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Antibacterial action of several tannins against *Staphylococcus aureus*

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We examined the antibacterial action of several tannins on plasma coagulation by *Staphylococcus aureus* and the effect of conventional chemotherapy combined with tannic acid below the MIC. Coagulation was inhibited in plasma containing tannic acid (100 mg/L), gallic acid (5000 mg/L), ellagic acid (5000 mg/L), (–)-epicatechin (1500 mg/L), (–)-epicatechin gallate (500 mg/L) or (–)-epigallocatechin gallate (200 mg/L) after incubation for 24 h. All tannins inhibited coagulation at a concentration below the MIC. The MICs of oxacillin and cefdinir for *S. aureus* were reduced to ≤0.06 mg/L in Mueller–Hinton agar plates with tannic acid (100 mg/L) at a concentration below the MIC. The antistaphylococcal activity of tannic acid was reduced in plates with 10% rabbit blood, but not in those with 10% rabbit plasma. Membranous structures formed in a culture medium containing equal proportions of plasma and tryptic soy broth after incubation for 24 h. The colony counts of *S. aureus* in membranous structures in the medium containing oxacillin (40 mg/L) and tannic acid (100 mg/L) were *c.* 10-fold lower than those in medium containing oxacillin (40 mg/L) alone ($P < 0.01$). Tannic acid merits further investigation as a possible adjuvant agent against *S. aureus* skin infections treated with β-lactam antibiotics.

Introduction

Tannins are water-soluble polyphenols that are commonly found in higher herbaceous and woody plants.[1] They can be classified into two categories: hydrolysable and non-hydrolysable (condensed). Tannic acid is an important gallotannin belonging to the hydrolysable class, while catechin belongs to the non-hydrolysable class.[2] Hydrolysable tannins are esters of phenolic acids and a polyol, usually glucose.[1,3] The phenolic acids are either gallic acid in gallotannins, or hexahydroxydiphenic acid in ellagitannins. The hexahydroxydiphenic acid of ellagitannins undergoes lactonization to produce ellagic acid.[3] Tannins have been reported to be bacteriostatic or bactericidal against *Staphylococcus aureus*.[4] Catechins from tea leaf are composed mainly of (–)-epicatechin (EC), (–)-epigallocatechin (EGC), (–)-epicatechin gallate (ECG) and (–)-epigallocatechin gallate (EGCG).[5–7] Oxacillin, ampicillin and cefalexin showed increased antibacterial activity against methicillin-resistant *S. aureus* (MRSA) in the presence of catechin below MIC.[8] ECG markedly lowered the MIC of oxacillin and other β-lactams, but not of other antimicrobial agents, for strains of MRSA.[9] In the presence of plasma *S. aureus* forms a fibrin-rich biofilm that is highly resistant to the immune system and conventional chemotherapy.[10] We reported previously that 5% zinc oxide,[11] 0.12% calcium oxide, 0.25% magnesium oxide,[12] 70% sucrose[13] and low pH[14] inhibit plasma coagulation by *S. aureus* cells, and that the formation of biofilms would probably also be inhibited under these conditions.[13,14] We further hypothesized that the inhibition of fibrin formation produced by *S. aureus* cells could be an important strategy against *S. aureus* infection in human skin. The purpose of the present study was to examine the antibacterial activity of several tannins on the coagulation of plasma by *S. aureus* and the effect of incubating *S. aureus* for 24 h in the combination of conventional chemotherapy and tannic acid below the MIC.

Materials and methods

Bacterial strains

Nine strains of *S. aureus*, isolated from furuncle lesions (coagulase type IV) and nine isolated from impetigo [five strains of coagulase type I (exfoliative toxin B producers)

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and four of coagulase type V (exfoliative toxin A producers)] were used to examine plasma coagulation and antimicrobial activities. Of the 18 strains of *S. aureus*, seven were methicillin susceptible (MSSA; oxacillin MIC $\leqslant$ 2 mg/L) and 11 were MRSA (oxacillin MIC $\geqslant$ 4 mg/L).

Bacterial suspension for inoculation

The strains of *S. aureus* were grown in 8 mL of tryptic soy broth (TSB; Nissui Pharmaceutical Co., Tokyo, Japan) at 37°C overnight without shaking. Following incubation, the bacterial cells were harvested by centrifugation at 6000**g** for 10 min at 4°C, then resuspended in sterile saline solution and centrifuged as described above. The process was repeated three times. The washed bacteria were resuspended in polypropylene microcentrifuge tubes (1 mL; Iuchi BioSystems, Tokyo, Japan) or tissue-culture dishes (35×10 mm^2; Becton Dickinson, NJ, USA) and were used in the following experiments.

Plasma coagulation under various concentrations of tannic acid, gallic acid, ellagic acid, (–)-epicatechin, (–)-epicatechin gallate and (–)-epigallocatechin gallate

Cell suspensions of *S. aureus* (*c.* 10^8 cfu) were used for inoculation into 0.5 mL of rabbit plasma (Denka Seiken, Tokyo, Japan) either alone (control) or supplemented with tannic acid (100 or 50 mg/L; Sigma, St Louis, MO, USA), gallic acid (5000 or 2500 mg/L; Sigma), ellagic acid (5000 or 2500 mg/L; Sigma), EC (1500 or 800 mg/L; Sigma), ECG (500 or 200 mg/L; Sigma) or EGCG (200 or 100 mg/L; Sigma) in microcentrifuge tubes. A clot (plasma coagulation) was looked for in the microcentrifuge tubes after incubation for 24 h at 37°C.

MICs of tannins and antibiotics

The MICs of tannic acid, gallic acid, ellagic acid, EC, ECG and EGCG against 18 *S. aureus* strains were examined in Mueller–Hinton agar (MHA; Difco, Detroit, MI, USA) using the agar plate method (inoculum size 10^6 cfu/mL). The MICs of oxacillin (Sigma) and cefdinir (Fujisawa Pharmaceutical, Osaka, Japan) for 18 strains of *S. aureus* were examined in MHA, MHA with 100 mg/L tannic acid, and MHA with 20 mg/L tannic acid. The MICs of tannic acid and oxacillin were also examined in MHA with 10% defibrinated rabbit blood (Japan Ram, Fukuyasu, Hiroshima, Japan) or 10% rabbit plasma.

Concentrations of ionic calcium and iron

The concentrations of ionic calcium of human serum (Sigma) either alone or supplemented with tannic acid (100 or 1000 mg/L) for 24 h at 37°C were measured by Scripps Reference Laboratories (SRL, Tokyo, Japan) using the ionic electrode method. The concentrations of iron in human serum either alone or supplemented with tannic acid (1000 mg/L) for 24 h at 37°C were measured by SRL using the International Standard Method.[15] The precipitates were removed by centrifugation at 500**g** for 20 min at room temperature, and the concentrations of the supernatants were examined. Because the concentrations of these in plasma and TSB could not be technically measured, we examined those of human serum.

Effect of tannic acid on membranous structures

Cell suspensions of a strain of *S. aureus* from impetigo, containing 6×10^7 cfu and of a strain of *S. aureus* from a furuncle containing 1.1×10^8 cfu were each inoculated into 4 mL of a culture medium [plasma TSB (Pl-TSB); ratio of rabbit plasma:TSB, 1:1] covering 1.77 cm^2 coverslips (Sumitomo Bakelite, Tokyo, Japan) in tissue culture dishes. The MICs of oxacillin, tannic acid and oxacillin with tannic acid (100 mg/L) for both strains were 32, 1000 and $\leqslant$0.06 mg/L, respectively. After incubation for 24 h at 37°C, membranous structures had formed on the coverslips. Coverslips with membranous structures were then placed into 4 mL of Pl-TSB either alone (control) or supplemented with oxacillin (40 mg/L), tannic acid (100 mg/L) or oxacillin (40 mg/L) and tannic acid (100 mg/L). We used tannic acid at a concentration of 100 mg/L, because tannic acid inhibited plasma coagulation of *S. aureus* cells and decreased the MIC of oxacillin at this concentration. After incubation for 24 h at 37°C, plasma coagulation was checked and the coverslips were gently washed five times with 1 mL of sterile saline. The coverslips were put in 5 mL of sterile saline and sonicated (Model M-225R, Ultrasonics Inc.) at 60% power for 60 s at 4°C. The number of organisms stripped from the coverslip was then counted (cfu).

Results

The 18 strains of *S. aureus* did not coagulate plasma containing tannic acid (100 mg/L), gallic acid (5000 mg/L), ellagic acid (5000 mg/L), EC (1500 mg/L), ECG (500 mg/L) or EGCG (200 mg/L) after incubation for 24 h at 37°C. All 18 strains of *S. aureus* did coagulate plasma alone after incubation for 24 h at 37°C.

Table 1 shows the MICs of tannic acid, gallic acid, ellagic acid, EC, ECG and EGCG for 18 *S. aureus* strains in MHA plates. The MIC of tannic acid for all 18 strains of *S. aureus* was >4500 mg/L in MHA with 10% defibrinated rabbit blood. The MIC of tannic acid was the same in the MHA both with and without 10% rabbit plasma.

Table 2 shows the MICs of oxacillin and cefdinir for 18 strains of *S. aureus* in MHA with or without tannic acid (100 mg/L). The MICs of oxacillin and cefdinir decreased to $\leqslant$0.06 mg/L in MHA with tannic acid (100 mg/L). MICs

Table 1. MICs (mg/L) of tannic acid, gallic acid, ellagic acid, EC, ECG and EGCG for *S. aureus* strains on MHA plates

	No. of strains with MIC			
	250 mg/L	1000 mg/L	8000 mg/L	Total
Tannic acid	14	4		18
Gallic acid			18	18
Ellagic acid			18	18
EC			18	18
ECG		18		18
EGCG	18			18

in MHA with tannic acid at 20 mg/L were the same as those in MHA plates without tannic acid. The MIC of oxacillin was not reduced in the MHA with tannic acid (100 mg/L) and 10% defibrinated rabbit blood, but decreased to ⩽0.06 mg/L in the MHA with tannic acid (100 mg/L) and 10% rabbit plasma.

The concentration of ionic calcium was 2.56 mEQ/L in human serum alone (control; normal range: 2.41–2.72 mEQ/L), 2.00 mEQ/L in human serum with tannic acid (100 mg/L) and 1.76 mEQ/L in human serum with tannic acid (1000 mg/L). The concentration of iron was 112 μg/dL in human serum alone (control) and 115 μg/dL in human serum supplemented with tannic acid (1000 mg/L).

The colony counts of *S. aureus* cells in membranous structures in Pl-TSB containing oxacillin (40 mg/L) and tannic acid (100 mg/L) were *c.* 10-fold lower than those in Pl-TSB alone and Pl-TSB broth containing oxacillin (40 mg/L; $P < 0.01$; Table 3).

Discussion

Tannic acid is present in many foods including tea, cocoa beans, grapes, strawberry and persimmon. Tannic acid is also categorized as a 'generally recognized as safe' (GRAS) food additive.[4] Sharquie *et al.*[16] reported that crude tea ointment was very effective, with a cure rate of 81.3% in patients with impetigo contagiosa. Tannins form chelates with metal ions and are therefore different from smaller phenols,[3] so we looked at the concentrations of ionic calcium and iron. The antimicrobial mechanisms of tannins can be summarized as follows. (i) The astringent property of the tannin may induce complexation with enzymes or substrates. Many microbial enzymes in raw culture filtrates or in purified forms are inhibited when mixed with tannins. (ii) A tannin's toxicity may be related to its action on the membranes of the microorganisms. (iii) Complexation of metal ions by tannins may account for tannin toxicity.[3] Tannic acid, but not gallic acid, was found to be inhibitory to the growth of intestinal bacteria such as *Bacteroides fragilis*, *Clostridium perfringens*, *Escherichia coli* and *Enterobacter cloacae* amongst others. Tannic acid has a much greater relative binding efficiency to iron than gallic acid. Tannic acid may work like a siderophore to chelate iron from the medium and make iron unavailable to microorganisms. Microorganisms growing under aerobic conditions need iron for a variety of functions, including reduction of the ribonucleotide precursor of DNA, formation of haem, and other essential purposes.[17] Chung *et al.*[17] reported that the inhibitory effect of tannic acid on the growth of intestinal bacteria may be caused by its strong iron-binding capacity. Chung *et al.*[4] also reported that tannic acid inhibited the growth of all 15 of the bacteria tested, but gallic acid and ellagic acid did not inhibit any of them. They concluded that the ester linkage between gallic acid and glucose (to form tannic acid) was important to the antimicrobial potential of these compounds.[4] Based on the serum iron concentration seen in the present study (see Results), we suggest that inhibition of the growth of *S. aureus* cells in the media with tannic acid was not due to a decrease in iron.

Ellagic acid was reported to accelerate blood clotting and has been used to control haemorrhage in animals.[3] However, in the present study, ellagic acid (5000 mg/L) below the MIC inhibited plasma coagulation by *S. aureus* cells rather than accelerating it.

Catechins are a major component of green tea (10–18% of the dry weight), with EGCG being the most important of the four classes of catechins.[6,7] Ikigai *et al.*[18] investigated the mode of antibacterial action of EGCG and EC and found that EGCG caused leakage of 5,6-carboxyfluorescein from phosphatidyl choline liposomes, while EC caused little damage to the membrane. They reported that bactericidal catechins primarily act on and damage the bacterial membrane.[18] Yam *et al.*[19] reported that gallocatechins and their gallates are the main chemical moieties responsible for the antibacterial activity of green tea extracts; the substances having epi configuration seem, in general, to be more active. Takahashi *et al.*[8] reported that the colony counts of MRSA did not decrease in media with catechin (100 mg/L) or oxacillin (5 mg/L) compared with an untreated control, but decreased to between 1/1000 and 1/10 000 in media with catechin (100 mg/L) and oxacillin (5 mg/L) after incubation for 24 h. In the present study, we confirmed that the MICs of oxacillin and cefdinir for *S. aureus* were markedly decreased in the MHA plates with tannic acid (100 mg/L) below the MIC. However, the MIC of roxithromycin against *S. aureus* strains did not decrease in the MHA plates with tannic acid (100 mg/L), and the MIC of oxacillin did not decrease in the MHA plates with gallic acid (100 mg/L; data not shown). Therefore, tannic acid at a sub-MIC seemed to act on the membranes of *S. aureus* cells. These findings indicate that β-lactam antibiotics have increased antistaphylococcal activity in the presence of tannic acid.

Table 2. MICs (mg/L) of oxacillin and cefdinir for *S. aureus* strains in MHA plates with or without tannic acid (100 mg/L)

	No. of strains with MIC (mg/L)												
	⩽0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	⩾128	Total
Oxacillin alone			6			1		2		5		4	18
Oxacillin + tannic acid (100 mg/L)	18												18
Cefdinir alone			3	4	2	2	1	2	1	3			18
Cefdinir + tannic acid (100 mg/L)	18												18

Table 3. Colony counts of *S. aureus* cells in membranous structures ($n = 5$) in Pl-TSB and plasma coagulation after incubation for 24 h

	Incubation times (h)				
	impetigo strain		furuncle strain		
Medium	0	24	0	24	Plasma coagulation
	5.61 ± 0.06		6.12 ± 0.07		
Pl-TSB broth alone (control)		5.67 ± 0.14		6.08 ± 0.12	positive
Pl-TSB broth + oxacillin (40 mg/L)		5.80 ± 0.12		5.97 ± 0.09	positive
Pl-TSB broth + tannic acid (100 mg/L)		5.35 ± 0.19		5.31 ± 0.11	negative
Pl-TSB broth + oxacillin (40 mg/L) + tannic acid (100 mg/L)		4.81 ± 0.12[a]		4.62 ± 0.1[a]	negative

Values are mean ± S.D. $\log_{10}$ cfu/1.77 cm^2 coverslip.
MICs of oxacillin, tannic acid and oxacillin with tannic acid (100 mg/L) for both strains were 32, 1000 and ⩽0.06 mg/L, respectively.
[a] $P < 0.01$ [compared with Pl-TSB alone (control) and Pl-TSB +oxacillin (40 mg/L)].

Staphylocoagulase is an extracellular protein produced by *S. aureus*. Thrombin–staphylocoagulase complexes appear to be formed immediately when prothrombin and staphylocoagulase are mixed. While this reaction is considered to be non-enzymic, thrombin–staphylocoagulase complexes do become enzymically active and initiate fibrin polymerization.[20] The conversion of fibrinogen to fibrin first involves cleavage by thrombin of a specific Ary–Gly bond in each of the α-A and β-B chains to release the fibrinopeptides A and B from the amino termini of the chains.[21] Fibrin monomers are thus formed and aggregate to soluble fibrin. Soluble fibrin is then converted in a final step to insoluble fibrin by an activated cross-linking enzyme, factor XIIIa, the fibrin-stabilizing factor fibrinoligase. The presence of ionic calcium promotes the reaction.[21] In the present study, all six tannins tested inhibited plasma coagulation of *S. aureus* at a concentration that was below the MIC. We suggest that inhibition of plasma coagulation by tannic acid is due to a decrease in the concentration of ionic calcium, inhibition of enzyme production and hindrance of the enzyme reaction. The mechanism of inhibition of plasma coagulation by tannic acid should be investigated further.

The presence of a fibrin-rich biofilm is a well-known factor responsible for prolonging *S. aureus* infections. The biofilm of *S. aureus* is reinforced with fibrin fibres, making it more resistant to physical effects than are other bacterial biofilms.[10] We reported previously that the attachment of *S. aureus* to coverslips, the conversion of fibrinogen to fibrin and the abundant production of glycocalyx by *S. aureus* are minimum requirements for the production of a mature biofilm on coverslips after 72 h.[22] Although the membranous structures in the present study following incubation for 24 h were similar to fibrin clots containing *S. aureus* and plasma components, the *S. aureus* in the membranous structures in plasma were resistant to imipenem at 40 × MIC and roxithromycin at 4 × MIC (data not shown). If plasma coagulation does not occur in the presence of some tannins, the formation of fibrin-rich membranous structures by *S. aureus* will probably be inhibited. The antistaphylococcal activity of oxacillin against membranous structures increased in Pl-TSB with tannic

acid (100 mg/L) below the MIC. We suggest that these phenomena are due to inhibition of fibrin formation and a marked increase in the antistaphylococcal activity of oxacillin by the addition of tannic acid (100 mg/L). These results indicate that tannic acid may be a useful adjuvant agent for the treatment of *S. aureus* skin infections in addition to β-lactam antibiotics, at least under *in vivo* conditions without blood. Given the apparent trend towards the evolution of resistant strains, further investigation of natural products having antistaphylococcal activities may reveal useful topical applications for clinical dermatology.

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