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In vitro drug interactions of gallates with antibiotics in *Staphylococcus Aureus*

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1. ABSTRACT

The antimicrobial activities of tetracycline, mupirocin, and fusidic acid are tested in combination with Epicatechin Gallate (ECG), and Ethyl Gallate (EG) using 2 Methicillin resistant (MRSA) and 2 Methicillin sensitive (MSSA) strains of *Staphylococcus aureus*. Sub-inhibitory concentration of EG at 256mg l^{-1} is found to be synergistic when used in combination with tetracycline, mupirocin, and fusidic acid; and a sub-inhibitory concentration of ECG at 32 mg l^{-1} is found to be synergistic with tetracycline in all the four *Staphylococcus aureus* strains tested. The synergistic combinations reduce the MICs of all the above three antibiotics by 4 fold. Combining ECG at 32mg l^{-1} with mupirocin, reduces the MIC of mupirocin by four fold in MSSA C1 strain. 74% of the combinations show consistent results in both time-kill assay and checkerboard method. The identified combinations may lead towards novel therapeutic interventions for treating MRSA infections.

2. INTRODUCTION

S. aureus is a gram positive bacterium that is prevalent in hospital settings such as intensive care units, burn units and surgical units. It infects patients who have impaired immune system such as those suffering from diabetes mellitus and Acquired Immunodeficiency Syndrome (AIDS). MRSA is regarded as the third most prevalent nosocomial gram positive pathogen and is the second most important bacterium in the etiology of both pneumonia and catheter-related bacteremia (1). The emergence of resistance to various antimicrobials is the main problem in chemotherapy of *S. aureus*. MRSA are reported to show resistance not only to methicillin, but also to a broad spectrum of beta-lactam antibiotics, tetracyclines, sulfonamides, trimethoprim, and aminoglycosides (2).

Currently MRSA infections are treated by glycopeptides, vancomycin and teicoplanin, and

combinations of fusidic acid, rifampicin, and mupirocin. These antibiotics are used mainly for treatment of superficial infections (fusidic acid) or for the eradication of MRSA in nasal passages (mupirocin). Until recently, vancomycin was the antibiotic of choice for MRSA, however, several MRSA strains resistant to vancomycin are prevalent. With the dearth of new antibiotics coming to the marketplace and the evolution of Multi Drug Resistant (MDR) bacteria, life-threatening *S. aureus* bacterial infection are common (3). Thus, there is a need to find new ways to control MRSA and embark on a continued search for novel antimicrobial compounds and combinations.

Concurrently, plants have provided a source of hope for novel drug compounds. Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have antimicrobial properties (4). Among the numerous anti-bacterial phytochemicals, the properties of tea tannins have been widely investigated because of the continual use of tea compounds over ages for the treatment of a wide variety of diseases, their diversity and their easy availability and accessibility. Epicatechin gallate (ECG), a catechin found in Japanese green tea, *Camellia sinensis* is reported to have antioxidant, anti-inflammatory and anti-microbial properties (5). ECG's anti-MRSA activity and its effects on the cell wall and cell surface of Staphylococcus strains have been studied and reported by Stapleton *et al.* (6, 7). Alongside, anti-bacterial and anti-MRSA activities of several Alkyl Gallates have been investigated both as single agent and in combination with beta-lactam antibiotics. It is also reported that Ethyl Gallate (EG) is able to intensify beta-lactam susceptibility in methicillin-resistant and methicillin-sensitive strains of *S. aureus* (8).

In this investigation, three non-beta-lactam antibiotics (mupirocin, tetracycline, and fusidic acid) are tested for their interaction with ECG and EG. Mupirocin is a unique antimicrobial mainly used for the decolonization of MRSA in the patient's nasal passages (9). Fusidic acid is a bacteriostatic antibiotic which is commonly used in combination therapy with rifampicin, and tetracycline is a bacteriostatic polyketide antibiotic commonly used in ointments (10). To the best of our knowledge, as of today, this is the first report on the interaction of non-beta-lactam antibiotics with EG and ECG. These experiments have been conducted with the hope that the identified synergistic combinations will provide novel therapeutic strategies for the treatment of infections caused by *S. aureus*. These combinations may also prevent the evolution of resistance that develops due to treatment with mupirocin, tetracycline or fusidic acid as single agent.

3. MATERIALS AND METHODS

3.1. Bacterial strains and growth conditions

The bacterial strains used in this experiment were - Methicillin Resistant *S. aureus* (MRSA); clinical strain MRSA C1, Methicillin Sensitive *S. aureus* - clinical strain MSSA C1, and a clinical strain of MSSA C2. Methicillin Resistant *S. aureus* ATCC 43300 was used as a control strain throughout the investigation. The bacteria were stored at -80°C in 15% glycerol broth. The growth media used were Iso Sensitest Broth and Iso Sensitest agar (ISB, Biomedia).

3.2. Reagents

The chemicals used were: Epicatechin Gallate: Potency: ≥98% (Sigma-Aldrich), Tetracycline ≥99% (Sigma-Aldrich), Mupirocin and Fusidic acid ≥99% (Fluka), and Ethyl Gallate 99% (Acros Organics). Stock Solutions were prepared and dilutions were made according to manufacturer's recommendations.

3.3. Methodology

3.3.1. Minimum Inhibitory Concentration (MIC) determination

Minimum inhibitory concentration was determined by microbroth dilution method performed in 96-well microtiter plates with an inoculum of 10^6 CFU/ml (11). Iso Sensitest Broth was used in this study. Microbroth dilution assay was done in accordance with the procedure recommended by the Clinical and Laboratory Standards Institute (CLSI) (12, 13). All MIC determinations were done in triplicate. The bacterial culture was incubated at 37°C for 24 hours. MIC was defined as the lowest concentration of antibiotic that inhibited the visible growth. MIC readings were done both visually and confirmed by optical density reading with spectrophotometer at 600nm.

3.3.1. Checkerboard assay

Antibiotic interactions were determined by the checkerboard assay. Checkerboard assay is an easy and one of the most frequently used method for assessing drug interactions (14) (2, 15). The bacterial concentration used for this analysis was 10^6 CFU/ml. The combinations were done using 96-well microtiter plates, and incubated at 37° C for 24 hours. The readings were done both visually and confirmed by optical density reading with spectrophotometer at 600nm.

Drug interactions (synergy, indifference or antagonism) were measured by determining the fractional inhibitory concentration (FIC), which is a ratio of the MIC of a drug in combination and MIC of the drug alone. The results were expressed as fractional inhibitory concentration (FIC) index equal to the sum of the FICs for each drug. If the FIC index is ≤ 0.5, the antimicrobial combination was interpreted as synergistic, between 0.5 and 4 as indifferent, and >4 as antagonistic.

3.3.1. Time kill assay

In the time kill method, bacterial strains were grown in presence and absence of antimicrobial agents (both alone and in combination with phytochemicals). Falcon tubes containing Drug A (Antibiotic – tetracycline, mupirocin, or fusidic acid), Drug B (Phytochemical – ECG or EG), Drug A+B, positive control (*S. aureus* + ISB), were inoculated with 1.5 x 10^6 CFU/ml of bacteria and incubated at 37°C for 24 hours, respectively. A falcon tube containing only the ISB was used as a negative control. Samples were withdrawn from each of the tubes at 4 hours, 8 hours and 24 hours and plated for the determination of

Table 1. Effect of ECG and EG on efficacies of different antibiotics against strains of *S. aureus*

Bacteria strain	Antibiotic	Antibiotic alone (mg l^{-1})	MIC mg l^{-1} Antibiotic with phytochemicals		Checkerboard FIC index		Checkerboard Interaction		Time Kill Interaction	
			EG (256mg l^{-1})	ECG (32mg l^{-1})	EG (256mg l^{-1})	ECG (32mg l^{-1})	EG (256mg l^{-1})	ECG (32mg l^{-1})	EG (256mg l^{-1})	ECG (32mg l^{-1})
MRSA C1	Tetracycline	1	0.25	0.25 4 0.03	0.5	0.5	S	S	S	S
	Mupirocin	4	1		0.5	1	S	I	S	ND
	Fusidic Acid	0.03	0.0075		0.5	1	S	I	S	ND
MRSA ATCC	Tetracycline	0.12	0.03	0.03 1 0.01	0.5	0.5	S	S	S	S
	Mupirocin	1	0.25		0.5	1	S	I	S	ND
	Fusidic Acid	0.01	0.0025		0.5	1	S	I	S	ND
MSSA C1	Tetracycline	0.25	0.06	0.06 0.06 0.015	0.5	0.5	S	S	S	S
	Mupirocin	1	0.25		0.5	0.25	S	S	S	ND
	Fusidic Acid	0.03	0.0075		0.5	0.75	S	I	S	ND
MSSA C2	Tetracycline	0.5	0.12	0.12	0.5	0.5	S	S	S	S
	Mupirocin	0.25	0.06	0.25	0.5	1	S	I	S	ND
	Fusidic Acid	0.25	0.06	0.06	0.5	0.5	S	S	S	ND

The MIC of EG was 1024 mg l^{-1} . The MIC of ECG was 128 mg l^{-1} .*S= synergy *I= Indifference ND- Not Determined (as combination is non-synergistic in checkerboard)

Table 2. Difference in viable colony counts at different time intervals expressed in $\log_{10}$ value

Phytochemical	Antibiotic	*S. aureus* strain	0 hrs	4 hrs	8 hrs	24 hrs
ECG	T	ATCC	0	1.1	5.6	1.5
		MRSA C1	0	2.2	2.2	5.3
		MSSA C1	0	3.2	4.2	1
		MSSA C2	0	1.3	3.5	1
EG	F	ATCC	0	0.2	1.6	5
		MRSA C1	0	1.8	3.4	4
		MSSA C1	0	0.6	0.8	1.1
		MSSA C2	0	2.2	2.1	1.7
EG	M	ATCC	0	2.3	3.7	1.55
		MRSA C1	0	1.2	2.86	5.6
		MSSA C1	0	0.95	2.97	3
		MSSA C2	0	2.5	2.8	2.9
EG	T	ATCC	0	2.4	4.1	2
		MRSA C1	0	2.5	2.4	9.4
		MSSA C1	0	3.8	5.3	6.8
		MSSA C2	0	2	1.6	3.4

T- Tetracycline, M- Mupirocin, F-Fusidic acid, EG- Ethyl Gallate, ECG- Epicatechin Gallate

viable colony counts. The viable colony counting (CFU/ml) was measured at 48 hours after incubation at 37°C. Synergy was defined as ≥2 $\log_{10}$ CFU/ml fold decrease by the combination when compared with the most active single agent at any time during the 24hour experiment. Antagonism was defined as a ≥2 $\log_{10}$ CFU/ml fold increase by the combination compared with the most active single agent. Time kill assay was performed as a confirmation, only for combinations that show significant interactions in the checkerboard assay.

4. RESULTS

4.1. MIC

The MIC values for all the antibiotics and phytochemicals are shown in the Table 1. MIC values of Epicatechin gallate (ECG) and Ethyl gallate (EG) were found to be 128 mg l^{-1} and 1024 mg l^{-1}, respectively. MIC values of fusidic acid, mupirocin and tetracycline were found to be 0.01- 0.03mg l^{-1}, 0.25-4mg $l^{-1}$and 0.12-1mg l^{-1}, respectively. It must be mentioned that many MRSA have tetracycline resistance due to *tet* gene and the strains under investigation are tetracycline sensitive. It is observed that MIC values of antibiotics are much lower than the MIC value of phytochemicals as expected. ECG has lower MIC value than EG (Table 1). This suggests for higher antimicrobial activity (~10 fold) of ECG as compared to EG, against all the *S. aureus* strains under investigation. These results are in accordance with previous findings by Stapleton *et al.* (6).

4.2. Checkerboard assay and time killing assay

The activity of antimicrobial combinations can be assessed *in vitro* using checkerboard and time-kill method. While most data available have been generated using checkerboard, some investigators have used time-kill in order to provide a more dynamic description of antimicrobial activity and interactions depending on time (14, 15). In checkerboard method, it is observed that addition of ¼ MIC of EG (256 mg l^{-1}) reduces MIC of all the three antibiotics i.e. fusidic acid (Protein synthesis inhibitor), mupirocin (Isoleucyl t-RNA synthetase inhibitor), and tetracycline (30S ribosome inhibitor) by four fold in all the strains of *S. aureus* under investigation. Also, the addition of ¼ MIC of ECG (32mg l^{-1}) causes a reduction (~4 fold) in MIC of tetracycline in all the *S. aureus* strains under investigation. However, it is interesting to observe that the addition of ¼ MIC of ECG

(32 mg l^{-1}) shows a synergistic effect with ¼ MIC of mupirocin only for the MSSA strain C1 and not the MRSA strains. It is also observed that ECG and fusidic acid are synergistic (¼ MIC + ¼ MIC) against only one of the strains (MSSA clinical C2). These data show that different drug combinations have different effect on different strains of *S. aureus*, which may depend on the genetic diversity of the organism. In other words the susceptibility of *S. aureus* strains to the identified combinations may vary. The MIC data, checkerboard assay results and time kill findings are summarized in Table 1.

All the synergistic combinations identified by checkerboard method when tested by time-kill method confirmed that EG can prolong and potentiate the bactericidal activity of tetracycline. The effect of the combination of EG and tetracycline peaked at 8 hours and lasted for 24 hours. ECG shows highest synergistic effect at 8 hours (Table 2). However, it cannot sustain the antibacterial action until 24 hours like EG. (Figure 1 A-P)

74% of the combinations show consistent results in both time-kill assay and checkerboard method.

5. DISCUSSION

This study for the first time shows that EG potentiates the activity of the non- beta-lactam antibiotics in both MSSA and MRSA, while ECG selectively potentiates the activity of non- beta-lactam antibiotics in MSSA. It is also found that tetracycline is synergistic with ECG and EG in both MSSA and MRSA. Thus, the presented data confirms that treatment with combinations of antibiotics and phytochemicals may improve therapeutic efficacy over single agent treatment (8, 16, 17). Of note, the MIC levels of both of the phytochemicals in this study are lower than the reported toxicity level for these chemicals. EG has relative toxicity of 1.5 and ECG has relative toxicity of 2 in the toxicity studies graded from 0 being very safe and 3 being very toxic (18). Therefore, these two compounds have potential for use as antimicrobials in the routine treatments as they have the capability to increase the susceptibility of organism to antibiotics and are safe. Since, these combinations can help lower the dosage of conventional antibiotics, it is suggested that they may prevent the evolution of resistance.

Stapleton *et al.* observed that ECG mediates alterations to the physical nature of the bilayer, elicits the structural changes to wall's teichoic acid, which is the acid labile component of peptidogylcan and results in modulation of the cell-surface properties necessary to maintain the beta-lactam-resistant phenotype. Kubo *et al.* postulated that EG inhibits the respiratory chain of MRSA bacteria (6, 19). However, currently, the mechanism responsible for the synergism between the combinations is not known. Despite the lack of knowledge for the underlying mechanism of the synergistic effect of these combinations, the identified synergistic combinations could have potential value in treating life-threatening bacterial infections caused by *S. aureus*. They may also provide novel ways of combating drug resistance since phytochemicals are structurally different from antibiotics and often have different modes of action. These data encourage further studies with these agents plus other antimicrobial classes and *in vivo* animal experiments to further investigate these interesting finding before clinical tests can move forward.

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