Influence of Aqueous Green Tea Extract on the Antimicrobial Activity of some Antibiotics against Multiresistant Clinical Isolates

Nourhan H. Fanaki, Mervat A. Kassem*, Mohamed A. Fawzi and Fatma S.E. Dabbous

Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Alexandria University.

ABSTRACT

Green tea has been shown to have several antibacterial activities, limiting bacterial growth and acting in synergy with \( \beta \)-lactam antibiotics. Antibiotics belonging to different groups were tested separately and in combinations with green tea against different isolates using disc agar diffusion technique. The bactericidal activity of certain antibiotics against selected isolates was evaluated separately and in presence of green tea using surface viable count. Subinhibitory concentrations of green tea showed marked increase in the sensitivity of even the multiple resistant isolates to most of the antibiotics tested. Moreover, green tea enhanced the bactericidal activity of all tested antibiotics. Green tea had the ability to cure resistance to sensitivity of even the multiple resistant isolates to most of the antibiotics tested. Moreover, green tea had the ability to cure resistance to sensitivity of even the multiple resistant isolates to most of the antibiotics tested. Additionally, it was reported that green tea could reverse the methicillin resistance of MRSA by using surface viable count. Subinhibitory concentrations of green tea showed marked increase in the sensitivity of even the multiple resistant isolates to most of the antibiotics tested. Moreover, green tea enhanced the bactericidal activity of all tested antibiotics. Green tea had the ability to cure resistance to sensitivity of even the multiple resistant isolates to most of the antibiotics tested. Moreover, green tea could reverse the methicillin resistance of MRSA using surface viable count.

INTRODUCTION

Tea beverage is an infusion of variously processed leaves of one of the varieties of an evergreen shrub, *Camellia sinensis* family *Theaceae* \(^{(1)}\). It is consumed every day by billions of people worldwide demonstrating its safety \(^{(2)}\). Tea beverage, next to water, is the most popularly consumed beverage in the world due to its ability to revive, refresh and relax the body and mind \(^{(0, 4)}\). Tea is generally consumed in the form of black, oolong or green tea. Green tea differs from black tea in that an oxidation step, called "fermentation", occurs in the processing of the latter. Tea consists mainly of polyphenolic compounds, about 60 – 80%, that make up ~ 30% of the dry weight of flush \(^{(1)}\). Epigallocatechin gallate (EGCG) is the most abundant catechin in most green tea brands \(^{(4)}\).

Green tea has been shown to have a wide range of beneficial physiological and pharmacological effects. In addition, the antimicrobial activity of green tea, recognized about 90 years ago, is mainly due to EGCG, the main component of tea polyphenols \(^{(3, 5-7)}\). It was found that green tea extracts exhibited bacteriostatic and bactericidal activities against both methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) \(^{(8)}\), *S. epidermidis*, *Salmonella typhi*, *S. typhimurium*, *S. enteritidis*, *Shigella flexneri*, *Sh. dysenteriae*, *Bordetella pertussis* \(^{(5)}\) and *Vibrio spp*, including *V. cholerae* \(^{(7)}\). Synergistic bactericidal effects between \( \beta \)-lactams as ampicillin/sublactam or imipenem and EGCG against MRSA isolates were reported \(^{(10, 11)}\). In addition, it was reported that EGCG could reverse the methicillin resistance of MRSA by inhibiting the synthesis of PBPs \(^{(8)}\). Moreover, EGCG did not enhance only \( \beta \)-lactams activity but also it enhanced the activity of non-\( \beta \)-lactam cell wall biosynthesis inhibitors such as D-cycloserine \(^{(12)}\). The possibility to drink green tea with antibiotics could be so easily to happen. The effect of GT on the antimicrobial activity of some antibiotics has been reported in a limited number of studies \(^{(1)}\). Consequently, the main aim of our investigation was to evaluate the antimicrobial activity of the combinations of green tea and some selected antibiotics against multiresistant clinical isolates.

MATERIALS and METHODS

**Microorganisms:** The standards strains used in this study were: *Staphylococcus aureus* ATCC 6538P, *Escherichia coli* NCTC 10418 and *Pseudomonas aeruginosa* ATCC 9027. The twenty eight bacterial isolates used in this study were collected from different sources (urine 10, pus 9, blood 4, sputum 2, stool 2, and used contact lens 1). They were as follows: *S. aureus* (14), *S. epidermidis* (2), *S. saprophyticus* (1), *E. coli* (8), *Ps. aeruginosa* (2) and *Enterobacter sakazakii* (1).

**Culture media:** The following Oxoid-made media were used: Nutrient broth No. 2 (NB), Nutrient agar, and a chemically defined medium (CDM) pH adjusted to 6.8 ±0.1 \(^{(13)}\).


**Antibiotic Sensitivity Discs:** All antibiotic discs were the product of BIOANALYSE Tibbi Malzemeler San. Ve Tic. Ltd. Sti. Turkey.

**Antibiotics:** The antimicrobial agents used in this study were obtained from the corresponding pharmaceutical companies: Cefoperazone sodium (Pharco Pharmaceutical Co., Egypt), Cefuroxime sodium (Galaxo Wellcome, Egypt), Chloramphenicol (Alexandria Co., Egypt), Ciprofloxacin (Sreepathi Pharmaceutical Ltd,
Preparation of sterilized green tea extract (GT): Ten grams of ungrounded leaves of green tea were soaked in 100 ml boiling distilled water for 10 min. The extract was filtered from the leaves by means of gauze (14). The filtrate was then centrifuged using ML W T 51, GDR centrifuge at the highest speed (III) for 5 min. The supernatant was then collected and sterilized by filtration through a membrane filter of a 0.45 µm pore size. Only freshly prepared sterilized green tea extract (GT) was used (15).

1. Effect of different GT on the antimicrobial activity of different antibiotics using disc agar diffusion technique:
   It was adopted by Ghabashy A.A. (16) with some modifications as follows:
   Preparation of the agar plates: Sterile nutrient agar plates were prepared to contain final concentration equivalent to 1/4 MIC of GT when used to test Gram positive bacteria or 10 mg/ml in case of Gram negative bacteria, in addition to the control plates (free of GT).
   Preparation of inoculum: Each tested organism was subcultured in 3 ml sterile nutrient broth and the resultant microbial growth was firstly compared with 0.5 'McFarland Opacity Standard' which was equivalent to approximately 10⁸ cfu/ml and properly diluted, if necessary, to achieve the same turbidity of the standard.
   Procedure of the test: The inoculum was spread onto the surface of the nutrient plates by means of sterile cotton swabs. The plates were then left to dry aseptically at room temperature for few min. Control plate: was prepared by inoculating the nutrient agar plate with swab of tested microorganism.
   Combination plate: was prepared by inoculating the nutrient agar plate containing the corresponding concentration of the GT using swab of tested microorganism.
   The antibiotic discs were then distributed onto the surface of each inoculated plate. The space between the discs must be not narrower than 24 mm and the distance of the discs from the edge of the plate must be not less than 10 mm. The plates were then incubated at 37°C for 18 h and the average diameter of inhibition zones around the discs was determined and compared to the corresponding control plate. The results were then translated into susceptible (S), intermediate (I) or resistant (R) according to the published Tables of the National Committee of Clinical Laboratory Standards NCCLS 2002 (17). To insure the reproducibility of the results each experiment was repeated 3 times throughout this study.

2. Investigation of dynamics of the antimicrobial activity of GT-antibiotic combinations against selected bacterial isolates:
   For each case, 4 flasks were prepared each with a final volume of 9 ml, flask one contained the appropriate antibiotic concentration in sterile chemically defined media (CDM). The second contained the required concentration of GT in CDM. The third contained a combination of the antibiotic and the GT in the same medium while the forth flask had 9 ml CDM and was considered as control.
   At zero time, all flasks were inoculated each with 1 ml of the tested bacterial suspension containing about 10⁸ cfu/ml. The flasks were mixed well and placed in shaking water bath (25 strokes/min) at 37°C. Samples were withdrawn from each flask at 0, 1, 2, 4 and 6 h and the samples were then ten fold serially diluted with sterile saline. 40 µl samples of the different dilutions were dropped separately onto the surface of overdried nutrient agar plates. The plates were then incubated at 37°C for 24 h. The number of colonies was determined and the average number of survivors was then calculated (18).

3. Effect of GT on the modulation of antibiotic resistance in certain bacterial isolates:
   3.1. Curing of some antibiotic-resistance bacterial isolates with GT:
   Bacterial suspensions were prepared of the corresponding overnight cultures to contain about 10⁸ cfu/ml for each isolate. Aliquots of 100 µl of each suspension were then spread separately over the surface of overdried nutrient agar plates which were then incubated at 37°C for 24 h. Copies of the control plates with separate colonies of untreated Staphylococcus and Gram negative isolates were transferred to nutrient agar plates containing 1 mg/ml and 10 mg/ml, respectively using sterile tooth picks and incubated overnight at 37°C. These plates were considered as cured plates. Control plates lacking GT were prepared for each isolate along with the cured plates.
   Two sets of nutrient agar plates containing two fold serial dilutions of selected antibiotics (cefoperazone, cefuroxime, chloramphenicol or tetracycline HCl) were freshly prepared. The highest concentration used was always 2 fold higher than the antibiotic resistance breakpoint against the tested microorganism.
   Each developed colony in the cured plates as well as its corresponding colony in control plates was transferred individually on the surface of nutrient agar plates containing serial dilutions of the selected antibiotic using the same tooth pick. The plates were incubated at 37°C for 24 h. The
values of MIC of each antibiotic to each colony were recorded for both untreated and treated isolates (19).

3.2. Direct inhibitory effect of GT on isolated β-lactamases of S. aureus isolate using nitrocefin method:
Preparation of cell suspension: S. aureus isolate, S4 was subcultured on nutrient agar slant and the resultant growth was washed up aseptically by 2 ml phosphate buffer (pH 7). The content of microbial culture was vortexed and transferred aseptically to a sterile empty test tube.

Production of β-lactamases: The microbial suspension was mixed with equal volume of penicillin G solution (12 mg/ml dissolved in sterile phosphate buffer pH 7). The mixture was then incubated at 37°C for 1 h (20). After incubation, the suspension was centrifuged for 15 min. The supernatant was considered as stock solution of β-lactamases.

Procedure of test: The stock β-lactamases solution was distributed in 0.5 ml portions in 3 empty sterile test tubes. The first tube was preincubated for 30 min with 1 mg/ml GT, the second with 1/2 mg/ml of GT while the third received sterile distilled water and was considered as control. Nitrocefin solution (500 µg/ml) was then added as a substrate to each test tube. The resultant color change was recorded by detecting the absorbance at 492 nm after 30 min with spectrophotometer (Spekol II, Carl Zeiss, Jena, Germany) (21). The percentage of inhibition of enzyme activity was determined graphically according to the calibration curve of β-lactamases which was discussed in the next section (22). Moreover, the absorbance of mixture of 1 mg/ml GT and nitrocefin solution (500 µg/ml) was measured to insure the absence of any interference.

Construction of standard curve of β-lactamases: Stock β-lactamases was 2 fold serially diluted with phosphate buffer pH 7. Nitrocefin (500 µg/ml) was then added and the color change was measured as mentioned earlier. The measured absorbance was plotted against log concentration of β-lactamases and a regression line was constructed.

3.3. Effect of subinhibitory concentrations of GT on the modulation of tetracycline HCl susceptibility of some staphylococcus spp isolates:
3.3.1. Effect of omeprazole, proton pump inhibitor, and GT on the antimicrobial activity of tetracycline HCl using broth dilution technique:
The MICs of tetracycline HCl against 10 Staphylococcus spp isolates were determined in absence and presence of 100 µg/ml omeprazole as well as GT (0.75 mg/ml) using broth dilution method (23). The 200 µg/ml tetracycline HCl stock solution was 2 fold serially diluted in sterile distilled water and distributed in 0.5 ml portions in test tubes containing 0.5 ml of either omeprazole solution or GT. Proper control tubes were included in each set for each isolate. Aliquots of 1 ml double strength NB inoculated with the test organism (10⁵ cfu/ml) were added to the test tubes giving final volume of 2 ml. The tubes were well shaken, then incubated at 37°C for 24 h. The MIC values were determined by the visual inspection for turbidity.

3.3.2. Direct inhibitory effect of GT on the tetracycline HCl efflux pump of staphylococcus spp:
Suspensions of 3 selected Staphylococcus isolates, S4, S12 and S16 each containing about 10⁷ cfu/ml were preincubated at room temperature for 15 min in the absence and presence of 1 mg/ml. Each of them was then loaded with 100 µg/ml tetracycline HCl for additional 15 min. The bacterial suspensions were then centrifuged and the resultant pellets were resuspended in 2 ml of Mg²⁺ buffer (24). The released fluorescence was immediately recorded quantitatively using spectrofluorometer at 400 nm as excitation wave length and 520 nm as emission wave length (25).

RESULTS and DISCUSSION

1. Effect of GT on the antimicrobial activity of different antibiotics against tested bacteria using disc agar diffusion technique:
Tea intake is second to water in terms of worldwide popularity as a beverage (4). Green tea contains ~ 30 % polyphenolic compounds of which 60-80% catechins (1, 3). EGCG is the most abundant catechin in green tea (4). In addition to its antimicrobial activity, green tea has been shown to have a wide range of beneficial physiological and pharmacological effects (4). As a popular habit in Egypt, it was noticed that there is unintentional co-administration of antibiotics with a cup of tea that may occur frequently every day. It was also noticed that several researchers studied the combinations of green tea or its active components, EGCG and ECG, with some antimicrobial agents (10, 11). Therefore, it was interesting to investigate the effect of green tea on the antimicrobial activity of antibiotics of different groups against selected microorganisms.
Antibiotics belonging to different groups with different mechanisms and targets of action were tested in this study. Their antimicrobial activity against Staphylococcus spp and Gram negative isolates and their available standard strains was tested in absence and presence of 1/4 MIC and 10
mg/ml of GT, respectively, using the disc agar diffusion technique. The selected concentrations of GT were 1/4 MIC and 10 mg/ml since the use of 1/2 MIC and 20 mg/ml resulted in too wide inhibition zones surrounding the discs, which interfered with each others.

Twenty-eight bacterial isolates, 17 *Staphylococcus* spp, 7 *E. coli*, one Ent. sakazakii and 3 *Ps. aeruginosa*, in addition to three standard strains (S₁, E₁, and P₁) were involved in this experiment. A total of 16 and 13 of the commonly used antibiotics were employed in case of *Staphylococcus* spp and Gram negative bacteria, respectively.

The data in Tables 1 and 2 showed that the presence of GT caused a marked increase in the sensitivity of multiple resistant *Staphylococcus* spp. as well as multiple resistant Gram negative isolates to most of the antibiotics tested. The sensitivity of 72% (13 out of 18) of tested *Staphylococcus* spp to different antibiotics was increased (up to 85%). The sensitivity of 50% percent of tested *Staphylococcus* spp responded positively to the antimicrobial activity of tetracycline and showed up to 52% increase in their percentage relative difference in inhibition zone diameter, Table 1. These results may be explained by what was reported by Roccaro et al that subinhibitory concentrations of EGCG could inhibit Tet (K) efflux activity in *Staphylococcus* spp which \(^{(23)}\).

Our results showed maximum enhancement upon testing the antimicrobial activity of amoxicillin against *S. aureus* isolate, S₁₄ (85%) followed by that of cefuroxime against isolate S₄ (64%) as shown in Table 1. Such enhancement could be attributed to the effect of GT on the microbial cell wall and cytoplasmic membrane. For this reason green tea may increase the permeability of antibiotics into microbial cells causing change in the response of microbial cells to different antibiotics \(^{(26, 27, 29)}\).

The chemical interaction or complex formation between GT and tested antibiotics was also expected to be one of the possible mechanisms of enhancement or reduction of the antimicrobial activity of the tested antibiotics. Therefore, a spectrophotometric analysis was done and the results showed that the presence of GT did not affect the UV-Visible spectra of all tested antibiotics. These results indicated that there was no chemical interaction or complex formation between green tea and any of the tested antibiotics (data not shown).

On the other hand, the antimicrobial activity of 62% of the tested antibiotics was enhanced by 12% and up to 360% in presence of GT when tested against Gram negative bacteria. In addition, 81.8% of the tested isolates showed an increase in their sensitivity to aztreonam, cefoperazone and imipenem up to 300%, 100% and 230%, respectively. The maximum increase in the sensitivity was shown with isolate, P₁ to chloramphenicol and aztreonam which increased by 360% and 300%, respectively, (Table 2). Consequently, the resistance of isolate P₁ to chloramphenicol was switched to become sensitive. These results may be explained by the probability of the loss of bacterial inactivation of chloramphenicol by chloramphenicol acetyltransferase enzyme since Dashwood et al \(^{(30)}\) reported that green tea caused in vitro inactivation of N, O-acetyltransferase enzymes which contribute to the metabolic activation of heterocyclic amines.

The enhanced antimicrobial activity of most of the tested β-lactams could be explained by several mechanisms including the following:

- Both β-lactams and EGCG, the main component of green tea extract, attack directly or indirectly the same microbial target, the cell wall \(^{(12)}\). This hypothesis was emphasized by the synergistic effect of EGCG when combined with non β-lactam inhibitors of cell wall biosynthesis \(^{(12)}\). That can explain the increase in the antimicrobial activity of vancomycin against some *Staphylococcus* spp in the presence of GT (Table 1).
- It was reported that GT inhibited the synthesis of PBP1, PBP2 by > 90% and, to some extent, PBP3. However, PBP2 was not affected \(^{(5)}\).
- Diluted tea extract caused some inhibition of induction and prevented the excretion of β-lactamases into supernatant fraction \(^{(5)}\). Moreover, there was in vitro evidence that EGCG inhibit directly penicillinase activity thus was restoring the antibacterial activity of penicillins \(^{(21)}\). This may explain why, isolates S₆, S₁₂ and S₁₄ showed enhanced sensitivity to amoxicillin but not to amoxicillin-clavulanic acid in this study (Table 1).

Consequently, the direct inhibitory effect of subinhibitory concentrations of GT on isolated β-lactamases was studied.
Table 1: The antimicrobial activity of different antibiotics against tested \textit{Staphylococcus} spp* in absence and presence of GT using disc agar diffusion technique

<table>
<thead>
<tr>
<th>Bacterial code</th>
<th>Antibiotic abbreviations</th>
<th>% relative difference in inhibition zone diameter ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AK AMC AX C CE CEP CXM DA E F NOR RA SXT TE VA</td>
<td></td>
</tr>
<tr>
<td>( \text{S}_1 )</td>
<td>-11 +14 ..... +23 .....  +1% .....  +1% .....  +1% .....  + % .....  + % .....  + % .....  + %</td>
<td>( \text{S}_2 )</td>
</tr>
</tbody>
</table>

* Eighteen strains of \textit{Staphylococcus} spp were tested against sixteen antibiotics.

# Antibiotics abbreviations: as mentioned under Materials and Methods.

‡ % relative difference in inhibition zone diameter = \([a_c - a_o] / a_o \times 100\)

\( a_c \): inhibition zone diameter in plates inoculated with bacterial cells and containing the corresponding concentration of GT.

\( a_o \): inhibition zone diameter in plates inoculated with the corresponding bacterial cells.

…… : either no change or \( \pm 2 \) mm change in % relative difference inhibition zone diameter, + : increase in % relative difference inhibition zone diameter, - : decrease in % relative difference inhibition zone diameter
Table 2: Antimicrobial activity of different antibiotics against tested Gram negative bacteria* in absence and presence of GT using disc agar diffusion technique

<table>
<thead>
<tr>
<th>Bacterial code</th>
<th>AK</th>
<th>AMC</th>
<th>ATM</th>
<th>C</th>
<th>CE</th>
<th>CEP</th>
<th>CXM</th>
<th>IPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>E₁</td>
<td>.....</td>
<td>.....</td>
<td>-28</td>
<td>+25</td>
<td>.....</td>
<td>.....</td>
<td>.....</td>
<td>.....</td>
</tr>
<tr>
<td>E₂</td>
<td>.....</td>
<td>+125</td>
<td>+18</td>
<td>.....</td>
<td>.....</td>
<td>+66</td>
<td>+21</td>
<td>-10</td>
</tr>
<tr>
<td>E₃</td>
<td>.....</td>
<td>+33</td>
<td>+33</td>
<td>+12</td>
<td>.....</td>
<td>+100</td>
<td>-25</td>
<td>+35</td>
</tr>
<tr>
<td>E₄</td>
<td>.....</td>
<td>+86</td>
<td>+21</td>
<td>.....</td>
<td>.....</td>
<td>+25</td>
<td>+20</td>
<td>+50</td>
</tr>
<tr>
<td>E₅</td>
<td>.....</td>
<td>+35</td>
<td>+44</td>
<td>+14</td>
<td>+25</td>
<td>+21</td>
<td>.....</td>
<td>+40</td>
</tr>
<tr>
<td>E₆</td>
<td>.....</td>
<td>+55</td>
<td>+76</td>
<td>+17</td>
<td>+46</td>
<td>+28</td>
<td>.....</td>
<td>+42</td>
</tr>
<tr>
<td>E₇</td>
<td>+31</td>
<td>+33</td>
<td>+45</td>
<td>.....</td>
<td>+60</td>
<td>.....</td>
<td>+37</td>
<td></td>
</tr>
<tr>
<td>Ent</td>
<td>.....</td>
<td>.....</td>
<td>.....</td>
<td>.....</td>
<td>.....</td>
<td>.....</td>
<td>+100</td>
<td></td>
</tr>
<tr>
<td>P₁</td>
<td>.....</td>
<td>.....</td>
<td>+300</td>
<td>+360</td>
<td>.....</td>
<td>+100</td>
<td>.....</td>
<td>+42</td>
</tr>
<tr>
<td>P₂</td>
<td>.....</td>
<td>.....</td>
<td>+41</td>
<td>.....</td>
<td>.....</td>
<td>+54</td>
<td>-13</td>
<td>+20</td>
</tr>
<tr>
<td>P₃</td>
<td>.....</td>
<td>.....</td>
<td>+63</td>
<td>+35</td>
<td>.....</td>
<td>+45</td>
<td>.....</td>
<td>+230</td>
</tr>
</tbody>
</table>

* Thirteen strains of Gram negative bacteria were tested against thirteen antibiotics.
# , ‡ , ......, + and -  as described under Table 1.

2. Dynamics of the antimicrobial activity of GT-antibiotic combinations against some bacterial strains:
The bactericidal activity of each of erythromycin and ciprofloxacin against S. aureus isolate S₉, cefoperazone sodium against E. coli isolate E₈ and chloramphenicol against Ps. aeruginosa isolate P₂ was further evaluated in the presence of subinhibitory concentration of GT using the surface viable count.
Culture medium used in this experiment was CDM since nutrient broth was not suitable due to its high protein content to which tea polyphenols bound resulting in reduction of their concentrations

Erythromycin and GT concentration used were 125 µg/ml (twice MIC) and 0.75 mg/ml (1/8 MIC), respectively. Erythromycin alone showed more potent bactericidal effect against isolate, S₉ than that obtained in the presence of GT (1/8 MIC) alone. The combination of erythromycin and GT exerted a significant bactericidal activity along the 6 h of incubation. There was about 1 and 0.88 log decrease in the number of survivors compared to erythromycin alone at 4 h and 6 h-contact times, respectively (Fig. 1a).
When the ciprofloxacin-GT combination was tested against S. aureus isolate S₉, their respective concentrations were 4 (2 MIC) and 0.75 (1/4 MIC) mg/ml. The data in Fig. 1b showed that ciprofloxacin alone exerted better bactericidal activity than that of GT alone against isolate, S₉. The bactericidal effect of ciprofloxacin against isolate, S₉ was enhanced by the presence of GT after 1 h contact time compared to that of ciprofloxacin alone. The maximum decrease in the number of survivors was 1.3 log at 1 h-contact time (Fig. 1b).
Fig. 2a presented the bactericidal activity of GT-cefoperazone sodium combination tested against E. coli isolate E₃. Compared to control, 250 µg/ml cefoperazone sodium (equivalents to 12.5 MIC) caused about 3.8 log reduction in the number of survivors of isolate, E₃ after 4 h-contact while 10 mg/ml caused about 2.7 log reduction in the number of survivors. GT enhanced the bactericidal activity of cefoperazone sodium against isolate, E₃, where there was ~ 99.92% killing of the inoculum compared to cefoperazone alone at 4 h-contact time which caused 99.68% killing (Fig. 2a).
The bactericidal activity of GT-chloramphenicol combination against Ps. aeruginosa isolate, P₂ was shown in Fig. 2b. The concentrations of chloramphenicol and GT used in this experiment were 500 µg/ml and 5 mg/ml, respectively. Fig. 2b showed that GT exerted no bactericidal activity while chloramphenicol (4 MIC) alone caused almost 1.7 log reduction of survivors of isolate, P₂.
after 2 h of incubations. As shown from the figure, the combination caused >99.99% killing after 2 h-contact compared to chloramphenicol alone.

The results of these experiments revealed that GT enhanced bactericidal activity of the tested antibiotics at different contact time, where the number of survivors in the GT-antibiotic reaction mixtures was less than that of antibiotic alone by 0.3–5 log (Fig. 1-4). In 2002, Hu et al. (10,11) demonstrated synergistic bactericidal effects between either ampicillin/sulbactam or imipenem and EGCG against MRSA isolates. In addition, additive effect between EGCG and other antibiotics including tetracycline, chloramphenicol, streptomycin, erythromycin and rifampicin was noticed (31). In contrast to our results, Zhao et al. (12) observed no synergy between EGCG and ampicillin against E. coli and other Gram negative bacilli tested. The contradiction observed between the results could be attributed to the difference in medium tested, type of isolates tested and the form of green tea used, where they used pure active constituents but here aqueous extract of green tea was used.

![Figure 1: Dynamics of the antimicrobial activity of GT-erythromycin combination ‘a’ and GT-ciprofloxacin combination ‘b’ against S. aureus isolate S9.](image1)

![Figure 2: Dynamics of the antimicrobial activity of GT-cefoperazone sodium combination against E. coli isolate E3 ‘A’ and GT-chloramphenicol combination against Ps. aeruginosa isolate P2 ‘B’.](image2)
3. Effect of GT on the modulation of antibiotic resistance in certain bacterial isolates:

3.1. Curing of some antibiotic-resistance bacterial isolates with GT:
The data in Table 3 illustrated the effect of 24 h-treatment of some \textit{S. aureus} and Gram negative isolates with 1 and 10 mg/ml, respectively, on the loss of their antibiotic-resistance markers. The antibiotics tested were cefoperazone sodium against isolate, E3, cefuroxime sodium against isolates, S4 and S11, chloramphenicol against isolates, P1 and P2, and tetracycline HCl against isolate, S15. As seen from Table 3, the pretreatment of the selected isolates with GT did not affect their antibiotic resistance, except in the case of \textit{S. aureus} isolate, S4. Forty six colonies from the 60 GT-treated colonies of isolate, S4 were cured and lost their resistance to cefuroxime sodium, where the MIC of cefuroxime sodium against 10 of these colonies was reduced from ≥128 to 32 μg/ml while that of the remaining 36 colonies was reduced to ≤8 μg/ml.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Isolate code</th>
<th>Treatment*</th>
<th>Resistant colonies</th>
<th>% loss of resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cefoperazone sodium</td>
<td>E3</td>
<td>None</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td>Cefuroxime sodium</td>
<td>S4</td>
<td>None</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>14</td>
<td>76.6</td>
</tr>
<tr>
<td></td>
<td>S11</td>
<td>None</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>P1</td>
<td>None</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>None</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td>Tetracycline HCl</td>
<td>S15</td>
<td>None</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>60</td>
<td>0</td>
</tr>
</tbody>
</table>

* System:
None: cells not treated with GT.
A: cells treated with 10 mg/ml for 24 h at 37°C.
B: cells treated with 1 mg/ml for 24 h at 37°C.

3.2. Direct inhibitory effect of GT on isolated β-lactamases of \textit{S. aureus} isolate using nitrocefin method:
All isolates were first detected for their β-lactamases production using iodometric method and the results showed that all the tested \textit{Staphylococcus} isolates and isolate Ent were β-lactamases producers (data not shown). The nitrocefin method was then used for detecting the direct inhibitory effect of GT on β-lactamases production by \textit{S. aureus} S9. It was found that GT directly inhibited the activity of β-lactamases isolated from \textit{S. aureus} isolate S9 in a concentration-dependant manner (the higher the concentration of GT, the lower the absorbance at 492 nm, i.e. the higher the inhibitory effect of GT). The results showed, as calculated from the calibration curve, that 1 and 0.5 mg/ml caused 10 and 5.625 fold reduction of the isolated β-lactamases detected, respectively (Table 4). These results were in agreement with that stated by Zhao \textit{et al.} (31).
Table 4: Direct inhibitory effect of GT on isolated β-lactamases of *S. aureus* isolate using nitrocefin method:

<table>
<thead>
<tr>
<th>Concentration of GT (mg/ml)</th>
<th>Absorbance at 492 nm</th>
<th>Calculated concentration of β-lactamases (unit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.21</td>
<td>1.8</td>
</tr>
<tr>
<td>0.5</td>
<td>0.18</td>
<td>0.32</td>
</tr>
<tr>
<td>1</td>
<td>0.17</td>
<td>0.18</td>
</tr>
</tbody>
</table>

* β-lactamases were isolated from *S. aureus* isolate, S9.  
# Concentrations of β-lactamases were calculated from the equation of calibration curve (not shown).

3.3. Effect of subinhibitory concentrations of GT on the modulation of tetracycline HCl susceptibility of some *staphylococcus* spp isolates:

3.3.1. Effect of omeprazole, proton pump inhibitor, and GT on the antimicrobial activity of tetracycline HCl using broth dilution technique:

The antimicrobial activity of tetracycline HCl alone, tetracycline HCl-omeprazole and tetracycline HCl-GT combinations against some *Staphylococcus* spp was determined using the broth dilution method. The concentrations of omeprazole, proton pump inhibitor, and GT used were 100 µg/ml and 0.75 mg/ml, respectively. The MIC values of tetracycline HCl alone, in presence of omeprazole and in presence of GT ranged from 1.5-25, 0.75-25 and 0.375-25 µg/ml, respectively. Only isolates, S4, S6 and S16 were affected by tetracycline HCl-omeprazole combination, where 2-4 fold reduction in MIC occurred compared to tetracycline HCl alone (Table 5). On the other hand, GT caused 2-4 fold reduction in the MIC of tetracycline HCl against most tested *Staphylococcus* spp (eight isolates) including the three previously mentioned isolates. These may be attributed to the proton pump inhibitory activity of omeprazole indicating the probability of the presence of Tet (K) efflux pump in these isolates. The results may also suggest that GT may have a proton pump inhibitory activity.

Table 5: Comparative effect of omeprazole (Omper)*, proton pump inhibitor, and GT # on the antimicrobial activity of tetracycline HCl (TE) against *Staphylococcus* spp using broth dilution method:

<table>
<thead>
<tr>
<th>Isolate code (spp)</th>
<th>TE</th>
<th>TE-Omper</th>
<th>TE-GT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Minimum inhibitory concentration (µg/ml)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S2 (<em>S. aureus</em>)</td>
<td>1.5</td>
<td>1.5</td>
<td>0.75</td>
</tr>
<tr>
<td>S4 (<em>S. aureus</em>)</td>
<td>1.5</td>
<td>0.75</td>
<td>0.75</td>
</tr>
<tr>
<td>S6 (<em>S. epidermidis</em>)</td>
<td>1.5</td>
<td>0.75</td>
<td>0.375</td>
</tr>
<tr>
<td>S7 (<em>S. aureus</em>)</td>
<td>1.5</td>
<td>1.5</td>
<td>0.75</td>
</tr>
<tr>
<td>S8 (<em>S. aureus</em>)</td>
<td>1.5</td>
<td>1.5</td>
<td>0.75</td>
</tr>
<tr>
<td>S9 (<em>S. aureus</em>)</td>
<td>1.5</td>
<td>1.5</td>
<td>0.75</td>
</tr>
<tr>
<td>S10 (<em>S. aureus</em>)</td>
<td>1.5</td>
<td>1.5</td>
<td>0.75</td>
</tr>
<tr>
<td>S15 (<em>S. aureus</em>)</td>
<td>6.25</td>
<td>6.25</td>
<td>6.25</td>
</tr>
<tr>
<td>S16 (<em>S. epidermidis</em>)</td>
<td>25</td>
<td>6.25</td>
<td>6.25</td>
</tr>
<tr>
<td>S17 (<em>S. aureus</em>)</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
</tbody>
</table>

* Omper was used in a concentration of 100 µg/ml.  
# GT was used in a concentration of 0.75 mg/ml.
3.3.2. Direct inhibitory effect of GT on the tetracycline HCl efflux pump of *Staphylococcus* spp:
A quantitative experiment was conducted to measure the effect of 1mg/ml (subinhibitory concentration) on tetracycline efflux pump of some *Staphylococcus* spp \(^{(25)}\). Since the MIC values of tetracycline HCl against isolates, S4, S6, and S16 were reduced in presence of the proton pump inhibitor, omeprazole (Table 5), these isolates were selected for further investigation.

The quantity of tetracycline effluxed from *Staphylococcus* cells pretreated with GT for 15 minutes was measured spectrofluorometrically and compared to the control. The results (Fig. 3) revealed that fluorescence emitted by GT-pretreated cells decreased by about 350 units compared to the control. The decrease in the fluorescence emitted indicated the decrease in the quantity of tetracycline effluxed. These results were in agreement with that of Roccaro *et al* \(^{(25)}\).

![Fluorescence vs Time](image)

**Fig. 3:** Effect of 1 mg/ml of GT on the tetracycline efflux pump of some *Staphylococcus* spp isolates S\(_4\) (a), S\(_6\) (b), S\(_{16}\) (c) using spectrofluorometric technique

In conclusion, GT showed antibacterial activity against multiresistant clinical isolates. Moreover, the majority of GT-antibiotic combinations exhibited synergistic effect against tested isolates. Therefore, we recommend for GT-antibiotic combinations may be worthy of further *in vivo* evaluation in the future.

**REFERENCES**


