Anti-biofilm activities of quercetin and tannic acid against *Staphylococcus aureus*

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*Staphylococcus aureus* is a leading cause of nosocomial infections because of its resistance to diverse antibiotics. The formation of a biofilm is one of the mechanisms of drug resistance in *S. aureus*. The anti-biofilm abilities of 498 plant extracts against *S. aureus* were examined. Seventy-two plant extracts belonging to 59 genera and 38 families were found to significantly inhibit the formation of biofilms of *S. aureus* without affecting the growth of planktonic cells. The most active extract, from *Alnus japonica*, inhibited the formation of biofilms by three *S. aureus* strains by >70% at 20 μg ml⁻¹. Transcriptional analyses showed that extract of *A. japonica* repressed the intercellular adhesion genes *ica* and *icaD* most markedly. Quercetin and tannic acid are major anti-biofilm compounds in the extract of *A. japonica*. Additionally, the extract of *A. japonica* and its component compound quercetin, reduced hemolysis by *S. aureus*. This phenomenon was not observed in the treatment with tannic acid. This study suggests that various plant extracts, such as quercetin and tannic acid, could be used to inhibit the formation of recalcitrant biofilms of *S. aureus*.

**Keywords:** biofilm; plant extracts; quercetin; *Staphylococcus aureus*; tannic acid

**Introduction**

Bacteria attach preferentially to various surfaces using a self-produced extracellular polymeric matrix, to produce biofilms. These can be difficult to eradicate with biocides or antibiotics, and thus, biofilms composed of pathogenic bacteria can pose serious problems for human health (Costerton et al. 1999).

*Staphylococcus aureus* is an important human pathogen that often exhibits antibiotic resistance, and is responsible for numerous outbreaks of nosocomial infections (Lowy 1998). Furthermore, *S. aureus* can secrete exotoxins, such as hemolysin, enterotoxins, coagulase, TSST-1, and protein A, associated with specific diseases (Ohlsen et al. 1997). Because biofilms play critical roles in antibiotic resistance (Stewart & Costerton 2001), new biofilm-inhibiting compounds are urgently required against recalcitrant pathogens such as methicillin-resistant *S. aureus* (MRSA) and vancomycin-methicillin-resistant *S. aureus*.

Various approaches have been reported for treating staphylococcal biofilm infections involving matrix-degrading enzymes and small molecule approaches (Kiedrowski & Horswill 2011). The manipulation of staphylococcal disassembly mechanisms such as those involving intercellular adhesion proteins, agr quorum sensing, protease, DNase, *cis*-2-decenoic acid, 3-amino acids, phenol-soluble polypeptides, and other surface proteins or environmental factors have also been investigated (Boles & Horswill 2011). In particular, the intercellular adhesion (*ica*) locus is known to control positively the formation of biofilms of *S. aureus* (Cramton et al. 1999), and several chemicals that repress *ica* transcription, such as hydrogen peroxide (Glynn et al. 2009), sub-inhibitory povidone-iodine (Oduwole et al. 2010), and sulfhydryl compounds (Wu et al. 2011), have been reported to reduce the formation of biofilms by *Staphylococcus* spp.

Plants offer a rich source of antimicrobial agents and other pharmaceuticals (Cowan 1999; Zhao et al. 2005; Li & Vederas 2009). However, of the ~500,000 plant species, only 1% has been phytochemically investigated from the perspective of antimicrobial activity (Cowan 1999; Palombo 2009). Nevertheless, several plant-derived molecules have been reported to target the formation of biofilms by *S. aureus* with those identified including diterpenoids (Kužmina et al. 2007), oleic acid (Stenz et al. 2008), ellagic acid (Quave et al. 2012), esculetin and fisetin (Dürrig et al. 2010), 1,2,3,4,6-penta-O-galloyl-β-D-glucopyranose (Lin et al. 2011), and tannic acid (Payne et al. 2013).

In the present study, 498 medicinal plants were screened for their ability to inhibit the formation of biofilms of *S. aureus* without affecting cell growth. Attempts were made to identify anti-biofilm compounds in the most active plant extracts and to understand their mechanisms of action through transcriptional analysis. In addition, the hemolysis

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of human red blood cells by *S. aureus* in the presence of plant extracts was investigated.

Materials and methods

**Bacterial strains, materials, and measurement of growth rate**

Two methicillin-sensitive *S. aureus* strains (MSSA; ATCC 25923 and ATCC 6538), and one MRSA strain (ATCC BAA-1707) were used. All experiments were conducted in Luria-Bertani (LB) medium at 37 °C, with the exception of the biofilm assay for MRSA where the LB medium was supplemented with 0.2 % glucose. The 498 Asian medicinal plant extract library was obtained from the Korean Plant Extract Bank (http://www.extract.pdrc.re.kr/extract/f.htm, Daejeon, Republic of Korea). The plant library was publicly available and a list of the plants investigated is provided in Supplementary Table S1 with vendor IDs. [Supplementary material is available via a multimedia link on the online article webpage.] The plants tested were chosen because of their diversity and possible medicinal activities as determined by literature searches. The extraction procedure has been described previously (Cho et al. 2010). Briefly, each plant was dried at room temperature for 5 days away from direct sunlight, ground, extracted with 99.8% methanol at 50 °C, and extracts were vacuum-dried at 45 °C. Twenty milligram aliquots of methanol extracts were then prepared and stored at 4 °C until required. All dried plant extracts were dissolved in dimethyl sulfoxide. Nine plant compounds, namely, apigenine, betulinic acid, catechol, kaempferol, luteolin, quercetin, shikimic acid, cis-stilbene, and tannic acid were purchased from Sigma-Aldrich (St Louis, USA). For measurements of cell growth, optical densities (ODs) were measured at 600 nm (OD_{600}) using a spectrophotometer (UV-160, Shimadzu, Japan). Specific growth rates were determined from the linear portions (OD_{600} of between 0.2 and 0.7) of log absorbance vs time scatter-plots. Each experiment was performed using at least two independent cultures.

**Crystal violet biofilm assay and anti-biofilm screening**

A static biofilm formation assay was performed in 96-well polystyrene plates (SPL Life Sciences, Korea), as described previously (Pratt & Kolter 1998). Briefly, cells were inoculated into LB medium (total volume = 300 μl) at an initial turbidity of 0.05 (OD_{600}) and cultured with or without plant extracts for 24 h without shaking. Biofilms in 96-well plates were stained with crystal violet and subsequently dissolved in 95% ethanol before measuring the absorbance at 570 nm (OD_{570}) to quantify total biofilm formation. The growth of cells in the 96-well plates was also measured at OD_{620}. Plant extracts at 0.1 mg/ml^{-1} in four wells with two independent cultures were used for the initial anti-biofilm screening. In subsequent detailed analyses, the results were the average of at least 12 replicate wells.

**Isolation of RNA and real-time qRT-PCR**

To isolate total RNA, cells of *S. aureus* were cultured for 5 h with shaking, before the addition of plant extracts, quercetin, or tannic acid, and incubated for a further 2 h. Before taking samples, RNase inhibitor (RNAlater, Ambion, TX, USA) was added and cells were immediately chilled for 30 s in dry ice and 95% ethanol (to prevent RNA degradation), and centrifuged at 5,000 × g for 3 min. Cell pellets were immediately frozen in dry ice and stored at −80 °C. Total RNA was isolated using a Qiagen RNAeasy mini Kit (Valencia, CA, USA). To remove all DNA, purified RNA was treated for 15 min with 30 units of DNase I. qRT-PCR (quantitative real-time, reverse-transcription PCR) was used to investigate the transcriptions of aus, clp, scpA, splA, sspA, and tssA (protease genes), agrA (quorum-sensing gene), sigB (RNA polymerase sigma factor), sarA (accessory regulator A), polysaccharide intercellular adhesins (icaA, icaD), hla (α-hemolysin gene), and nucleases (nuc1, nuc2). Gene specific primers and 16s rRNA housekeeping gene primers were used (Supplemental Table S2). Real-time qRT-PCR was performed using the StepOne™ Real-Time PCR system (Applied Biosystems, Foster City, CA) and the SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA). At least two independent cultures were used per experiment.

**HPLC assay of quercetin and tannic acid**

Quercetin concentrations were measured by reverse-phase HPLC using a 4.6 × 250 mm ZORBAX Eclipse XDB-C18 column (Agilent Technology, Santa Clara, USA) and elution with a 1% formic acid aqueous solution, acetonitrile, and isopro pyl alcohol (70:22:8 ratio) as a mobile phase at a flow rate of 0.5 ml min^{-1} (Careri et al. 2003). Plant extracts and commercial standards of quercetin and tannic acid (Sigma-Aldrich) were dissolved in methanol and then filtered through a 0.2 μm syringe filter. Under these conditions, the retention time and absorbance maximum of quercetin were 23.9 min and 370 nm, respectively. Tannic acid concentrations were measured by reverse-phase HPLC using a 4.6 × 250 mm Agilent HC-C18 column (Agilent Technology, Santa Clara, USA) and elution with 5% acetic acid as the eluent at 1.5 ml min^{-1} (Mahendran et al. 2006). Under these conditions, the retention time and absorbance maximum of tannic acid were 3.6 min and 270 nm, respectively. The chromatographic peaks of quercetin and tannic acid were identified using retention times and by comparing UV/visible spectra with commercial standards.
Hemolysis assay

The hemolysis analysis was performed as described previously with some modifications (Larzábal et al. 2010). The lysis of human red blood cells was measured using cultures of S. aureus grown in the presence of plant extract, quercetin, or tannic acid. Briefly, S. aureus cells were diluted to 1:100 and cultured in LB medium with or without plant extracts or plant metabolites for 16 h at 250 rpm. Cultures of S. aureus (50 μl including cells and culture supernatant) were added to human red blood cells (previously separated by centrifugation at 900 x g for 5 min and washed (x3) with PBS buffer) and diluted in PBS buffer (330 μl red blood cells per 10 ml of PBS). To determine hemolytic activities, mixtures of blood and S. aureus were incubated at 37 °C for 1 h at 250 rpm. Supernatants were collected after centrifugation (16,600 x g for 10 min) and the ODs at 543 nm were measured.

Results

Screening the anti-biofilm activities of diverse plant extracts

To identify anti-biofilm compounds, methanol extracts from a total of 498 plants (331 genera, 466 species, including 20 different plant parts) were screened in 96-well plates. To minimize antimicrobial effects, 0.1 mg ml⁻¹ of each plant extract was used. Nevertheless, seven plant extracts showed antimicrobial activity. Specifically, the extracts of Callistemon lanceola, Cannabis sativa, Carex siderosticta, Chrysanthemum coronarium var. spathiosum, Magnolia obovata, Mallotus japonica, and Morus bombycis reduced the growth of cells of S. aureus by >50% cf. untreated controls (Supplementary Table S1). However, these seven antimicrobial extracts were not investigated further, because the objective of this study was to identify non-toxic biofilm inhibitors less prone to the development of drug resistance.

Overall, the 498 plant extracts controlled the formation of biofilms of S. aureus with widely different efficiencies (Figure 1). The 72 extracts that inhibited formation by >70% belonged to 59 genera and 38 families. The most active extracts were the methanol extracts of Abius japonica (a medicinal tree), C. lanceola (a hedgerow plant), Phyllanthus urinaria (chamberbitter), Reynoutria sachalinensis (giant knotweed), and Staphylea bumalda (a deciduous tree). These extracts at concentrations of 0.1 mg ml⁻¹ inhibited the formation of biofilm by S. aureus by >90% (Figure 1). The extract of C. lanceola further showed antimicrobial activity (Supplementary Table S1). Detailed information on the growth, and the formation of biofilms of S. aureus in the presence of the 498 plant extracts is provided in Supplementary Table S1.

Extract of A. japonica was used to study the mechanism controlling the inhibition of biofilm formation because it was the most active extract. At 20 μg ml⁻¹ extract of A. japonica inhibited the formation of biofilms of S. aureus by >70%; the formation of biofilm was inhibited in a dose dependent manner after treatment for 12 and 24 h (Figure 2A). In addition, extract of A. japonica effectively inhibited the formation of biofilms of MSSA ATCC 25923 and MRSA ATCC BAA-1707 (Figure 2C and D). In particular, at 5 μg ml⁻¹ extract of A. japonica inhibited the formation of MRSA biofilm by >90% (Figure 2D).

Effect of extract of A. japonica on the growth of S. aureus cells

The growth of cells of S. aureus was investigated in order to identify anti-biofilm compounds without antimicrobial activity. The presence of extract of A. japonica at concentrations up to 20 μg ml⁻¹ did not diminish the cell growth curve of S. aureus ATCC 6538, and at 50 μg ml⁻¹ it slightly delayed cell growth over the first 12 h (Figure 2B). The specific growth rate of S. aureus was 1.41 ± 0.06 h⁻¹ in the absence of plant extract, and 1.41 ± 0.06 and 1.19 ± 0.06 h⁻¹ in the presence of concentrations of 20 and 50 μg ml⁻¹ of extract, respectively. Because 50 μg ml⁻¹ of extract of A. japonica retarded cell growth, 20 μg ml⁻¹ of this extract was used to investigate the mechanism of biofilm inhibition.
Transcriptional changes induced in biofilm-related genes of S. aureus cells by extract of A. japonica

To investigate the genetic basis for the inhibition of biofilm formation, qRT-PCR was performed to observe the differential expressions of biofilm-related genes in cells of S. aureus treated with extract of A. japonica (20 μg ml⁻¹) and the null control. Noticeably, extract of A. japonica most markedly repressed the expression of genes associated with intercellular adhesion (icaA and icaD), the expression of several protease genes (aur, splA, and sspA), agrA (a quorum-sensing gene), and sigB (a virulence factor gene), but did not affect the expression of the housekeeping gene (16S ribosomal RNA) (Figure 3). Since down-regulation of the ica gene is a known mechanism causing an inhibition in the formation of biofilm in S. aureus (Cramton et al. 1999; O’Gara 2007), this result suggests that extract of A. japonica inhibits the formation of biofilm by S. aureus mainly by reducing the expression of the ica gene.

Characteristics of the anti-biofilm compounds in extract of A. japonica

To investigate the characteristics of the active compounds in extract of A. japonica, the extract was heat-treated at 100°C for 10 min. The anti-biofilm activity remained intact (data not shown), indicating that the component(s) responsible were not proteinaceous.

Since the genus Alnus has been reported to produce diverse metabolites, with more than 62 compounds identified (Tung, Kim, et al. 2010; Sati et al. 2011), nine
compounds were selected and screened for anti-biofilm activity against *S. aureus* ATCC 6538. Of the nine compounds, the flavonoid quercetin and tannic acid (both 20 μg/ml) were found to markedly inhibit the formation of biofilms by *S. aureus*, although kaempferol and luteolin (both flavonoids) also decreased the biofilm formation (Figure 4). During the preparation of this paper, it was reported that tannic acid inhibits the formation of biofilms of *S. aureus* (Payne et al. 2013), but the present study is the first to report that quercetin has anti-biofilm activity against this species.

**Quercetin and tannic acid in extract of *A. japonica* inhibited the formation of biofilms of *S. aureus***

The presence of quercetin and tannic acid in extract of *A. japonica* was confirmed by HPLC analysis, which showed that peaks from standard quercetin and standard tannic acid had corresponding peaks in the extract of *A. japonica* (Figure 5A and B). The concentrations of quercetin and tannic acid were 0.7 mg and 93 mg g⁻¹ of extract, respectively.

Further experiments showed that quercetin and tannic acid inhibited the formation of biofilms by the three strains of *S. aureus* in a dose-dependent manner (Figure 5C and D). In particular, quercetin (1 μg ml⁻¹) inhibited biofilm formation by MRSA by >80% and MSSA (ATCC 6538 and ATCC 25923) by >50% (Figure 5C). Tannic acid (20 μg ml⁻¹) inhibited the three *S. aureus* strains from forming biofilms by >50% (Figure 5D). However, the growth of bacterial cells was not affected by 5 μg ml⁻¹ of quercetin or 20 μg ml⁻¹ of tannic acid, although higher concentrations retarded growth slightly (Figure 5E). Furthermore, the effects of quercetin and tannic acid on the expression of genes relating to biofilm formation in *S. aureus* were similar to those of the extract of *A. japonica* (Figure 5F). For example, icaA and icaD were the most repressed (by >10-fold) by extract of *A. japonica*. Also, the quorum-sensing gene

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**Figure 3.** Transcriptional profiles of cells of *S. aureus* treated with extract of *A. japonica* cf. the non-treated control. *S. aureus* ATCC 6538 was cultivated in LB medium with or without extract of *A. japonica* at 20 μg ml⁻¹ for 2 h with shaking at 250 rpm. Transcriptional profiles were measured by qRT-PCR. Relative gene expressions represent transcriptional levels after treatment with extract of *A. japonica* vs non-treated controls (value of 1.0). The experiment was performed in duplicate. Error bars indicate SD.

**Figure 4.** Inhibitory effects of metabolites from *Alnus* sp. on the formation of biofilms of *S. aureus*. The formation of biofilm (OD₅₇₀) of *S. aureus* ATCC 6538 in 96-well plates was quantified in the presence of metabolites of *Alnus* sp. after 24 h without shaking. A minimum of two independent experiments were conducted (total 12 wells). Error bars indicate SD.
agrA, and two virulence-regulatory genes (sigB and sarA) were repressed by quercetin or tannic acid in an analogous manner to the extract of *A. japonica* (Figure 3). These results suggest that quercetin and tannic acid are largely responsible for the anti-biofilm activity of extract of *A. japonica*, and that quercetin is more potent than tannic acid.

**Extract of *A. japonica* and quercetin reduced hemolysis by *S. aureus***

Since *S. aureus* produces α-toxin that causes hemolysis (Song et al. 1996) and contributes to biofilm formation (Caiazza & O’Toole 2003), and because extract of *A. japonica* and its components were found to repress important regulatory genes (*agrA*, *sigB*, and *sarA*)
(Figure 5F), the effects of extract of A. japonica, quercetin, and tannic acid on blood hemolysis by S. aureus were investigated. Interestingly, both quercetin and extract of A. japonica inhibited the hemolysis of human red blood cells by S. aureus in a dose-dependent manner, whereas tannic acid did not (Figure 6). These results suggest that quercetin and extract of A. japonica would reduce the virulence of S. aureus and the ability of the bacterium to form biofilms.

Discussion
This study demonstrates that extracts of various medicinal plants exhibit anti-biofilm activity against S. aureus without inhibiting the growth of cells in planktonic culture. In addition, the study shows that quercetin and tannic acid are the major contributors to the anti-biofilm effect of the most active extract (A. japonica), and provides clues regarding the molecular mechanism involved.

A. japonica is a well-known traditional treatment for various conditions, such as fever, hemorrhage, diarrhea, hepatitis, and alcoholism (Tung, Kwon, et al. 2010; Sati et al. 2011). Furthermore, it has been reported that extract of A. japonica has antioxidant, antiviral, and hepatoprotective properties, and that these properties are due to its diverse phytochemical constituents which include tannins, flavonoids, diarylheptanoids, and triterpenoids (Tung, Kim, et al. 2010; Tung, Kwon, et al. 2010; Sati et al. 2011). Of the 62 known metabolites in A. japonica (Sati et al. 2011), nine were investigated in the present study. Quercetin and tannic acid were found to have high anti-biofilm activity against two strains of MSSA and a strain of MRSA (Figure 5). In addition, like quercetin, extract of A. japonica was found to possess anti-hemolysis activity (Figure 6). Although the exact mechanisms involved were not identified, this is the first report to show that extract of A. japonica and quercetin have anti-biofilm and anti-hemolysis activity.

Quercetin is widespread in the plant kingdom and is one of the most studied flavonoids, having been shown to have antioxidant, antiviral, and carcinostatic properties (Formica & Regelson 1995). Recently, in silico screening of 57,346 compounds found in Chinese medicinal plants demonstrated that the flavonoid fisetin (at below its MIC) was the most effective at inhibiting the formation of biofilms of S. aureus, and interestingly, fisetin was found to be 5-deoxyquercetin (Dürig et al. 2010). Thus, the present study supports previous findings that flavonoids, such as fisetin, quercetin, and kaempferol (Figure 4), have anti-biofilm activity against S. aureus. In addition, it was found previously that several flavonoids, such as, flavone and quercetin reduce hemolysis by S. aureus (Lee et al. 2012). The present study corroborates these findings, demonstrating that the extract of A. japonica, which contains quercetin, reduces the hemolytic activity of S. aureus.

It was suggested recently that tannic acid at 20 μM (34 μg ml⁻¹) inhibits the formation of biofilms of S. aureus using transglycosylase IsaA, and thus, the authors suggested that drinking tea could reduce MRSA nasal colonization (Payne et al. 2013). The present study shows that tannic acid represses the gene expression of the ica operon and other regulatory genes (Figure 5F). Because tannic acid is widespread in plants, other tannic acid-rich plants probably inhibit the formation of biofilms of S. aureus. For example, extract of P. urinaria, which also had anti-biofilm activity (Figure 1), contained 27 mg g⁻¹ tannic acid. However, tannic acid was not detected in extracts of R. sachalinensis or S. bumalda.
(Figure 1), which suggests that these plants have devised different means of inhibiting the formation of biofilms of *S. aureus*.

The genetic mechanism governing the formation of biofilms by *S. aureus* cells is a complex process that involves environmental factors, quorum sensing, protease, DNase, and other global regulators (Boles & Horswill 2011). Down-regulation of the expression of the *ica* gene is the best understood mechanism in the inhibition of biofilm formation (Cramton et al. 1999; O’Gara 2007), and the present observation of down-regulation of *ica* by extract of *A. japonica*, quercetin, or tannic acid supports these previous reports. Furthermore, since the *ica* locus is present in various strains of *Staphylococcus* (Cramton et al. 1999), repression of *ica* could possibly be used to inhibit the formation of biofilms of *S. aureus* and to reduce *S. aureus* infections.

Plants have developed sophisticated defense mechanisms that allow them to survive in their ecosystems, and hence, they represent a rich source of antimicrobial agents and other compounds of pharmaceutical interest (Cowan 1999; Zhao et al. 2005; Li & Vederas 2009). Over the past decade, the efficacies of several plant-derived inhibitors have been investigated to determine their potential for reducing the formation of biofilms of *S. aureus* (Cramton et al. 1999; Kuźma et al. 2007; Stenz et al. 2008; Dürig et al. 2010; Payne et al. 2013) and other pathogenic bacteria. This study demonstrates that various plant extracts, notably quercetin and tannic acid, can inhibit the formation of biofilm by recalcitrant *S. aureus*. This study is one of the most extensive performed to date to identify anti-biofilm agents from plants, and provides comprehensive data on this topic. Furthermore, the results strongly support the notion that plants are an important resource of biofilm inhibitors.

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