Synergism and Postantibiotic Effect of Green Tea Extract and Imipenem Against Methicillin-resistant *Staphylococcus aureus*

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**ABSTRACT**  
This study aimed to evaluate the antibacterial activity of imipenem-green tea extract combination against methicillin-resistant *Staphylococcus aureus* (MRSA) isolates using disc diffusion technique, checkerboard titration method and time-killing assay. In addition, for the first time the postantibiotic effect (PAE) of green tea either alone or plus imipenem was explored. Presence of subinhibitory concentration of green tea extract (0.25×MIC) enhanced the susceptibility of ten MRSA clinical isolates to imipenem as expressed by the percentage of relative inhibition zone diameter (% RIZD) which ranged from 140 to 254%. The fractional inhibitory concentration (FIC) indices of imipenem-green tea extract combinations were from 0.156 to 0.5. For MRSA-S2 strain, combination of imipenem and green tea extract, at 0.25×MIC each, resulted in 99.99% killing after 24 h incubation. Moreover, the PAE values obtained *in vitro* were 1.5-2.8 h for imipenem and 2.2-3.55 h for green tea extract, while higher values of 2.9-4.7 h were obtained for the combination of imipenem and green tea extract. In conclusion, the combined effect of imipenem and green tea extract seems to be significantly synergistic and may be eligible for further evaluation *in vivo* against MRSA infections. In addition, extension of the PAE duration of imipenem-green tea extract combination compared with imipenem alone may have significant impact on the effect of consumption of green tea extract on the dosing regimen of imipenem.

**Key words:** Medicinal plants, postantibiotic effect, checkerboard, time-killing assay

**INTRODUCTION**  
Green tea extract from the leaves of *Camellia sinensis* has been shown to have a wide range of antimicrobial activity (Chou et al., 1999). It exhibited antibacterial effects against *Streptococcus mutans* (Smullen et al., 2007), *Pseudomonas aeruginosa* (Jazani et al., 2007a), *Acinetobacter* sp. (Jazani et al., 2007b), methicillin-resistant *Staphylococcus aureus*, MRSA (Peng et al., 2010), *Yersinia enterocolitica*, *Salmonella typhi* and *Shigella dysenteriae* (Tiwari et al., 2005). The antibacterial activity of green tea was found to be mainly due to the high content of catechins specially epigallocatechin gallate, EGCg (Song and Seong, 2007). Tea catechins have been reported to have antibacterial activity against *Helicobacter pylori* (Yanagawa et al., 2003; Takabayashi et al., 2004), bacteria causing food-borne disease (Taguri et al., 2004), *Stenotrophomonas maltophilia* (Gordon and Wareham, 2010) and MRSA (Cho et al., 2008).

MRSA have emerged as serious pathogens in the nosocomial and community setting (Morell and Balkin, 2010). It is considered a serious problem due to its multi-drug resistant properties. Therapeutic options for treating MRSA infections are limited because most MRSA strains...
are resistant to most common antimicrobial agents (Ippolito et al., 2010). New chemotherapeutic agents or strategies are urgently needed to control such multidrug-resistant bacteria (Jasmine et al., 2007; Sittiwat and Puangpmongkhot, 2008; Anam et al., 2010). Much attention was focused on the utilization of green tea extract or catechins as alternative to or synergistic enhancers of antimicrobial agents to overcome MRSA infections (Lai and Roy, 2004; Hemaiswarya et al., 2008).

The antimicrobial activity of green tea extract in combination with amoxicillin (Peng et al., 2010), ciprofloxacin (Jazani et al., 2007c), chloramphenicol, gentamicin and nalidixic acid (Tiwari et al., 2005) was previously studied. In the present study, the antibacterial activity of imipenem-green tea extract combination against MRSA was evaluated and the PAE of green tea extract either alone or in combination with imipenem against MRSA was studied.

MATERIALS AND METHODS

Bacterial isolates, culture media and antimicrobial agents: Ten clinical isolates of MRSA were collected from two hospitals in Al-Ahsa, eastern region of Saudi Arabia during a period of three months (December 2009-February 2010). All strains were maintained on Mueller-Hinton (MH) agar plates. *Staphylococcus aureus* ATCC 25923 was included in the experiments as a control strain. All antimicrobial assays were carried out in Mueller-Hinton broth (MHB, Oxoid Ltd, UK). Antibiotic discs were purchased from Oxoid Ltd, UK. Imipenem/Cilastatin was purchased from Merck Sharp and Dome, BV, USA.

Preparation of the green tea extract: Aqueous green tea extract was prepared by the method described by Tiwari et al. (2005) with some modifications. Fifty milliliter boiling distilled water was added to 5 g of Chinese green tea leaves (Twinings, UK). After standing for 10 min, the extract was sterilized by filtration through 0.45 μm membrane filter. Filtered sterilized green tea extract was stored at 4°C.

Determination of the minimum inhibitory concentration (MIC) of green tea extract: The MIC of green tea extract was determined according to Peng et al. (2010) with some modifications. The 10% sterile green tea extract was twofold serially diluted using sterile distilled water. Ten millilitre of each dilution was aseptically well mixed with 10 mL double strength sterile molten MH agar. The mixtures were poured in sterile Petri dishes. Ten microlitre of a final inoculum (10⁶ cfu mL⁻¹) of tested strains were spotted onto the surface of the solidified plates. The plates were then incubated at 37°C for 24 h. The MIC was defined as the lowest concentration of green tea extract at which no visible growth was observed.

Determination of the MIC of imipenem: MICs of imipenem against tested strains were determined by broth dilution method using MHB in accordance with CLSI (2006). The experiments were carried out in triplicate. The MIC was defined as the lowest antibiotic concentration that completely prevented visible growth after incubation at 37°C for 24 h.

Disc diffusion assay: The effect of subinhibitory concentration (0.25×MIC) of green tea extract on the antibacterial activity of imipenem was assessed by determination of the percentage of relative inhibition zone diameter (% RIZD) using agar diffusion method as described by Rojas et al. (2006) using the following formula:
Growth pattern of MRSA-S2 in presence of subinhibitory concentrations of imipenem and green tea extract: The dynamic of growth of MRSA-S2 in presence of subinhibitory concentrations of imipenem and green tea extract was studied. The 0.25×MIC imipenem and green tea either alone or in combination were prepared in MHB inoculated with MRSA-S2. The final inoculum was $10^6$ cfu mL$^{-1}$. Control lacking imipenem and green tea extract was prepared. The optical density (OD) at 600 nm was determined at zero time and after 2, 4, 6 and 24 h incubation at 37°C in a rotary shaker.

Checkerboard titration method: The combination between imipenem and green tea extract was tested by checkerboard titration method (Krogstad and Moeller, 1986).

Time-killing assay: Combined bactericidal effects of imipenem and green tea extract were assessed against MRSA-S2, for which the MIC values of imipenem and green tea extract were 32 µg mL$^{-1}$ and 3.125 mg mL$^{-1}$, respectively. About $10^6$ cfu mL$^{-1}$ of MRSA-S2 were incubated at 37°C in presence of imipenem together with green tea extract at 0.25×MIC each. Suitable controls lacking imipenem and green tea extract were included in the experiment. Aliquots of bacterial cultures were plated onto MH agar at 0 h and after 2, 4, 6 and 24 h. Colonies were counted after 24 h incubation at 37°C. The rate of killing was determined by plotting the log number of survivors per mL (log mL$^{-1}$) against the time. Synergy was defined as a ≥100-fold increase in the bacterial killing after 24 h in presence of imipenem-green tea extract combination compared with the most active antibacterial agent alone.

Postantibiotic effect: PAE of imipenem, green tea extract and imipenem-green tea combinations at three different concentrations (1, 2 or 3×MIC) were determined against MRSA-S2 in MHB by the method described by Craig and Gudmundsson (1996). The PAE was calculated

$$PAE = T - C$$

where, $T$ is the time required for the viable counts of the exposed bacteria to increase by $1 \log_{10}$ above the counts observed immediately after washing and $C$ is the corresponding time for unexposed controls.

RESULTS

All tested MRSA isolates were resistant to imipenem. The results of the MIC for both imipenem and green tea extract are summarized in Table 1. The MIC values of imipenem ranged from 16-256 µg mL$^{-1}$ while the MIC values of green tea extract ranged from 0.78 to 6.25 mg mL$^{-1}$.

Presence of subinhibitory concentration of green tea extract (0.25×MIC) enhanced the susceptibility of MRSA isolates to imipenem as expressed by percentage relative inhibition zone diameter (%) RIZD mentioned in Table 2. The synergy between imipenem and green tea extract against MRSA-isolates was confirmed by determination of FIC indices depicted in Table 2. The FIC indices were from 0.156 to 0.5.

The growth pattern of MRSA-S2 in presence of subinhibitory concentrations of imipenem and/or green tea extract was shown in Fig. 1. Presence of 0.25×MIC green tea partially inhibited the growth of the tested MRSA while imipenem-green tea extract combination completely prevented
Table 1: Minimum inhibitory concentrations (MICs) of green tea extract and imipenem against ten MRSA isolates (S1-S10) and *Staphylococcus aureus* ATCC 29213

<table>
<thead>
<tr>
<th>Strain</th>
<th>Green tea extract (μg mL⁻¹)</th>
<th>Imipenem (μg mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRSA-S1</td>
<td>0.780</td>
<td>64</td>
</tr>
<tr>
<td>MRSA-S2</td>
<td>3.125</td>
<td>32</td>
</tr>
<tr>
<td>MRSA-S3</td>
<td>6.250</td>
<td>32</td>
</tr>
<tr>
<td>MRSA-S4</td>
<td>1.560</td>
<td>16</td>
</tr>
<tr>
<td>MRSA-S5</td>
<td>3.125</td>
<td>32</td>
</tr>
<tr>
<td>MRSA-S6</td>
<td>6.250</td>
<td>256</td>
</tr>
<tr>
<td>MRSA-S7</td>
<td>1.560</td>
<td>32</td>
</tr>
<tr>
<td>MRSA-S8</td>
<td>3.125</td>
<td>64</td>
</tr>
<tr>
<td>MRSA-S9</td>
<td>6.250</td>
<td>64</td>
</tr>
<tr>
<td>MRSA-S10</td>
<td>3.125</td>
<td>32</td>
</tr>
<tr>
<td>ATCC 29213</td>
<td>0.780</td>
<td>0.0156</td>
</tr>
</tbody>
</table>

Table 2: Percentage of relative inhibition zone diameter (% RIZD) and fractional inhibitory concentration (FIC) indices of imipenem-green tea extract combinations against ten MRSA isolates (S1-S10) and *Staphylococcus aureus* ATCC 29213

<table>
<thead>
<tr>
<th>Strain</th>
<th>% RIZD</th>
<th>FIC indices</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRSA-S1</td>
<td>157</td>
<td>0.50</td>
</tr>
<tr>
<td>MRSA-S2</td>
<td>254</td>
<td>0.196</td>
</tr>
<tr>
<td>MRSA-S3</td>
<td>173</td>
<td>0.50</td>
</tr>
<tr>
<td>MRSA-S4</td>
<td>140</td>
<td>0.50</td>
</tr>
<tr>
<td>MRSA-S5</td>
<td>200</td>
<td>0.312</td>
</tr>
<tr>
<td>MRSA-S6</td>
<td>236</td>
<td>0.50</td>
</tr>
<tr>
<td>MRSA-S7</td>
<td>184</td>
<td>0.312</td>
</tr>
<tr>
<td>MRSA-S8</td>
<td>250</td>
<td>0.196</td>
</tr>
<tr>
<td>MRSA-S9</td>
<td>150</td>
<td>0.50</td>
</tr>
<tr>
<td>MRSA-S10</td>
<td>147</td>
<td>0.50</td>
</tr>
<tr>
<td>ATCC 29213</td>
<td>119</td>
<td>0.196</td>
</tr>
</tbody>
</table>

Fig. 1: Dynamics of growth of MRSA-S2 in presence of: imipenem alone at 0.25×MIC; green tea extract alone at 0.25×MIC; imipenem plus green tea extract at 0.25×MIC each; untreated control; OD: optical density
Table 3: Postantibiotic effects (PAEs) of green tea extract and imipenem against MRSA-S2

<table>
<thead>
<tr>
<th>Concentration</th>
<th>PAE (h) Green tea extract</th>
<th>PAE (h) Imipenem</th>
<th>PAE (h) Green tea extract+Imipenem</th>
</tr>
</thead>
<tbody>
<tr>
<td>1×MIC</td>
<td>2.20</td>
<td>1.5</td>
<td>2.9 (1×MIC each)</td>
</tr>
<tr>
<td>2×MIC</td>
<td>2.65</td>
<td>2.3</td>
<td>3.25 (2×MIC each)</td>
</tr>
<tr>
<td>3×MIC</td>
<td>3.55</td>
<td>2.8</td>
<td>4.7 (3×MIC each)</td>
</tr>
</tbody>
</table>

Fig. 2: Time-kill curves of synergistic anti-MRSA-2 effects of: imipenem alone at 0.25×MIC; green tea extract alone at 0.25×MIC; imipenem plus green tea extract at 0.25×MIC each; untreated control; CFU: colony forming unit.

the growth (Fig. 1). The time-kill kinetics of imipenem, green tea extract and their combination were performed against MRSA-S2 and the results are shown in Fig. 2. The results showed a potent synergic biocidal effect of imipenem plus green tea extract combination (0.25×MIC each) against MRSA-S2. This combination resulted in 99.99% killing after 24 h incubation as shown in Fig. 2.

The PAE durations of imipenem, green tea extract and imipenem-green tea extract combinations were determined at three different concentrations; 1, 2 and 3×MIC. PAE values obtained were 1.5-2.8 h for imipenem and 2.2-3.55 h for green tea extract against MRSA-S2 (Table 3). On the other hand, values of 2.9-4.7 h were obtained when imipenem was combined with green tea extract (Table 3) which were notably long.

DISCUSSION

Tea, one of the most popular beverages in the world, is consumed every day by billions of peoples. The safe consumption of tea for thousands of years indicates low toxicity of tea and its constituents (Cabrera et al., 2006). Green tea catechins have been reported to have antibacterial activity, especially against MRSA (Hamilton-Miller and Shah, 2000). MRSA has become a major community and nosocomial pathogen in the past two decades. New chemotherapeutic agents and approaches are needed to combat MRSA (Ippolito et al., 2010).

In the present study, synergic effects of imipenem and green tea extract combinations against MRSA were observed after calculation of the FIC indices (≤0.5). These results are in agreement to the marked reduction of MIC of different β-lactams reported in presence of green tea extract.
in vitro (Peng et al., 2010). In addition, Hu et al. (2002) showed that EGCg synergizes the activity of β-lactams against MRSA. This synergistic effect may be mainly because both imipenem and green tea extract attack the same site, the cell wall (Zhao et al., 2001). In addition, Shimamura et al. (2007) showed that EGCg binds directly to peptidoglycan layer and interferes with the synthesis of cell wall.

The time-kill kinetics study showed that presence of subinhibitory concentration of green tea extract (0.25×MIC) reversed the high level resistance of MRSA-S2 to imipenem leading to 99.99% killing after 24 h incubation. These results are in accordance with the results of Hu et al. (2002) who showed that imipenem-EGCg combination completely inhibited the growth of MRSA in 24 h.

The present study is the first that evaluated the PAE of green tea extract either alone or in combination with antimicrobial agents. An important finding reported in this study was the long PAE exhibited by green tea compared with imipenem. In addition, combination of imipenem and green tea extract resulted in a significant extension of the PAE duration compared with imipenem alone. This finding may have significant impact on the effect of consumption of green tea extract or catechins on the dosing regimen of imipenem.

From a clinical point of view, it is hard to predict synergistic effects in vivo on the basis of presented in vitro evidence alone because it is difficult to estimate the in vivo concentration of EGCg after tea has been drunk (Hu et al., 2002). In addition, previous reports showed that the in vivo efficacy of green tea extract appears hindered by limited absorption of EGCg and binding with different biological macromolecules (Shimamura et al., 2007). Recently published report showed that green tea extract weakens the antibacterial activity of amoxicillin in MRSA infected mice (Peng et al., 2010). On contract, Isogai et al. (2001) reported that combination of green tea extract and levofloxacin have shown synergistic effect against enterohemorrhagic Escherichia coli in an infected mouse model. Moreover, recent study demonstrated that encapsulation of catechins in chitosan nanoparticles enhances their intestinal absorption and is a promising strategy for improving their bioavailability (Dube et al., 2010). Therefore, in vivo studies should be done for conformation of the synergy between imipenem and green tea extract specially for treatment of topical and digestive tract infections caused by MRSA.

CONCLUSION

In conclusion, green tea extract exerts multiple effects against MRSA, including independent growth-inhibiting activity, as well as cooperative bactericidal and growth-inhibiting effects in combination with imipenem. This potent synergy between green tea extract and imipenem suggests a possible clinical use for treatment of MRSA infections and could be useful in overcoming emerging resistant strains. Moreover, association of imipenem and green tea extract was found to prolong the PAE in vitro which may help imipenem to be dosed less frequently in the future after further clinical trails.

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REFERENCES


