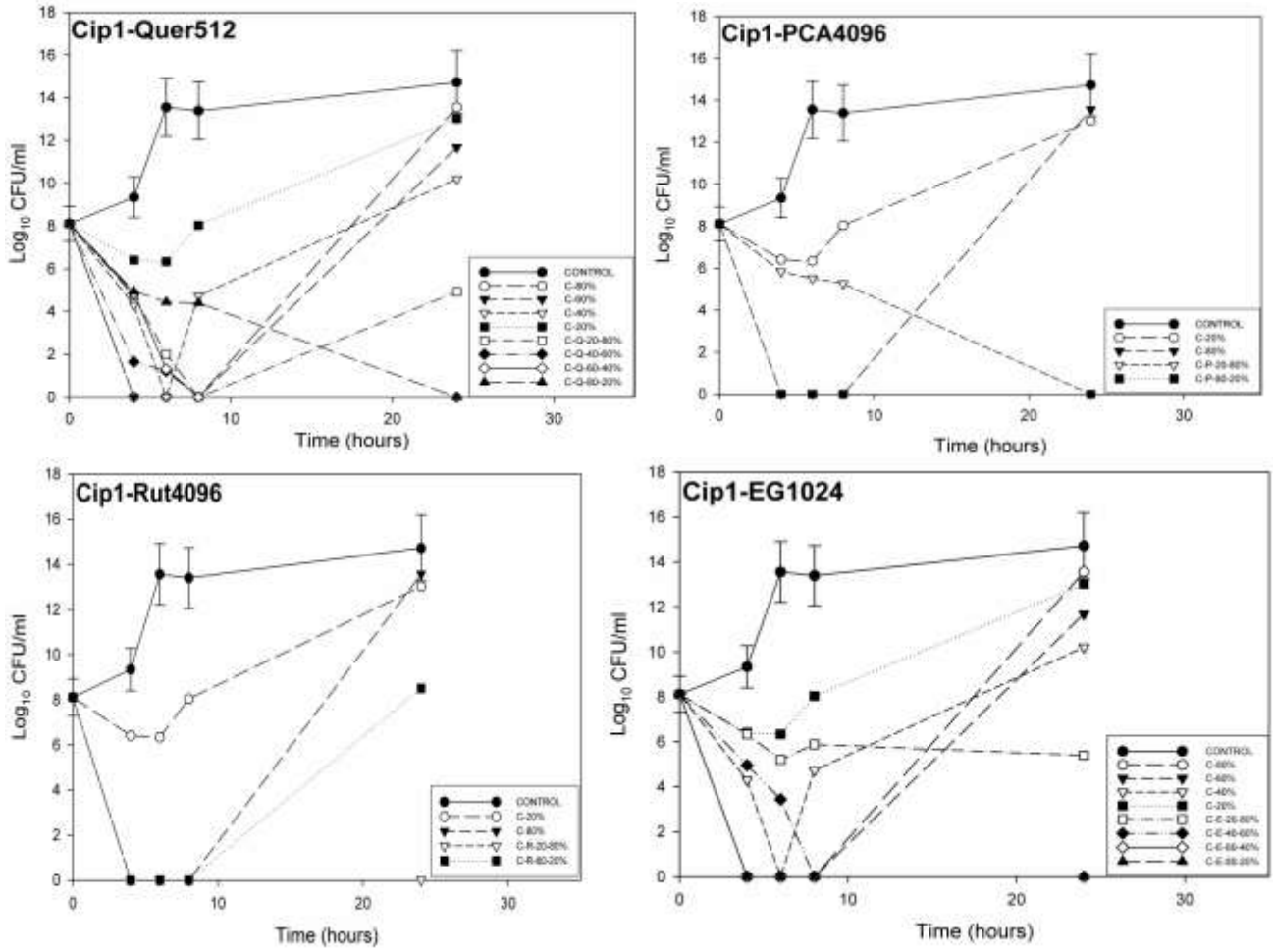

Figure 2. Time-kill plots of ciprofloxacin with a phytochemical in working combinations. (A) Ciprofloxacin in combination with quercetin (1 µg/mL – Cip1, 512 µg/mL – Quer512), (B) with protocatechuic acid (4096 µg/mL – PCA4096), (C) with rutin (4096 µg/mL – Rut4096) and (D) ethyl gallate (1024 µg/mL – EG1024).. All percentage mixtures given in the form of antibiotic to phytochemical percentage (C-Q, C-P, C-R, C-E).

there was no genetic variation found in isolates (MIC ≥ 8 µg/mL) exposed to ciprofloxacin-phytochemical combination.

DISCUSSION

In this study, the authors used ciprofloxacin that belongs to the fluoroquinolone family. It is a broad spectrum antibiotic that inhibit bacterial enzyme, DNA topoisomerase II, important for DNA replication and cell division. However, due to its increased and over use in the past, many bacterial species including *S. aureus* have acquired ciprofloxacin resistance (Campion et al., 2004; Nakanishi et al., 1991). Reduction of antibiotic dosages may contribute to minimizing the side effects associated with many antibiotics and in some cases also play a role in delaying the occurrence of resistance (Amyes et al., 2007). The reduction of antibiotic dose can be augmented by alternative antimicrobial agents in combination with it to give plausible synergy. In the present work, phytochemicals were used in combination with the antibiotic with the aim of facilitating the reduction of synthetic drug dose, thereby reducing toxicity and adverse interactions; at the same time, modulating development of adaptive resistance.

Similar to the quinolones, quercetin and rutin (plant flavanoids) target type II DNA topoisomerases and induces DNA cleavage. These are therefore, also referred to as topoisomerase poisons (Plaper et al., 2003). As seen from the results, the potentiating activity of quercetin with ciprofloxacin can be attributed to the high selectivity of quercetin for prokaryotic DNA gyrase, while ciprofloxacin primarily target topoisomerase IV in *S.*

Table 2. Mutation frequency of T_{360} samples of *S. aureus* exposed to ciprofloxacin alone and in combination with phytochemicals. The strain names are represented as drug, to which it was exposed to for 15 days, followed by the percentage of its starting MIC. The MICs given in the table were determined after 15 days of drug exposure.

Control	Strain	MIC of Ciprofloxacin (µg/ml)	Mutation frequency	
			2× MIC	4× MIC
	MSSA (ATCC 29213)	**1**	**4×10^{-7}**	**$< 10^{-9}$**
Ciprofloxacin only				
Ciprofloxacin MIC and sub-MIC concentrations	Cip100	16	10^{-1}	10^{-6}
	Cip80	8	10^{-1}	9×10^{-7}
	Cip60	8	10^{-1}	9×10^{-7}
	Cip50	8	10^{-1}	2×10^{-7}
	Cip40	8	10^{-1}	3×10^{-7}
	Cip20	4	10^{-1}	5×10^{-7}
Ciprofloxacin in combination				
With PCA	Cip20-PCA80	1	$< 10^{-9}$	$< 10^{-9}$
	Cip40-PCA60	1	$< 10^{-9}$	$< 10^{-9}$
	Cip80-PCA20	2	1.5×10^{-6}	$< 10^{-9}$
With Rutin	Cip20-Rut80	2	1.6×10^{-6}	$< 10^{-9}$
	Cip80-Rut20	2	2×10^{-6}	$< 10^{-9}$
With Quercetin	Cip20-Quer80	1	$< 10^{-9}$	$< 10^{-9}$
	Cip40-Quer60	2	$< 10^{-9}$	$< 10^{-9}$
	Cip60-Quer40	2	1.6×10^{-6}	$< 10^{-9}$
	Cip80-Quer20	4	3×10^{-6}	$< 10^{-9}$
With EG	Cip20-EG80	2	2.3×10^{-6}	$< 10^{-9}$
	Cip40-EG60	2	1.5×10^{-6}	$< 10^{-9}$
	Cip60-EG40	2	$< 10^{-9}$	$< 10^{-9}$
	Cip80-EG20	4	3×10^{-6}	$< 10^{-9}$

aureus (Bearden and Danziger, 2001; Hilliard et al., 1996). This brings about cell lysis due to irreversible DNA damage upon replication and hence, gives a bactericidal effect. This was also observed in the long term bacterial physiological analysis (Figure 2 and Tables 2 to 3). The low mutation frequency and non-mutation selection for some combinations are suggestive of delayed mutation. This delay can be due to the fact that both the drugs work on different and essential targets of the bacterial cell to bring about cell death. Ciprofloxacin resistance in *S. aureus* is mainly due to altered target, usually mutation in genes coding enzyme DNA gyrase and by multi-drug resistant or MDR efflux pumps (Bearden and Danziger, 2001; Hooper, 2002). As seen in the DNA evaluation of the ciprofloxacin exposed strains, a point mutation was observed in one of the genes coding the topoisomerase II protein sub-units. Quercetin in some studies has also shown to make the bacterial cell wall more permeable, allowing the drugs to enter through the cell wall (Mirzoeva et al., 1997). This explains the potentiating activity of the plant medicine on the antibiotic action.

In the case of ciprofloxacin with rutin, the same potentiation was not observed (Figure 2 and Table 2) even though rutin belongs to the same class of plant flavanoids. Here, the primary target for both drugs is bacterial toposisomerase IV, making it a competitive site (Bernard et al., 1997). Hence, even at high concentrations of rutin (3277 µg/mL or 80% MIC), potentiating activity was not observed at any combination.

The activity of ciprofloxacin with EG was comparable to that of ciprofloxacin with quercetin, in terms of bactericidal activity, potentiating activity and exhibiting lower mutation frequency. In general, alkyl gallates have an amphipathic molecular structure with a hydrophobic alkyl part (tail) and a hydrophilic pyrogallol moiety (head). They are known to disrupt the fluidity of the lipid bilayer of the bacterial cell membrane. Due to their molecular structure, they act as surfactants and inhibit the electron transport chain of the membrane, thereby inhibiting the bacterial respiratory system (Kubo et al., 2002, 2003). Since they do not have to enter the cell for their action, they are usually not affected by the mechanisms that cause

Table 3. Ciprofloxacin MIC (µg/mL) for mutants selected from *S. aureus* drug exposed strains. The mutants were selected from 2× and 4× respective MICs of T_{360} samples.

Strain	× MIC	MIC of selected mutant strains (µg/mL)
Control (MSSA ATCC 29213)	1	2
Cip100	2	64
Cip80	2	32
Cip60	2	32
Cip50	2	64
Cip40	1	32
Cip20	1	8
Cip20PCA80	1	2
Cip40PCA60	1	2
Cip80PCA20	2	4
Cip20Rut80	2	8
Cip80Rut20	2	16
Cip20Quer80	-	1
Cip40Quer60	2	2
Cip60Quer40	2	2
Cip80Quer20	1	8
Cip20EG80	2	2
Cip40EG60	2	2
Cip60EG40	1	2
Cip80EG20	2	4

resistance in bacteria. By further addition of ciprofloxacin, the potentiation might have taken place due to disturbance in the fluid membrane by EG, allowing easy access for the antibiotic to its intracellular target. The low mutation frequency and absence of any genetic variation in the T_{360} sample also supports the potentiation of ciprofloxacin by EG.

The activity of ciprofloxacin with PCA also showed bactericidal effect in both short term and the long term studies. The MIC was consistent with the parent strain throughout the 15 days and no mutants were selected at higher MIC values from these samples. The molecular mechanism of action for PCA and related compounds is not completely understood, but their antibacterial properties have been demonstrated by several researchers (Liu et al., 2005; Rahman et al., 2005).

The results of the present study of antibiotic potentiation by phytochemicals and their role in the delay of adaptive resistance or mutational events in *S. aureus* suggest a possible incorporation of these ubiquitous elements of nature into the treatment of resistant bacterial infection by common antibiotics. However, more research and *in-vivo* trials need to be conducted with these combinations. Even though plant-derived antimicrobials have been shown to be effective both *in-vitro* and *in-vivo*, and known for their resistance modulating capabilities, they are not yet available in mainstream medicine (Lewis and Ausubel, 2006; Simoes et al., 2009). Plant antibacterials tend to be less potent as compared to most antibiotics and unable to be used alone for treatment of infections. As also seen in this paper, their MIC is 10 to 12 fold higher than ciprofloxacin. For them to be used in a clinical scenario, the pharmacokinetic and pharmacodynamic (PK/PD) models of the drug must be taken into account. With such high MIC values *in vitro*, the bioavailability of the drug *in vivo* as effective dose becomes difficult to reach. Many academic researchers have reviewed attractive potential of plant-derived antimicrobials, and have suggested that they cannot be used alone (Alviano and Alviano, 2009; Lewis and Ausubel, 2006; Sibanda and Okoh, 2007; Simoes et al., 2009). Plant antimicrobials with their narrow spectrum of action can target multiple sites in a bacterial cell, many of which are potentiating targets for a selected antibiotic. Their addition, as resistance modifying agents can be beneficial when used in combination with a strong antibiotic. The bacterial cell acquires resistance against the antibiotic within a few days, but the addition of phytochemicals can make the treatment constructive for long term.

Conclusions

In this paper, the authors have demonstrated the use of phytochemicals as resistance modulating agents in

combination with test antibiotic, ciprofloxacin against common nosocomial bacteria, *S. aureus*. It was observed in the-long term study that *S. aureus* developed ciprofloxacin resistance in as few as four days of continuous exposure to the antibiotic. The T_{360} samples exposed only to ciprofloxacin were genetically mutated to a similar ciprofloxacin-resistant strain shown by Sutandar and co-workers (2008). This was further validated by disk diffusion tests, PAP tests and mutant selection studies. The mutation frequencies of ciprofloxacin exposed strains were much higher when compared with those of the combinations. The results suggested a delay, if not total reversal, in the process of acquiring ciprofloxacin-resistance with the addition of phytochemicals as modulators or potentiating agents.

The microchannel monitoring system used in the study was capable of expediting the process of simultaneous drug combination investigations. This system may be useful to enhance the study of antimicrobial combinations on bacterial pathogens by taking into consideration other combination ratios not usually investigated in accordance with the existing methods of FICI.

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REFERENCES

Alviano DS, Alviano CS (2009). Plant extracts: Search for new alternatives to treat microbial diseases. Curr. Pharm. Biotech. 10(1):106-121.

Amyes SGB, Walsh FM, Bradley JS (2007). Best in class: A good principle for antibiotic usage to limit resistance development? J. Antimicrob. Chemother. 59(5):825-826.

Andrews JM (2001). Determination of minimum inhibitory concentrations. J. Antimicrob. Chemother. 48(SUPPL. 1): 5-16.

Arora S, Baptista C, Lim CS (2011). Maggot metabolites and their combinatory effects with antibiotic on *Staphylococcus aureus*. Ann. Clin. Microbiol. Antimicrob. 10(1).1-6

Arora S, Lim CS, Foo JY, Sakharkar MK, Dixit P, Liu AQ, Miao JM (2009). Microchip system for monitoring microbial physiological behaviour under drug influences. Proceedings of the Institution of Mechanical Engineers, Part H: J. Eng. Med. 223(6):777-786.

Barrett JF (2005). Can biotech deliver new antibiotics? Curr. Opin. Microbiol. 8(5):498-503.

Bax R, Mullan N, Verhoef J (2000). The millennium bugs - The need for and development of new antibacterials. Int. J. Antimicrob. Agents. 16(1): 51-59.

Bearden DT, Danziger LH (2001). Mechanism of action of and resistance to quinolones. Pharmacotherapy. 21(10 II SUPPL.).

Bernard FX, Sable S, Cameron B, Provost J, Desnottes JF, Crouzet J, topoisomerase IV. Antimicrob. Agents Chemother. 41(5):992-998.

Campion JJ, McNamara PJ, Evans ME (2004). Evolution of ciprofloxacin-resistant *Staphylococcus aureus* in in vitro pharmacokinetic environments. Antimicrob. Agents Chemother. 48(12):4733-4744.

Cottarel G, Wierzbowski J (2007). Combination drugs, an emerging option for antibacterial therapy. Trends Biotechnol. 25(12):547-555.

Cowan MM (1999). Plant products as antimicrobial agents. Clin. Microbiol. Rev. 12(4):564-582.

Cragg GM, Newman DJ (2005). Plants as a source of anti-cancer agents. J. Ethnopharmacol. 100(1-2):72-79.

Cushnie TPT, Lamb AJ (2005). Antimicrobial activity of flavonoids. Int. J. Antimicrob. Agents. 26(5):343-356.

Fiore C, Eisenhut M, Krausse R, Ragazzi E, Pellati D, Armanini D, Bielenberg J (2008). Antiviral effects of Glycyrrhiza species. Phytother. Res. 22(2):141-148.

Gibbons S (2004). Anti-*staphylococcal* plant natural products. Nat. Prod. Rep. 21(2):263-277.

Grundmann H, Aires-de-Sousa M, Boyce J, Tiemersma E (2006). Emergence and resurgence of meticillin-resistant *Staphylococcus aureus* as a public-health threat. Lancet. 368(9538):874-885.

Hilliard JJ, Krause HM, Bernstein JI, Fernandez JA, Nguyen V, Ohemeng KA, Barrett JF (1996). A comparison of active site binding of 4-quinolones and novel flavone gyrase inhibitors to DNA gyrase. Adv. Exp. Med. Biol. 390:59-70.

Hooper DC (2002). Fluoroquinolone resistance among Gram-positive cocci. Lancet Infect. Dis. 2(9):530-538.

Kubo I, Fujita KI, Nihei KI (2002). Anti-*Salmonella* activity of alkyl gallates. J. Agric. Food Chem. 50(23):6692-6696.

Kubo I, Fujita KI, Nihei KI (2003). Molecular design of multifunctional antibacterial agents against methicillin resistant *Staphylococcus aureus* (MRSA). Bioorg. Med. Chem. 11(19):4255-4262.

Kyaw BM, Arora S, Win KN, Danie LCS (2011). Prevention of emergence of fusidic acid and rifampicin resistance in *Staphylococcus aureus* using phytochemicals. Afr. J. Microbiol. Res. 5(22):3684-3692.

Levy SB, Marshall B (2004). Antibacterial resistance worldwide: Causes, challenges and responses. Nat. Med. 10(S12):S122-S129.

Lewis K, Ausubel FM (2006). Prospects for plant-derived antibacterials. Nat. Biotechnol. 24(12):1504-1507.

Lindsay JA, Holden MTG (2004). *Staphylococcus aureus*: Superbug, super genome? Trends Microbiol. 12(8):378-385.

Liu KS, Tsao SM, Yin MC (2005). In vitro antibacterial activity of roselle calyx and protocatechuic acid. Phytother. Res. 19(11): 942-945.

Livermore DM (2000). Antibiotic resistance in staphylococci. Int. J. Antimicrob. Agents. 16(S1):S3-S10.

Lorian V (2005). Antibiotics in Laboratory Medicine PA 19106, Lippincott William and Wilkins.

Marquez B, Neuville L, Moreau NJ, Genet JP, Dos Santos AF, De Andrade MCC, Sant'Ana AEG (2005). Multidrug resistance reversal agent from Jatropha elliptica. Phytochemistry. 66(15):1804-1811.

Mirzoeva OK, Grishanin RN, Calder PC (1997). Antimicrobial action of propolis and some of its components: The effects on growth, membrane potential and motility of bacteria. Microbiol. Res. 152(3):239-246.

Moellering RC (1983). Rationale for use of antimicrobial combinations. Am. J. Med. 75(2 A):4-8.

Nakanishi N, Yoshida S, Wakebe H, Inoue M, Yamaguchi T, Mitsuhashi S (1991). Mechanisms of clinical resistance to fluoroquinolones in *Staphylococcus aureus*. Antimicrob. Agents Chemother. 35(12):2562-2567.

Norrby SR, Nord CE, Finch R (2005). Lack of development of new antimicrobial drugs: A potential serious threat to public health. Lancet Infect. Dis. 5(2):115-119.

Pillai SK, Moellering RCJ, Eliopoulos GM (2005). Antimicrobial Combinations. Antibiotics in Laboratory Medicine. Lorian V, Lippincott Williams and Wilkins. pp. 365-440.

Plaper A, Golob M, Hafner I, Oblak M, Å olmajer T, Jerala R (2003). Characterization of quercetin binding site on DNA gyrase. Biochem. Biophys. Res. Commun. 306(2):530-536.

Rahman MM, Lopa SS, Sadik G, Harun Or R, Islam R, Khondkar P,

Alam AHMK, Rashid MA (2005). Antibacterial and cytotoxic compounds from the bark of Cananga odorata. Fitoterapia. 76(7-8):758-761.
Raos JL, Recio MC (2005). Medicinal plants and antimicrobial activity. J. Ethnopharmacol. 100(1-2): 80-84.
Sakharkar MK, Jayaraman P, Soe WM, Chow VTK, Sing LC, Sakharkar KR (2009). In vitro activity of antibiotics and phytochemicals against *Pseudomonas aeruginosa*. J. Microbiol. Immunol. Infect. 42(5):364-370.
Sarris J (2007). Herbal medicines in the treatment of psychiatric disorders: A systematic review. Phytother. Res. 21(8):703-716.
Schwalbe R, Steele-Moore L, Goodwin AC (2007). Antimicrobial Susceptibility Testing Protocols, CRC Press, Taylor and Francis Group.
Shibata H, Kondo K, Katsuyama R, Kawazoe K, Sato Y, Murakami K, Takaishi Y, Arakaki N, Higuti T (2005). Alkyl gallates, intensifiers of B-lactam susceptibility in methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. 49(2):549-555.
Sibanda T, Okoh AI (2007). The challenges of overcoming antibiotic resistance: Plant extracts as potential sources of antimicrobial and resistance modifying agents. Afr J Biotechnol. 6(25): 2886-2896.
Simoes M, Bennett RN and Rosa EA (2009). Understanding antimicrobial activities of phytochemicals against multidrug resistant bacteria and biofilms. Nat. Prod. Rep. 26(6):746-757.
Standards NCCL (1992). Methods for determining bactericidal activity of antimicrobial agents. National Committee for Clinical Laboratory Standards**:** M26-T.
Standards NCCL (2000). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, National Committee for Clinical Laboratory Standards: M7-A5.
Stapleton PD, Shah S, Anderson JC, Hara Y, Hamilton-Miller JMT, Taylor PW (2004a). Modulation of B-lactam resistance in *Staphylococcus aureus* by catechins and gallates. Int. J. Antimicrob. Agents. 23(5):462-467.
Stapleton PD, Shah S, Hamilton-Miller JMT, Hara Y, Nagaoka Y, Kumagai A, Uesato S, Taylor PW (2004b). Anti-*Staphylococcus aureus* activity and oxacillin resistance modulating capacity of 3-O-acyl-catechins. Int. J. Antimicrob. Agents. 24(4):374-380.
Sutandar A, Lim CS, Hsu LY (2008). System for real-time monitoring of mutation-in-progress. J. Appl. Microbiol. 104(5):1400-1407.
Tegos G, Stermitz FR, Lomovskaya O, Lewis K (2002). Multidrug pump inhibitors uncover remarkable activity of plant antimicrobials. Antimicrob. Agents Chemother. 46(10):3133-3141.