# Antimicrobial effects of epigallocatechingallate and epicatechins of green tea on planktonic and biofilm forms of *staphylococcus aureus*, including MRSA

Mostafa Mahmoud[1], Fahd Alkhaleefah[2], and Doaa Mohammed Sherif[3]

[1]. Department of Medical Microbiology and Immunology, Faculty of Medicine, Ain Shams University, Egypt.
[2]. Department of Biological Sciences, Faculty of Science, University of Hull, Cottingham Road, Hull, UK.
[3]. Department of Medical Microbiology and Immunology, Faculty of Medicine, Mansoura University, Egypt.
mamostafa67@yahoo.com

**Abstract:** *Staphylococcus aureus* (SA) is a common microorganism responsible for many types of infections. Resistance to conventional drugs is rapidly acquired by SA, especially strains such as methicillin-resistant (MRSA), vancomycin intermediate- *Staphylococcus aureus* (VISA) and vancomycin-resistant (VRSA). The study was conducted to assess the effects of two green tea polyphenolic catechin compounds, epigallocatechingallate (EGCG) and epicatechins (EC), on 120 non-repetitive strains of SA (90 MRSA and 30 methicillin-sensetive *SA "*MSSA*"*). After determination of the EGCG minimal inhibitory concentrations (MICs), 12 conventional antimicrobial agents were tested for their effect on staphylococcal strains alone and in combination with the sub-MIC of EGCG. Eighteen of the Innovotech MBEC kits were used to induce biolfims over its pegs which were then challenged with EGCG to test for its effects upon biofilm and planktonic bacterial forms. Results showed that EGCG has antibacterial effects against all the tested strains of staphylococci with mean MIC of 64-256 µg/ml (mostly 256 µg/ml) upon planktonic bacteria and minimal biofilm eradication concentration (MBEC) of 512 µg/ml actericidal upon biofilms. No antibacterial effects for EC were detected. There was positive synergy with penicillin (and ciprofloxacin upon MRSA), and additive effects with EGCG and clarithromycin and gentamycin. These results prove the in vitro antibacterial effects of EGCG of green tea upon both planktonin and biofilm MRSA and MSSA, however further clinical studies are in need.
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## 1. Introduction

The development of bacterial resistance to traditional antibacterial drugs has led researchers to consider the use of other compounds with antibiotic action e.g. natural tea plant extract (Stapleton and Taylor, 2002).

Tea which is the most non-alcoholic popular beverage, after water, and is consumed regularly worldwide has around six types, all of them originating from a single plant called *Camellia sinensis*. These types differ according to how the fresh leaves are processed and scented. The three most commonly used teas are black (fully fermented with low quality of polyphenols), oolong (partially fermented), and green tea which is obtained by the application of steam, then drying or pan-frying to inactivate the enzyme polyphenol oxidase, resulting in a number of polyphenols in a monomeric form (Ng et al., 2008; Bansala et al., 2013). The phenolic catechins present in green tea include; epicatechin (EC), epigallocatechin-3-gallate(EGCG), epicatechin -3-gallate (ECG), epigallocatechin (EGC), gallocatechin (GC) and catechin. Among all tea polyphenols, the most predominating catechin is the EGCG, which represents around 65% of the total catechins, with 100-200 mg of it in a cup of green tea, epigallocatechin-3-gallate (EGCG) (Zaveri, 2006). It has been shown that green tea has antibacterial effects noted many years ago (Cushnie and Lamb, 2005), antiviral effects, including against HIV (Li et al., 2000), anti-ageing (Esposito et al., 2002) and anti-inflammatory effects (Dona et al., 2003; Bansala et al., 2013). Green tea also has antioxidant effects which help to protect from cancers, cardiovascular and neurological diseases (Yang et al., 2002; Petti and Scully, 2009). The EGCG content of white tea is greater than that of green tea and black tea (Tanaka et al., 2010).

*Staphylococcus aureus* which is present as commensal microflora in many parts of the human body including the skin, external orifices, and the upper respiratory tract so, it act as one of the most opportunistic pathogens and is the most common infectious agent encountered by human beings. Almost every individual will experience at least one skin infection due to this microorganism (Elston and Barlow, 2009).

Methicillin-resistant Staphylococcus aureus (MRSA) has become an increasingly important pathogen in both hospital and community settings. Although vancomycin is generally the first choice for treatment of MRSA infections, vancomycin-resistant S. aureus has been reported in several countries, including the United States. Therefore, researchers began to investigate natural plant oils and other alternative lines of treatment in search for effective antibacterial agents to combat such resistant bacteria (Tohidpour et al., 2010). The antibacterial effects of honey were trialled in the treatment of infections (Maeda et al., 2008) and tea tree oil and tea extracts have been used in several countries (Warnke et al., 2009).

The formation of bacterial biofilms which is a conglomerate bacterial communities that live within a self-produced extracellular matrix formed from polymeric materials and firmly attached to the surface (Bridier et al., 2010), is also considered to be one of the virulence factors of *Staphylococcus aureus,* as it protects the organism from the effects of antimicrobials and disinfectants (Diemond-Hernández et al., 2010). Bacteria present and entrapped in a biofilm differ from free-living bacteria (planktonic) in being highly resistant to antimicrobial drugs, highly resistant to the protective actions of the host immune response due to its isolation in the biofilm and more resistant to the action of disinfectants when the biofilm is formed over an inanimate surface (Costerton et al., 2005; Kalishwaralal et al., 2010). The formation of biofilm is also of crucial importance in those infections caused by implants, prosthesis or body surface applied devices e.g. intravenous cannulae or catheters, due to colonization of these items and subsequent infections (Sepandj et al., 2003; Kalishwaralal et al., 2010). The National Institute of Health in the USA has stated that more than 80% of microbial infections are due to biofilm formation. Also, the persistence and chronicity of bacterial infections occurring in endocarditis, otitis media, osteomyelitis, and biliary tract infections are mainly due to the formation of bacterial biofilms (Cogan, 2006; Shafahi and Vafai, 2010).

Some commercially available systems are used to induce the formation of bacterial biofilms including the MBEC™ assay, which allows microorganisms to grow and form films over 96 identical pegs protruding down from a plastic lid. These pegs can be then fitted to the commercial 96-well microplates in which different concentrations of antimicrobial, metal, or biocide compounds can be applied and tested for their killing activities upon the formed biofilms over the peg. (Ceri et al., 1999; Olson et al., 2002).

The aim of this study was to detect the possible antibacterial activity of two components of green tea extracts; epicatechin (EC) and epigallocatechin-3-gallate (EGCG) on *Staphylococcus aureus* including MRSA, both the free-living (planktonic) and biofilm types. It also aimed to reveal if there is any possible synergism between these two components and some of the more commonly used conventional antibacterial drugs used in the treatment of infections caused by these microorganisms.

## 2. Materials and Methods

**Source and storage of the microorganisms:** One hundred twenty non-repetitive strains of SA (each one isolated from different patient with a specific number; 90 MRSA and 30 MSSA) strains were identified as MRSA by being resistant to cefoxitin, and/or oxacillin and were isolated from different pathological materials from patients attending AL-Iman (215-bed) and Al-Dawadmi (200-bed) General Hospitals, that belongs to Saudi Ministry of Health, the first hospital is in Riyadh while the second is around 300 KM west to Riyadh. The strains were identified by Microscan WalkAway 96 SI system using Positive Combo panel type 21 (Siemens Healthcare Diagnostics Inc. USA) during the period from the years 2009-2012. The identified strains were labeled and kept frozen in cryovials at -70 $^{o}$C till time of need. The used quality control strains were ATCC# 25923 (MSSA) and ATCC# 25913 (MRSA) from BD (UK). When indicated, fresh culture was done from cryogenic stocks upon nutrient agar plates (NA) Oxoid (UK) following standard bacteriological techniques.

**EGCG source and stock solution preparation:** both EGCG and EC (with 99% purity) were purchased from Sigma-Aldrich® Chemical Co. Inc. (UK). Both substances were in the form of dry powder stored at 5-8 $^{o}$C until needed. Stock solutions of EGCG and EC were made up with 100% ethanol.

**MIC determination by Disc diffusion method:** The working dilutions of both EGCG and EC was done at various concentrations; (1024, 512, 256, 128, 64, and 32 µg/ml) according to methods used by some researchers (CLSI, 2012a; Blanco et al., 2005). For disc preparation, 10 µL from the corresponding working dilution were pipette upon a 6.5 mm blank paper susceptibility disc (Mast Group Ltd, Merseyside, UK) and allowed to dry in sterile desiccators. The concentrations used in disc diffusion study were (32, 64, 128, 256, and 512 µg/ml for EGCG. These discs were used and tested in triplicates in same conditions as antimicrobial discs. As ethanol has antibacterial action in higher concentrations, discs containing ethanol only without EGCG or EC were inoculated as control discs. As EC failed to produce inhibition zone at these concentrations, higher contents were used than in EGCG. The minimum

inhibitory concentration (MIC) of each of EGCG or EC was defined as the lowest concentration of either, showing no visible growth (Ando et al., 1999). All tests were carried out in triplicate and the average one was recorded.

**Synergy testing:** Twelve antimicrobial discs were tested against all 120 studied isolates by the disc diffusion method according to the CLSI technique (CLSI, 2012a) included; penicillin (P, 1 unit), vancomycin (VA, 5µg), Gentamycin (CN, 10 µg), fusidic acid (FD, 10µg), clarithromycin (CLR, 2 µg), linezolid (LZD, 10 µg), ciprofloxacin (CIP, 1 µg), Neomycin (N, 10 µg), Rifampicin (RD, 2 µg), Trimethoprim (W, 5 µg), Tetracycline (TE, 10 µg) and mupirocin (MUP, 5 µg) all were supplied from Oxoid (Basingstoke, Hampshire, UK). These antimicrobial discs were used alone and in combination with the EGCG in sub-MIC concentrations (32, 64, and 128 µg/ml) to detect synergism between them by adding 10 µl of EGCG suspension to the antimicrobial discs (Blanco et al., 2005). No synergy test was done with EC as it failed to inhibit bacterial growth at higher concentrations.

**Determination of the MIC and the MBC of EGCG in the broth, for all strains of planktonic bacteria:** Different concentrations of EGCG, based upon the primary results of disc diffusion (1024, 512, 256, 128, 64, 32, and 16 µg/ml) were prepared and mixed with the culture broth in sterile Eppendorfs. Then the susceptibility testing was run in duplicate in microtitre tray using total volume of 200 µL and applying standard bacteriological methods (CLSI, 2012b). The optical density (OD) of the turbidity was read using a BioTek reader at a wavelength of 630 nm to determine the MIC of EGCG. After reading the ODs, subcultures from all wells of most isolates (with MIC of 256 and 64 µg/ml) were made on duplicate MHA plates and incubated at 37 $^{o}$C for up to 24 hours for visible colonial growth, in order to determine the MBC. Controls for the organism and the EGCG were included in each test.

**Biofilm formation:** To produce the staphylococcal bacterial biofilms, 18 isolates of the fresh subcultured isolates (12 MRSA and 6 MSSA) selected from those having EGCG MIC of 64 µg/µl in both disc diffusion and microdilution methods. They were grown and adjusted to match 1.0 McFarland standard turbidity and then diluted 1:30 with Muller-Hinton broth media (Oxoid, UK) in biological safety cabinet Class II A2. Then 22 ml of this solution were loaded to the troughs of the Innovotech MBEC High-throughput (HTP) Assay device (BioSurface Technology Corporation, Bozeman, USA). This device is a modification of the original Calgary biofilm device. Each strain was loaded into a single device containing the trough and 96 protruding pegs that can be fit in the 96-microwell plate. The device was incubated at 37 $^{o}$C for 48 hours in rocking table of 3-5 rocks/minute and angle of 10$^{o}$ to assist in the formation of biofilms on the polystyrene pegs (Ceri et al., 1999; Olson et al., 2002).

**Biofilm proving:** To prove the formation of biofilms for the 18 tested isolates, some of the polystyrene pegs of the Inovotech MBEC™-HTP device assay tray were removed from the lid using sterile forceps. The pegs were then washed to remove planktonic bacteria by sequential dipping of them into a set of 6 adjacent sterile Eppendorf tubes, containing 1 ml of sterile distilled water "rinsing tubes".

After washing steps the pegs containing the potential biofilms were sonicated, placed into a new set of Eppendorf tubes containing 1 ml sterile distilled water for 30 minutes to disrupt the biofilms. Then, after sonication, the contents of tubes containing the disrupted biofilm were then serially diluted 1:10 in 2 sets of 8 Eppendorf tubes (sets A and B "dilution tubes").

Using sterile tips and automatic pipette, 20 µl from each Eppendorf tube (of both rinsing and dilution tubes) were spot inoculated in duplicate into MHA plates by dropping from 2.5 cm and leaving them to dry, incubated at 37 $^{o}$C for 24 hours. The colonies were counted for each plate and each dilution, to gain information about the viable bacteria in the biofilm (dilution steps) and the planktonic (rinsing) steps.

**EGCG challenge:** The required EGCG concentrations in MH broth were prepared in the wells of a sterile microplate corresponding to the required formed films over the pegs of the MBEC tray. The final volume in each well was 200 µl with concentrations of 1024, 512, 256, 128, and 64 µg/ml in duplicate with control wells containing pegs not exposed to EGCG. The peg tray lid was covered and the microtitre tray wrapped in parafilm and incubated for 24 hours at 37 °C. Then assessment and measurement of the turbidity of the wells of challenge plate by BioTek reader at 630 nm was done to determine the MIC of planktonic bacteria i.e. is the concentration giving no visible growth.

**Biofilm recovery:** The recovery of the formed biofilms after exposure to EGCG was done by washing the peg tray lid twice for 2 minutes to remove planktonic bacteria, using 2 microtitre trays containing sterile distilled water. Then the pegs exposed to EGCG solutions and control pegs (not exposed to EGCG) were sonicated for 15 minutes into another new sterile microplate containing MHB, incubated for 24 h at 37 $^{o}$C (Recovery plate). Measurement of the turbidity of the recovery plate at 630 nm determines the minimum biofilm eradication concentrations (MBEC) of EGCG upon SA biofilms.

The MBEC value is the lowest dilution that prevents re-growth of bacteria from the treated biofilm (Sepandj et al., 2004).

For determination of the viable bacterial count after EGCG challenge and recovery, spot plating upon MHA plates was done from all wells.

**Statistical analysis:** analysis of the resulting data was carried out using the SPSS version 11 applying the student t-test for paired samples and ANOVA test for multiple variants. The result was considered significant when $p < 0.05$.

## 3. Results

**Determination of MIC of EC by disc diffusion method:** There was no inhibition zone around any of the tested EC discs used in all concentrations up to the 1024 µg/µl disc. Furthermore 30 µl from this concentration was used with no inhibition zone. The concentrations could not be increased as this would have meant a higher volume of ethanol, which is bactericidal in such concentrations and produced inhibition of growth. Also, the paper disc cannot withstand a higher volume of EC as this leads to clumping of the disc, thus affecting the results. Testing for antibacterial activity for EC was hampered by these conditions and the experiment was stopped at this level

**Determination of MIC of EGCG by disc diffusion method:** The inhibition zones for EGCG upon all tested isolates (table 1) ranged from 64-256 µg/µl. With the 256 µg/µl being the most common one.

**Table 1.** Mean diameters of inhibition zones (in mm) produced by EGCG against the 120 strains of staphylococci and ATCC strains.

| No. of strains | EGCG disc contents (in µg/ml) | | | | |
|---|---|---|---|---|---|
| | 32 | 64 | 128 | 256 | 512 |
| 57 MRSA | 0 | 0 | 0 | 13 | 26 |
| 17 MRSA | 0 | 0 | 11 | 18 | 22 |
| 16 MRSA | 0 | 12 | 18 | 25 | 32 |
| 15 MSSA | 0 | 0 | 0 | 13 | 19 |
| 7 MSSA | 0 | 0 | 11 | 15 | 19 |
| 8 MSSA | 0 | 10 | 16 | 25 | 32 |
| Total No. (%) | 0 | 24 (20%) | 24 (20%) | 72 (60%) | 0 |
| ATCC# 25923 | 0 | 0 | 12 | 18 | 29 |
| ATCC# 25913 | 0 | 0 | 13 | 22 | 30 |

BY Disc Diffusion Method; out of 90 MRSA isolates 57 isolates (83.3%) showed MIC at for EGCG at 256 µg/ml, and out of 30 MSSA isolates 15 (50%) had MIC for EGCG at 256 µg/ml, however, other strains showed lower MIC.

N.B. As 10 µl of the corresponding dilution were used per disc, so, the used 10 µl ethanol control disc also showed no inhibition zones indicating relation of the inhibition to EGCG effect. 2 different ATCC strains were included for growth and test control.

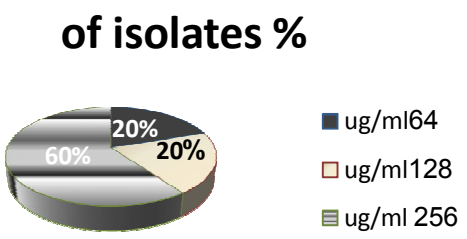


Figure 1. The percentages of MIC to various concentrations of EGCG upon studied MRSA and MSSA. This figure showed that 60% of all isolates used had MIC rates of MIC for EGCG at 256 µg/ml which may be used as a guide for therapeutic dose determination.

The results for conventional antimicrobial susceptibility testing of the tested strains were interpreted according to the CLSI recommendations **(CLSI, 2006)** table 2.

Table 2. Results susceptibility testing for conventional Antimicrobial discs by disc diffusion.

| AB disc | MRSA (90 strains) | | MSSA (30 strains) | |
|---|---|---|---|---|
| | S (%) | R (%) | S (%) | R (%) |
| Cefoxitin (FOX),10 µg | 0 | 90 (100%) | 30 (100%) | 0 |
| Penicillin (P) 1 unit | 0 | 90 (100%) | 8 (26.7%) | 22 (73.3%) |
| Vancomycin (VA) 5 µg | 90 (100%) | 0 | 30 (100%) | 0 |
| Gentamycin (CN) 10 µg | 58 (64.4%) | 32 (35.6%) | 28 (93.3%) | 2 (6.7%) |
| Fusidic acid (FD) 10 µg | 82 (91.1%) | 8 (8.9%) | 29 (96.7%) | 1 (3.3%) |
| Clarithromycin (CLR) 2 µg | 81 (90%) | 9 (10%) | 29 (96.7%) | 1 (3.3%) |
| Linezolid (LZD) 10 µg | 90 (100%) | 0 | 30 (100%) | 0 |
| Ciprofloxacin (CIP) 1 µg | 2 (2.2%) | 88 (97.8%) | 30 (100%) | 0 |
| Neomycin (N)10 µg | 81 (90%) | 9 (10%) | 28 (93.3%) | 2 (6.7%) |
| Rifampicin (RD) 2 µg | 90 (100%) | 0 | 30 (100%) | 0 |
| Trimethoprim (W) 5 µg | 87 (96.7%) | 3 (3.3% | 29 (96.7%) | 1 (3.3%) |
| Tetracycline (TE) 10 µg | 87 (96.7%) | 3 (3.3%) | 28 (93.3%) | 2 (6.7%) |
| Mupirocin (MUP) 5 µg | 68 (75.6%) | 22 (24.4%) | 26 (86.7%) | 4 (13.3%) |

AB=antimicrobial disc, S=susceptible, R=Resistant.

**Synergy test results:** Combination of 10 µl of EGCG in sub-MIC concentrations to the same antimicrobial discs was done and results are shown below (table 3, 4). The results are either synergetic i.e. EGCG induce susceptibility to the resistant antimicrobial, antagonism i.e. decreasing the zone size of susceptible antimicrobial, additive i.e. increasing the zone for susceptible antimicrobial, or no effect in the zone size.

Table 3. The results of synergy testing of EGCG and antimicrobials upon MRSA strain.

| | 32 µg/ml EGCG (16 MRSA) | | | | 64 µg/ml EGCG (17 MRSA) | | | | 128 µg/ml EGCG (57 MRSA) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Before | After | P | Sig. | Before | After | P | Sig. | Before | After | P | Sig. |
| P 1 | 9 | 27 | 0.02 | S | 8 | 28 | 0.04 | S | 5 | 26 | 0.01 | S |
| VA 5 | 17 | 11 | 1.2 | IS | 18 | 12 | 2.2 | IS | 16 | 10 | 1.6 | IS |
| CN 10 | 17 | 19 | 1.6 | IS | 20 | 26 | 1.3 | IS | 20 | 27 | 0.09 | IS |
| FD 10 | 32 | 32 | 1.5 | IS | 25 | 24 | 1.4 | IS | 31 | 30 | 2.4 | IS |
| CLR 2 | 18 | 24 | 0.08 | IS | 17 | 23 | 0.9 | IS | 9 | 20 | 0.04 | S |
| LZD 10 | 31 | 30 | 3.4 | IS | 30 | 31 | 3.4 | IS | 29 | 29 | 2.4 | IS |
| CIP 1 | 5 | 17 | 0.05 | S | 4 | 17 | 0.03 | S | 6 | 16 | 0.03 | S |
| N 10 | 23 | 20 | 1.6 | IS | 24 | 20 | 1.1 | IS | 22 | 21 | 2.2 | IS |
| RD 2 | 34 | 33 | 3.4 | IS | 33 | 34 | 3.2 | IS | 34 | 33 | 3.0 | IS |
| W 5 | 25 | 24 | 2.4 | IS | 29 | 30 | 2.4 | IS | 26 | 25 | 3.1 | IS |
| TE 10 | 33 | 33 | 3.7 | IS | 32 | 33 | 3.2 | IS | 34 | 34 | 2.3 | IS |
| MUP 5 | 34 | 33 | 3.4 | IS | 27 | 26 | 3.5 | IS | 33 | 34 | 3.7 | IS |

There are significant (S) synergistic effects when EGCG in sub-MIC concentrations was combined with penicillin and ciprofloxacin (MRSA resistant to it), while additive effect when combined with clarithromycin and gentamycin and antagonistic effects when combined with Vancomycin and neomycin. Other combinations were insignificant (IS).

Table 4. The results of synergy testing of EGCG and antimicrobials upon MSSA.

| | 32 **µg/ml** EGCG (8 MSSA) | | | | 64 **µg/ml** EGCG (7 MSSA) | | | | 128 **µg/ml** EGCG (15 MSSA) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Before** | **After** | P | Sig. | Before | After | P | Sig. | Before | After | P | Sig. |
| P 1 | 9 | 30 | 0.02 | S | 17 | 29 | 0.04 | S | 17 | 31 | 0.05 | S |
| VA 5 | 18 | 15 | 3.2 | IS | 19 | 14 | 2.2 | IS | 17 | 13 | 2.4 | IS |
| CN 10 | 19 | 31 | 0.04 | S | 18 | 29 | 0.04 | S | 18 | 28 | 0.03 | S |
| FD 10 | 26 | 25 | 1.5 | IS | 24 | 25 | 1.4 | IS | 29 | 30 | 2.4 | IS |
| CLR 2 | 18 | 21 | 1.3 | IS | 18 | 20 | 2.2 | S | 17 | 21 | 3.2 | IS |
| LZD 10 | 30 | 31 | 3.7 | IS | 32 | 30 | 3.1 | IS | 28 | 28 | 3.4 | IS |
| CIP 1 | 22 | 28 | 0.08 | IS | 23 | 28 | 0.09 | IS | 21 | 27 | 1.3 | IS |
| N 10 | 24 | 23 | 1.6 | IS | 25 | 26 | 2.3 | IS | 23 | 23 | 1.8 | IS |
| RD 2 | 34 | 35 | 3.3 | IS | 32 | 33 | 3.0 | IS | 36 | 35 | 3.5 | IS |
| W 5 | 29 | 28 | 2.8 | IS | 29 | 29 | 2.3 | IS | 26 | 27 | 3.7 | IS |
| TE 10 | 30 | 31 | 3.5 | IS | 29 | 30 | 3.2 | IS | 33 | 34 | 2.4 | IS |
| MUP 5 | 33 | 33 | 3.6 | IS | 33 | 32 | 3.2 | IS | 32 | 32 | 3.6 | IS |

There are also significant (S) synergistic effects when EGCG was combined with penicillin, and antagonistic with vancomycin. While additive effects when combined with clarithromycin, gentamycin and ciprofloxacin, the additive effect was significant in case of gentamycin. While other combinations produced insignificant effects (IS).

Table 5. Determination of MIC of EGCG by disc diffusion and broth microdilution methods upon all tested strains.

| EGCG Conc. (µg/ml) | Method | MRSA strains (%) | MSSA strains (%) | P | Sig. |
|---|---|---|---|---|---|
| 16 | DD | NA | NA | NA | NA |
| | BMD | 0 | 0 | | |
| 32 | DD | 0 | 0 | 4.5 | IS |
| | BMD | 0 | 1 (0.8%) | | |
| 64 | DD | 16 (12.3%) | 8 (6.2%) | 3.9 | IS |
| | BMD | 17 (13.1%) | 7 (5.4%) | | |
| 128 | DD | 17 (13.1%) | 7 (5.4%) | 3.6 | IS |
| | BMD | 19 (14.6%) | 8 (6.2%) | | |
| 256 | DD | 57 (43.9%) | 15 (11.5%) | 2.8 | IS |
| | BMD | 54 (41.5%) | 14 (10.8%) | | |
| 512 | DD | 0 | 0 | NA | NA |
| | BMD | 0 | 0 | | |
| 1024 | DD | 0 | 0 | NA | NA |
| | BMD | 0 | 0 | | |

Few strains showed different MIC reactions by broth microdilution (BMD) method from that previously tested by the disc diffusion (DD) method. However, these differences were statistically insignificant indicating that both methods were reliable in detection of the MIC of EGCG upon studied isolates. Furthermore, the ranges of MICs were between 64-256 µg/ml and for most of the studied strains it was at the 256 µg/ml.

**Table 6.** The mean results of subculturing form isolates having EGCG MICs of 256 and 64 µg/ml by broth microdilution method for determination of MBC for planktonic bacteria (Viable counts).

| EGCG Conc. µg/ml | MRSA (71 isolates) | | | | MSSA (21 isolates) | | | |
|---|---|---|---|---|---|---|---|---|
| | 64 µg/ml (17) | | 256 µg/ml (54) | | 64 µg/ml (7) | | 256 µg/ml (14) | |
| | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC |
| 1024 | - | - | - | - | - | - | - | - |
| 512 | - | - | - | - | - | - | - | - |
| 256 | - | - | - | - | - | - | - | - |
| 128 | - | - | * | + | - | - | * | + |
| 64 | - | + | * | ++ | - | + | * | ++ |
| 32 | * | ++ | * | ++ | * | ++ | * | +++ |
| 16 | * | +++ | * | +++ | * | ++ | * | +++ |

Key: - = no growth, + = slight growth (1-10 colonies/spot), ++ = moderate growth (11-20 colonies/spot), +++ = heavy growth (uncountable colonies/spot), * = turbidity in the wells.

The MIC was similar to the MBC at the 256 µg/ml for both the MRSA and MSSA, however, at 64 µg/ml there was some growth in most of the wells after 24 h incubation indicating that at higher concentration ECGC was acting as bactericidal while at lower concentration it was acting as bacteriostatic.

**Table 7**. The results for biofilm proving after spot subculturing upon MHA for 24 h from some pegs from each of the 18 tested isolates after washing (rinsing) and 1:10 serial dilution in 8 tubes after sonication (dilution).

| | Tube No. | MRSA (12 strains) | MSSA (6 strains) |
|---|---|---|---|
| Washing step | 1 | +++ | +++ |
| | 2 | +++ | +++ |
| | 3 | +++ | ++ |
| | 4 | ++ | + |
| | 5 | ++ | + |
| | 6 | + | - |
| Biofilm **A** Proving (dilution tubes) | 1 (sonicated biofilm) | +++ | +++ |
| | 2 ($10^{-1}$) | +++ | ++ |
| | 3 ($10^{-2}$) | ++ | ++ |
| | 4 ($10^{-3}$) | + | + |
| | 5 ($10^{-4}$) | - | - |
| | 6 ($10^{-5}$) | - | - |
| | 7 ($10^{-6}$) | - | - |
| | 8 ($10^{-7}$) | - | - |
| Biofilm **B** Proving (dilution tubes) | 1 (sonicated biofilm) | +++ | ++ |
| | 2 ($10^{-1}$) | +++ | + |
| | 3 ($10^{-2}$) | ++ | - |
| | 4 ($10^{-3}$) | + | - |
| | 5 ($10^{-4}$) | - | - |
| | 6 ($10^{-5}$) | - | - |
| | 7 ($10^{-6}$) | - | - |
| | 8 ($10^{-7}$) | - | - |

Key: - =no growth, +=slight growth (1-10 colonies/ spot), ++=moderate growth (11-20 colonies/spot), +++=heavy growth (uncountable colonies/spot).

After washing of the biofilms culture form these tubes showed descending grades of growth which in tube 6 of MSSA. Following sonication, the densities of inocula were heaver in the first dilution tubes till it disappeared indicating that sonication brought the bacteria from the biofilm to grow and this is the prove for the formation of bacterial biofilms within the examined pegs.

Table 8. Results of MIC for planktonic bacteria within the challenge plate and the MBEC of biofilm bacteria of the recovery plates measured at 630 nm

| EGCG Conc. µg/ml | MIC (challenge plate) | | MBEC (recovery, sonicated plate) | |
|---|---|---|---|---|
| | MRSA (12) | MSSA (6) | MRSA (12) | MSSA (6) |
| 64 | 11 | 5 | 0 | 0 |
| 128 | 1 | 1 | 0 | 0 |
| 256 | 0 | 0 | 2 | 1 |
| 512 | 0 | 0 | 10 | 5 |
| 1024 | 0 | 0 | 0 | 0 |

Although all studied isolates were selected from those having MIC of 64 µg/ml, 1 isolate from each group failed to be inhibited at that concentration during the EGCG challenge testing. However, the MIC was the same for remaining strains. The MBEC (biofilms) was 6-fold higher than the MIC for most of the strains (10 MRSA and 5 MSSA), while it was 4-fold higher for 3 strains.

Table 6. The mean results of MBEC spot subculturing form 18 biofilm recovery after EGCG challenge testing and sonication for both MRSA and MSSA strains in duplicate (on MHA plates) after incubation for 24 hours at 37 °C for determination of MBEC for biofilm bacteria (Viable counts).

| **EGCG Conc. µg/ml** | **MRSA (12 isolates)** | | | | **MSSA (6 isolates)** | | | |
|---|---|---|---|---|---|---|---|---|
| | 64 µg/ml (10) | | 128 µg/ml (2) | | 64 µg/ml (5) | | 128 µg/ml (1) | |
| | MIC | MBEC | MIC | MBEC | MIC | MBEC | MIC | MBEC |
| **1024** | - | - | - | - | - | - | - | - |
| **512** | - | + | - | - | - | + | - | - |
| **256** | * | + | - | + | * | + | - | - |
| **128** | * | ++ | * | ++ | * | ++ | * | + |
| **64** | * | +++ | * | ++ | * | ++ | * | ++ |
| **32** | * | +++ | * | +++ | * | +++ | * | +++ |
| **16** | * | +++ | * | +++ | * | +++ | * | +++ |

Key: - = no growth, + = slight growth (1-10 colonies/ spot), ++ = moderate growth (11-20 colonies/spot), +++ = heavy growth (uncountable colonies/spot), * = turbidity in the wells.

Despite the fact that all studied strains for the biofilm production were selected from those having MIC of 64 µg/ml by the disc diffusion method, they showed higher MBEC at 512 µg/ml for most isolates (10 MRSA and 5 MSSA). Growth was observed for some isolates indicating that biofilm bacteria are more resistant to EGCG and it reacted as being bacteriostatic at these concentrations upon biofilm bacteria.

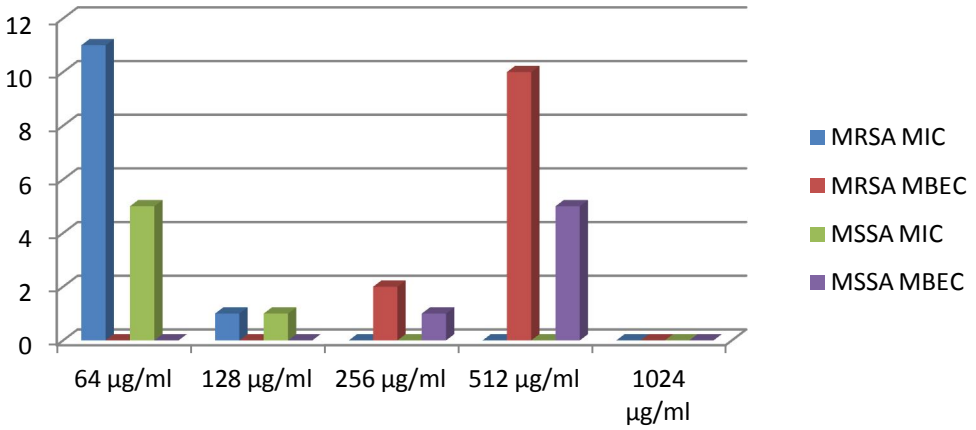


Figure 2. Although all strains were having MIC of 64 µg/ml by disc and broth methods, 1 strain of each had higher MIC for planktonic at challenge testing. MBECs were 6-fold increased (512 µg/ml) for most of the strains (10 MRSA and 5 MSSA) indicating higher resistance of bacteria within biofilm.

## 4. Discussion

The standard assay for testing the antibiotic susceptibility of bacteria is the MIC, which tests the sensitivity of the bacteria in their planktonic phase. The MIC is of limited value in determining the true antibiotic susceptibility of bacteria in their biofilm phase. The MBEC assay, on the other hand, allows direct determination of the bacteria in their biofilm phase of development (Sepandj et al., 2004). The MICs of EGCG for all studied strains in the current study were between 64-256 µg/ml, with the 256 µg/ml being the most common MIC (Table 1, 5). These concentrations are somewhat higher than that reported by other researchers, e.g. Kumar and colleagues (2012) reported MICs of 10, 20 and 30 µg, Zhao and colleagues (2001) reported MIC of EGCG for MRSA and MSSA as 100 µg/ml or less. Also, Hatano and colleagues (2008) reported an MIC of 64 µg/ml for EGCG upon four MRSA and MSSA strains which is similar to MIC of few isolates in the current study. However, Blanco and colleagues (2005) detected higher MIC for EGCG of 125-500 µg/ml.

The antibacterial mechanisms of EGCG are attributed to its direct attachment to the peptidoglycan layer of the bacterial cell wall (Zhao et al., 2001), leading to damage of the peptide cross-linking and damage to the cell wall, this action was reported to be non-specific with limited species selectivity (Shimamura et al., 2007). This may explain the difference in MIC reporting amongst different studies. Another factor is the accumulation of the autolysin enzymes within the cell wall, leading to inhibition of cell separation during growth and formation of defective multicellular forms of bacteria (Stapleton et al., 2007). In addition to the methods used may attribute to the different results as most studies used the broth dilution method and few (Kumar et al., 2012) used the disc diffusion method with lower MIC reporting. However, in the current study both method gave the nearly the same results with no statistical significance.

In the current study, the use of even higher concentrations of EC (up to 3.1 mg/disc) did not produce any inhibitory action upon the examined strains. This finding exclude the antibacterial action of EC upon *Staphylococcus aureus* so, it was of no value to study its synergy action. Similar finding was reported by Stapleton and colleagues (2007) for EC alone or combined with oxacillin. Other literaures reported no antimicrobial effects for EC (Rozoy et al., 2013).

Significant synergy was produced when EGCG in sub-inhibitory concentrations was combined with penicillin in both MRSA and MSSA (tables 3, 4) isolates and also with ciprofloxacin in MRSA strains. This synergy is explained by the combined action of both EGCG and β-lactam upon the same target, peptidoglycan, leading to more damaging effects upon bacterial cell wall (Zhao et al., 2001; Hu et al., 2002; Shimamura et al., 2007). Another explanation for the synergy between EGCG and penicillin is the inhibition of penicillinase enzyme produced by the two compounds at the same time, leading to augmentation of action (Hemaiswarya et al., 2008).

Additive effects i.e. increase in size of the inhibition zone of the already susceptible strains of SA to certain antimicrobial after the addition of EGCG, was observed with both gentamycin and clarithromycin (both of them are protein synthesis inhibitors). Similar findings were reported by Shimamura and colleagues (2007), and were explained by the increased permeability of the bacterial cell wall/membrane to the antimicrobial with the combined usage of EGCG. However, in the current study there were unexplained antagonistic effects with neomycin and EGCG in some strains. The different results regarding the combination effects was also reported by Yanagawa and colleagues (2003) who reported synergy in some isolates of H. pylori (80%) of strains after combination of neomycin and EGCG and antagonism in the rest of isolates. Furthermore, this antagonistic effect with combination of neomycin and EGCG may be explained by the precipitation of neomycin after its peptide binding by EGCG (Hu et al. 2002) and this also was the proposed mechanism for antagonism obtained in current study with vancomycin (Shimamura et al., 2007). Furthermore studies regarding this mechanism need to be done.

The results regarding synergy, addition, indifferent or antagonism are variable in different studies. For example, there was no different effect when EGCG was combined with tetracycline in the current study. However, Roccaro and colleagues (2004) reported improved MIC for strains resistant to tetracycline by EGCG due to the permissive effects on efflux pumps. This was not evident here in the current study mostly as most of the studied strains were susceptible to tetracycline.

In the current study the MIC and MBC or MBEC were similar at higher concentrations. However, they differ at lower concentrations of EGCG with presence of growth in some of the inhibited clear tubes indicating that EGCG acted as both bactericidal and bacteriostatic. These findings here coincide with other studies that reported EGCG is bactericidal in certain concentrations and can be bacteriostatic in other concentrations (Kono et al., 1994; Roccaro et al., 2004). The antimicrobial effects of EGCG were studied mostly *in vitro*, however, the in vivo effects of these molecules in inhibiting the bacterial growth may be affect by the presence of

various proteins (Shimamura et al., 2007) and needs to be further studied in details.

The higher MBEC of EGCG upon biofilm bacterial strains (512 µg/ml) than planktonic one (64 µg/ml) explains the resistant pattern and the more fastidious character of biofilm bacteria. The inhibitory effects of EGCG upon bacteria in the biofilm may be attributed to the inhibition of biofilm formation by the inhibition of the growth and slime formation of *SA*, and by its combining with the peptidoglycan, leading to breakage and damage of the bacterial cell wall (Blanco et al., 2005).

## 5. Conclusion

The findings in the current study prove that the green tea extract EGCG acts as an *in vitro* antibacterial agent against resistant microorganisms like MRSA and non-resistant ones like MSSA. It also gives evidence of the potential synergy produced when EGCG is in combination with previously resistant antimicrobials against *SA*, e.g. penicillin and ciprofloxacin, while antagonism with vancomycin. EGCG has also been shown to have a role in killing *SA* entrapped inside biofilms. The MIC of EGCG within biofilm is higher than with planktonic bacteria, therefore MBEC testing for biofilm producing clinical isolates is recommended as MIC results are not reliable and show lower values. Detailed molecular investigation of the mechanisms of EGCG is necessary, together with application of this experiment *in vivo* upon lab animals to confirm these findings.

Practical application of these findings is highly recommended. EGCG can be added to the relevant antimicrobials to augment their actions, after studying the pharmacokinetics and pharmacodynamics of combinations. However, any further investigation of the antibacterial activity of EC would not be profitable.

**Corresponding Author:**
Dr. Mostafa Mahmoud
Department of Microbiology & Immunolgy, Faculty of Medicine, Ain Shams University, Cairo, Egypt.
E-mail: mamostafa67@yahoo.com

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