Article

Enhancement effects of antimicrobial activities of \( \beta \)-lactam antibiotics by combination with persimmon tannin against \( \beta \)-lactamase-producing \textit{Staphylococcus aureus}

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Background \( \beta \)-lactamase-producing \textit{Staphylococcus aureus} is one of the most important bacterial pathogens. Combinations of \( \beta \)-lactam antibiotics with \( \beta \)-lactamase inhibitors such as sulbactam and tazobactam are useful therapeutic methods for combating infections of \( \beta \)-lactamase-producing bacteria. However, bacterial strains which have acquired inhibitor resistance have appeared so new therapeutic agents or new approaches are urgently needed for \( \beta \)-lactamase-producing bacteria.

Objective We investigated the antibacterial activity of persimmon tannin derived from the \textit{Diospyros kaki} cultivar "mishirazu" against \( \beta \)-lactamase-producing \textit{S. aureus} strains, and the enhancement effects of the antimicrobial activity of \( \beta \)-lactam antibiotics against those strains by combining them with persimmon tannin. The possibility of this combination as a new therapeutic agent against \( \beta \)-lactamase-producing bacteria was examined.

Methods The enhancement effects of the antimicrobial activities of \( \beta \)-lactam antibiotics in combination with persimmon tannin were tested by using an MBC (minimum bactericidal concentration) assay.

Results The antimicrobial activities of \( \beta \)-lactam antibiotics against \( \beta \)-lactamase-producing \textit{S. aureus} strains were obviously enhanced by the combination with persimmon tannin. Furthermore, it was clarified that the enhancement effect by persimmon tannin was due to the decomposition control of \( \beta \)-lactam antibiotics by \( \beta \)-lactamase.

Conclusion The combined persimmon tannin/\( \beta \)-lactam antibiotic is expected to be a new therapeutic method and/or a new therapeutic agent against infectious diseases caused by microorganisms producing \( \beta \)-lactamase.

Key words \( \beta \)-lactamase-producing \textit{Staphylococcus aureus}, persimmon tannin, \textit{Diospyros kaki}, antimicrobial activity, \textit{blaZ} gene
I. Introduction

*Staphylococcus aureus* is one of the most important bacterial pathogens (Eykyn *et al.*; 1990, Schaberg *et al.*; 1991). It causes skin infections, osteoarthritis, and respiratory tract infections in the community. Although β-lactam antibiotics (such as penicillin) are effective against the *S. aureus* infection; penicillin-resistant *S. aureus* strains were found to produce a β-lactamase (penicillinase) that inactivated the antibiotics (Kirby;1944, Spink and Ferris.;1945). The β-lactamase-producing strains have a *blaZ* gene, which encodes the β-lactamase enzyme (Okamoto *et al.*; 1996), and the strains were universally present in hospitals by the early 1950s. The emergence and spread of β-lactamase-producing bacterial strains have diminished the usefulness of the β-lactam antibiotics (Medeiros.; 1984). Combinations of β-lactam antibiotics (Medeiros.; 1984). Combinations of β-lactam antibiotics with sulbactam, tazobactam and clavulanic acid, which are β-lactamase inhibitors, are useful therapeutic methods for treating infections of β-lactamase-producing bacteria (Rizwi *et al.*; 1989, Maddux.; 1991). However, it was later reported that bacterial strains which had acquired inhibitor resistance had appeared (Blasquez *et al.*; 1993, Chaibi *et al.*; 1999). New therapeutic agents or new approaches are urgently needed for this antibiotic-resistant bacteria.

The scientific name of persimmons is *Diospyros kaki*. The genus *Diospyros* is widely distributed from tropical to temperature regions, mostly found in the humid tropics of Asia, Africa, and Central and South America (Whitmore.; 1978). They are especially well-known as a Japanese fruit. This fruit generally contains a large amount of tannin. Persimmon tannin has been used as a domestic medicine for burns, chilblains and stomach ulcers in Japan (Yoshimura.; 2002). Furthermore, the persimmon tannin is reported to have antibacterial activity (Inoue *et al.*; 1981, Nishiyama and Kozaki.;1984, Yoshioka *et al.*; 2005). It contains a condensed form of catechin gallate, gallo catechin gallate and catechin. A certain catechin, especially epigallocatechin gallate derived from green tea, is also well known to have antibacterial activity (Toda *et al.*; 1991, Ikigai *et al.*; 1993), and synergistically enhances the antimicrobial activity of β-lactam antibiotics (Yam *et al.*; 1998, Zhao *et al.*; 2001, 2002, Stapleton *et al.*; 2004, Horie *et al.*; 2009).

In this study, we investigated the antibacterial activity of persimmon tannin derived from the *D. kaki* cultivar "mishirazu" against β-lactamase-producing *S. aureus* strains, and the enhancement effects of the antimicrobial activity of β-lactam antibiotics against those strains by combining them with persimmon tannin.

II. Materials and Methods

1) Bacterial strains

The bacterial strains used in this study are listed in Table 2. The *Staphylococcus aureus* NBRC12732, NBRC14462, *Streptococcus mutans* NBRC13955, *Bacillus cereus* NBRC13494, *Escherichia coli* NBRC14237, *Salmonella enterica* serovar Typhimurium (S. Typhimurium) NBRC13245 and *Pseudomonas aeruginosa* NBRC12582 strains were obtained from the National Institute of Technology and Evaluation Biological Research Center, Chiba, Japan. The *S. aureus* SA-22 and SA-24 strains were isolated from healthy adult volunteer.

2) Persimmon tannin, antibiotics and susceptibility testing

Purified persimmon tannin was kindly supplied from Ms. Yuko Goto, Aizu-Wakamatsu Technical Support Centre, Fukushima Technology Center, Fukushima, Japan. Preparation of purified persimmon tannin was performed based on the standard protocol (Kojima *et al.*; 2006). Briefly, immature persimmon fruits, *Diospyros kaki* cultivar "mishirazu", were squeezed by a juicer. The obtained juice was heated at 75° C for 15 min, and then centrifuged at 4,700g for 20 min. The tannin was purified by using the ion exchange resin DIAION HP20 (Mitsubishi Chemistry Co., Ltd.). The tannin fractions were concentrated with an evaporator, and the products were dissolved in distilled water. The amount of tannin obtained was measured by the Folin-Denis standard method (Tsushida.; 2000).

Oxacillin (MIPIC) was obtained from Wako Pure Chemical Industries, Ltd. Benzil penicillin (PCG) and ampicillin (AMP) were obtained from Nacalai Tesque, Inc.
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MIC (minimum inhibitory concentration) was determined by a liquid microdilution method in 96-well microtiter plates according to the protocol recommended by the National Committee for Clinical Laboratory Standards (National Committee for Clinical Laboratory Standards; 1997). However, in this study, an accurate measurement by the MIC method was difficult because of turbidity from the extraction of tannin. Therefore, the MBC (minimum bactericidal concentration) was determined based on the MIC method as follows. Two-fold serially diluted antibiotics or persimmon tannin were prepared by using the Sensitivity Test broth (ST-broth, Nissui Pharmaceutical Co., Ltd.) and approximately 5 x 10⁴ CFU bacteria were inoculated. When the enhancement effects of the antimicrobial activity of the antibiotics used in combination with persimmon tannin were investigated, ST-broths which contained persimmon tannin at 56, 112 or 223 µg/mL were used for the preparation of two-fold serially diluted antibiotics. After cultivation at 35°C for 24h under an aerobic condition, each 2 µL of culture supernatant was inoculated in other 96-well plates containing the ST-broth. Cultivation was performed at 35°C for 8h and for 24h under an aerobic condition. The MBC was determined as the lowest concentration of antibiotic at which the bacteria were not able to grow.

3) PCR

PCR primers for the blaZ gene (Okamoto et al.: 1996) are described in Table 1. The PCR was performed using a DNA thermal cycler, model TP600 (Takara Bio Inc.), with 30 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 62°C, and extension for 30 s at 72°C. The PCR products were analyzed on 1.2% agarose gels and visualized by CYBR Safe DNA gel staining (Invitrogen). A 325-base-pair fragment of the blaZ gene was amplified by using the primers described above.

4) Inhibition assay of persimmon tannin against β-lactamase

Approximately 5 x 10⁴ CFU penicillin-susceptible S. aureus NBRC12732 or NBRC14462 strains were inoculated in 96-well microtiter plates which contained 0.006U/mL of β-lactamase (Calbiochem, EMD Bioscience, Inc.) and 56, 112 or 223 µg/mL of persimmon tannin in the presence of two-fold serial dilutions of PCG. After cultivation at 35°C for 24h, each 2 µL of culture supernatant was inoculated in other 96-well plates containing the ST-broth. After inoculation at 35°C for 8h and for 24h, the MBC was determined.

III. Results

1) PCR analysis of blaZ gene in S. aureus

A PCR assay of the blaZ gene employing the primer pair described in Table 1 produced a DNA product of the predicted DNA size (Fig.1). DNA fragments of 325 bp of the blaZ gene were amplified from the S. aureus SA-22 and SA-24 strains. It was estimated that both strains would produce the β-lactamase enzyme and be resistant to β-lactam antibiotics. On the other hand, the DNA fragment derived from the blaZ gene was not amplified from the S. aureus NBRC12732 and NBRC14462 strains. Both strains were estimated to be susceptible to β-lactam antibiotics.

Table 1 PCR primers used for detection of blaZ genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Positions</th>
</tr>
</thead>
<tbody>
<tr>
<td>blaZ</td>
<td>BlaF (sense)</td>
<td>5'-ACT CTT TGG CAT GTG AAC TG-3'</td>
<td>5458-5477</td>
</tr>
<tr>
<td></td>
<td>BlaR (antisense)</td>
<td>5'-AAT CCT GCA AGA AGA GTT AG-3'</td>
<td>5172-5153</td>
</tr>
</tbody>
</table>

Fig. 1 PCR analysis of blaZ gene in S. aureus. Lane 1, 100-bp DNA ladder (molecular weight marker); lane 2, S. aureus SA-22; lane 3, S. aureus SA-24; lane 4, S. aureus NBRC12732; lane 5, S. aureus NBRC14462. Expected size of PCR products (325 bp) is shown by arrows.
2) MBCs of persimmon tannin against Gram-positive and Gram-negative bacteria

The MBCs of persimmon tannin were measured to confirm the antimicrobial activities of the tannin against Gram-positive and Gram-negative bacteria (Table 2). Persimmon tannin exhibited antimicrobial activities against Gram-positive bacteria S. aureus, S. mutans and B. cereus (each MBC: 445 μg/mL). However, antimicrobial activities against Gram-negative bacteria E. coli, S. Typhimurium and P. aeruginosa were hardly observed (MBC: 3,560 μg/mL or more). Persimmon tannin was shown to have high specificity to bacteria species in its antimicrobial activity.

Table 2 MBC of persimmon tannin against Gram-positive and Gram-negative bacteria

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Strain</th>
<th>MBC (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus</td>
<td>NBRC12732</td>
<td>445</td>
</tr>
<tr>
<td>S. aureus</td>
<td>NBRC14462</td>
<td>445</td>
</tr>
<tr>
<td>Gram-positive</td>
<td>S. aureus</td>
<td>445</td>
</tr>
<tr>
<td>S. aureus</td>
<td>SA-22</td>
<td>445</td>
</tr>
<tr>
<td>S. aureus</td>
<td>SA-24</td>
<td>445</td>
</tr>
<tr>
<td>S. mutans</td>
<td>NBRC13995</td>
<td>445</td>
</tr>
<tr>
<td>B. cereus</td>
<td>NBRC14949</td>
<td>445</td>
</tr>
<tr>
<td>E. coli</td>
<td>NBRC14237</td>
<td>&gt;7130</td>
</tr>
<tr>
<td>Gram-negative</td>
<td>S. Typhimurium</td>
<td>7130</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>NBRC12582</td>
<td>3560</td>
</tr>
</tbody>
</table>

3) Enhancement of antimicrobial activity of the antibiotics by persimmon tannin

The MBCs of β-lactam antibiotics against the S. aureus SA-22, SA-24, NBRC12732 and NBRC14462 strains are shown in Table 3. Three kinds of β-lactam antibiotics, PCG, AMP and MPIPC, showed high antimicrobial activities against the NBRC12732 and NBRC14462 strains. However, PCG and AMP hardly showed any activity against the SA-22 and SA-24 strains which were detected in the blaz gene described in Fig. 1. Since the strains were highly susceptible to MPIPC (MPIPC is not decomposed by β-lactamase), it was expected that both strains would produce the β-lactamase enzyme.

The enhancement effects of the antimicrobial activity of the β-lactam antibiotics against S. aureus by combining them with persimmon tannin are shown in Table 4. Persimmon tannin was used in a concentration by which the proliferation of S. aureus was not inhibited (223, 112 or 56 μg/mL, half, quarter or 1/8 of the MBC). The antimicrobial activities of two β-lactams (PCG and AMP) were hardly observed to work against the S. aureus SA-22 and SA-24 strains during the 24 h bacteria cultivation period. On the other hand, the antimicrobial activities of the β-lactam antibiotics against both strains were obviously enhanced in combination with 223 μg/mL of persimmon tannin (Table 4). Also, in combination with 112 μg/mL of persimmon tannin, the enhancement effects of the antimicrobial activity of the β-lactam antibiotics were shown, especially in the 8 h bacteria cultivation period. However, the enhancement effect was hardly observed with 56 μg/mL of persimmon tannin.

Table 3 MBC of β-lactam antibiotics against S. aureus

<table>
<thead>
<tr>
<th>S. aureus</th>
<th>MBC (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCG</td>
</tr>
<tr>
<td>SA-22</td>
<td>32</td>
</tr>
<tr>
<td>SA-24</td>
<td>&gt;128</td>
</tr>
<tr>
<td>NBRC12732</td>
<td>2</td>
</tr>
<tr>
<td>NBRC14462</td>
<td>&lt;0.125</td>
</tr>
</tbody>
</table>

PCG, benzyl penicillin (U/mL); AMP, ampicillin (μg/mL); MPIPC, oxacillin (μg/mL)

Table 4 Effect of persimmon tannin in sensitizing β-lactamase producing S. aureus to β-lactam antibiotics

<table>
<thead>
<tr>
<th>S. aureus</th>
<th>Combination with persimmon tannin (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>223</td>
</tr>
<tr>
<td></td>
<td>PCG</td>
</tr>
<tr>
<td>SA-22</td>
<td>32</td>
</tr>
<tr>
<td>SA-24</td>
<td>&gt;128</td>
</tr>
<tr>
<td>SA-24</td>
<td>16</td>
</tr>
<tr>
<td>SA-24</td>
<td>&gt;128</td>
</tr>
</tbody>
</table>

PCG, benzyl penicillin (U/mL); AMP, ampicillin (μg/mL)

4) Inhibition effect of persimmon tannin against β-lactamase activity

To elucidate the mechanism in the enhancement effects of the antimicrobial activity of the β-lactam antibiotics by persimmon tannin, the inhibition effect of persimmon tannin against β-lactamase activity was examined. The MBC of PCG for the S. aureus NBRC12732 and NBRC14462 strains which were PCG-susceptible rose remarkably from 2, <0.125 U/mL to >128, >128 U/mL, respectively, by using an ST-broth containing 0.006 U/mL of...
Enhancement of antimicrobial activities by persimmon tannin

positive bacteria (S. aureus, S. mutans and B. cereus), but was hardly observed to Gram-negative bacteria (Table 2). Although it was not clear that persimmon tannin showed Gram-positive-specific antimicrobial activity, this is possibly related to the difference in the structure of the cell wall between Gram-positive and Gram-negative bacteria because the epigallocatechin gallate, which is one of the tannin derived from green tea, enhanced the antimicrobial activity of \( \beta \)-lactam antibiotics against methicillin-resistant S. aureus (Yam et al.; 1998, Stapleton et al.; 2004, Horie et al.; 2009), and inhibits the synthesis of peptidoglycan on the cell wall of bacteria (Zhao et al.; 2001). It is thought that persimmon tannin has inhibition activity as well as green tea tannin.

Bacterial strains producing \( \beta \)-lactamase acquire resistance to many \( \beta \)-lactam antibiotics used to treat infectious diseases caused by S. aureus or other microorganisms. In this study, the S. aureus SA-22 and SA-24 strains showed properties of resistance to \( \beta \)-lactam antibiotics (Table 3), and these strains had the \( \text{blaZ} \) gene, which encodes \( \beta \)-lactamase (Fig.1). The antimicrobial activities of the \( \beta \)-lactam antibiotics PCG and AMP used in combination with persimmon tannin were demonstrated to be enhanced against both strains. Furthermore, one of the mechanisms in the enhancement effect of the antimicrobial activity of persimmon tannin is elucidated. Persimmon tannin blocked the \( \beta \)-lactamase activity in a dose-dependent manner. The enhancement effect of the antimicrobial activity was due to the decomposition control of \( \beta \)-lactam antibiotics by \( \beta \)-lactamase. Interestingly, the \( \beta \)-lactamase (Calbiochem, EMD Bioscience, Inc.) used in this assay is derived from B. cereus strain (569/H9), therefore, the inhibition effect of persimmon tannin against \( \beta \)-lactamase activity might be shown not only for S. aureus but also for other bacteria which produce \( \beta \)-lactamase.

However, there is another possibility that the enhancement effect of the antimicrobial activity by persimmon tannin is due to the synergistic effect of \( \beta \)-lactam antibiotics and persimmon tannin, since it is thought that the target of the \( \beta \)-lactams and persimmon tannin is the peptidoglycan on the bacterial cell wall. Further analyses of the specificity

**Fig. 2** Inhibition effect of persimmon tannin against \( \beta \)-lactamase activity. Approximately \( 5 \times 10^6 \) CFU penicillin-susceptible S. aureus MRBC12732 or MRBC14462 strains were inoculated in 96-well microtiter plates containing 0.006 U/mL of \( \beta \)-lactamase and 56, 112 or 223 \( \mu \)g/mL of persimmon tannin in the presence of two-fold serial dilutions of benzil penicillin. After cultivation at 35°C for 24h, each 2 \( \mu \)L of culture supernatant was inoculated in other 96-well plates containing ST-broths. After inoculation at 35°C for 8h (A) and 24h (B), the MBC was determined. \( \beta \)-lac.: \( \beta \)-lactamase. PT: persimmon tannin

\( \beta \)-lactamase (Fig.2A, B). However, persimmon tannin blocked the \( \beta \)-lactamase activity in a dose-dependent manner. Even with 56 \( \mu \)g/mL of persimmon tannin, which is the lowest amount showing an inhibition effect against \( \beta \)-lactamase activity in the 8h bacteria cultivation period, the MBCs of PCG were restored from >128 U/mL to 16 U/mL (MRBC12732) and to 32 U/mL (MRBC14462) (Fig. 2A).

**IV. Discussion**

In this study, the antimicrobial activity of persimmon tannin was demonstrated against Gram-
to antibiotics and to bacteria species are necessary to clarify the mechanism of the enhancement effect in combination with persimmon tannin.

Persimmon tannin is the main component of *Diospyros kaki*. The combined persimmon tannin/β-lactam antibiotic is expected to be a new therapeutic method which possesses high safety against infectious diseases caused by β-lactamase-producing bacteria. Moreover, it is expected that a new therapeutic agent which shows an inhibition effect against β-lactamase activity may be developed based on the above findings.

V. Conclusion

The antimicrobial activity of persimmon tannin was demonstrated against Gram-positive bacteria, but was hardly observed against Gram-negative bacteria. Moreover, the antimicrobial activities of the β-lactam antibiotics against β-lactamase-producing *S. aureus* strains were obviously enhanced by the combination with persimmon tannin. The enhancement effect of the antimicrobial activity by persimmon tannin was due to the decomposition control of β-lactam antibiotics by β-lactamase. The combined persimmon tannin/β-lactam antibiotic is expected to be a new therapeutic method and/or a new therapeutic agent against infectious diseases caused by microorganisms producing β-lactamase.

VI. Acknowledgement

We are grateful to Ms. Yuko Goto (Aizu-Wakamatsu Technical Support Centre, Fukushima Technology Center, Fukushima, Japan) for supplying purified persimmon tannin. This work was supported by an Ohu University Joint Research Fund.

References


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Enhancement of antimicrobial activities by persimmon tannin


Whitmore TC. Flowering plants of the world.


(Summary)

柿由来タンニンによるβ－ラクタマーゼ産生黄色ブドウ球菌に対するペニシリン系抗生物質の抗菌活性増強効果

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岩間 正典1）、山田 明6）

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3）奥羽大学歯学部口腔病態解析制御学講座歯科薬理学， 4）奥羽大学薬学部生薬学分野
5）奥羽大学薬学部有機化学分野， 6）滋賀県立大学人間看護学部

背景 β-ラクタマーゼ産生黄色ブドウ球菌は、多くの
β-ラクタム系抗生物質に対して耐性である。更にこ
の細菌においては、スルラクタムやタポラクタムのよ
うなβ-ラクタマーゼインヒビターと、β-ラクタム系
抗生物質を組み合わせて用いても、効果を示さないイ
ンヒビター耐性菌も出現している。β-ラクタマーゼ
産生細菌による感染症に対し、新しい治療薬や治療方
法の開発が急務となっている。

目的 柿由来タンニンが、β-ラクタマーゼ産生細菌に
に対する新しい医薬品となる可能性について検討を行
うため、同タンニンのβ-ラクタマーゼ産生黄色ブドウ
球菌に対する抗菌活性、並びに同タンニンの同細菌に
に対する、β-ラクタム系抗生物質の抗菌活性を増強す
る効果について解析を行った。

方法 柿由来タンニンの抗菌活性およびβ-ラクタム系
抗生物質の抗菌活性増強効果は、MIC（minimum
inhibitory concentration）法を基にしたMBC（minimum
bactericidal concentration）法で試験し
た。また、柿由来タンニンのβ-ラクタマーゼに対す
る活性阻害効果に関しても、同様にMBC法で試験し
た。

結果 β-ラクタマーゼ産生黄色ブドウ球菌に対してほ
とんど抗菌活性を示さなかったβ-ラクタム系抗生物
質が、柿由来タンニンと併用することで顕著な抗菌活
性を示した。この効果は、同タンニンによるβ-ラク
タマーゼの活性阻害効果によるものであることが、強
く示唆された。

結論 柿由来タンニンは、単独あるいはβ-ラクタム系
抗生物質と組み合わせて用いることにより、β-ラク
タマーゼ産生黄色ブドウ球菌による感染症に対して、
高い安全性と有効性をもった新しい医薬品や治療方法
の開発に結びつく可能性が期待される。

キーワード β-ラクタマーゼ産生黄色ブドウ球菌、柿
由来タンニン、Diospyros kaki、抗菌活性、blaZ遺
伝子