

Supplementary Information

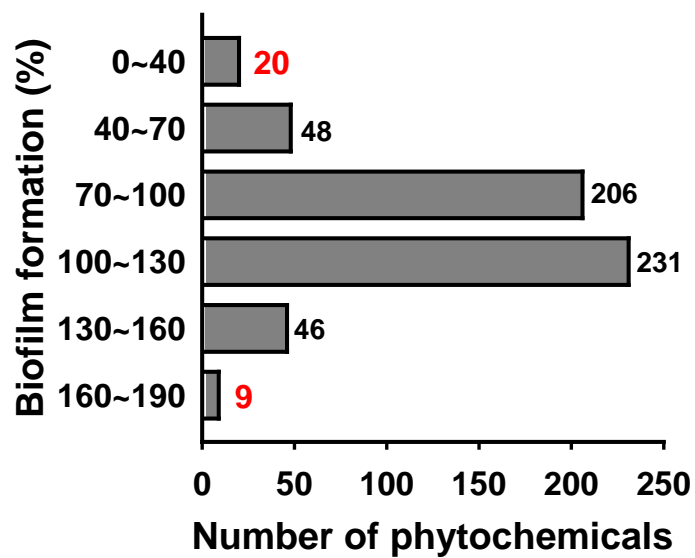
Calcium-chelating alizarin and anthraquinones inhibit biofilm formation and the hemolytic activity of *Staphylococcus aureus*

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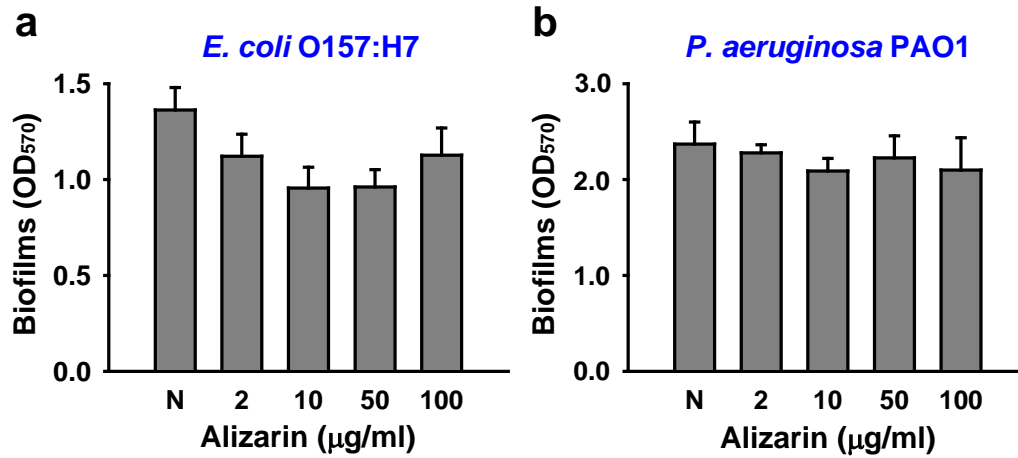
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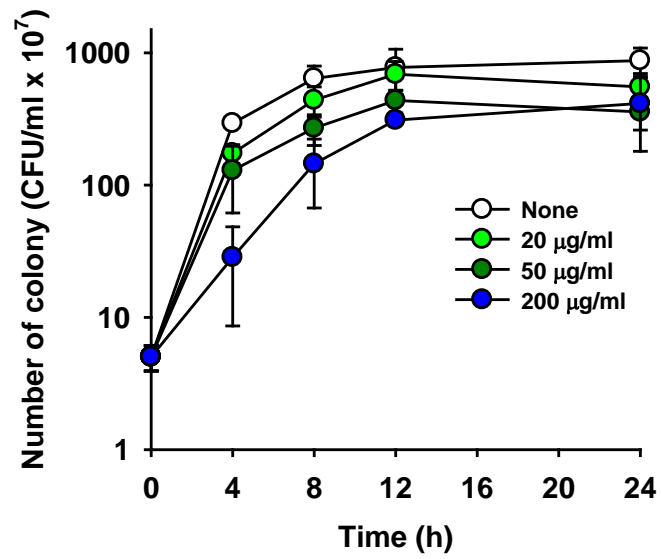
Supplementary Figure S1. Histogram of *S. aureus* biofilm formation in the presence of each of the 560 phytochemicals. Biofilm screening for *S. aureus* MSSA 6538 was performed at a concentration of 10 $\mu\text{g/ml}$ in 96-well plates after culture for 24 h. Numbers on the right indicate numbers of phytochemicals. Biofilm formation (%) on the Y-axis represents changes in biofilm formation, that is, biofilm formation in the presence of a phytochemical is expressed as a percentage of biofilm formation in its absence.



Supplementary Figure S2. Biofilm formation by *Escherichia coli* O157:H7 (a) or *Pseudomonas aeruginosa* PAO1 (b) in the presence of alizarin in LB medium after culture for 24 h. Two independent experiments were conducted (total 12 wells); error bars indicate standard deviations.

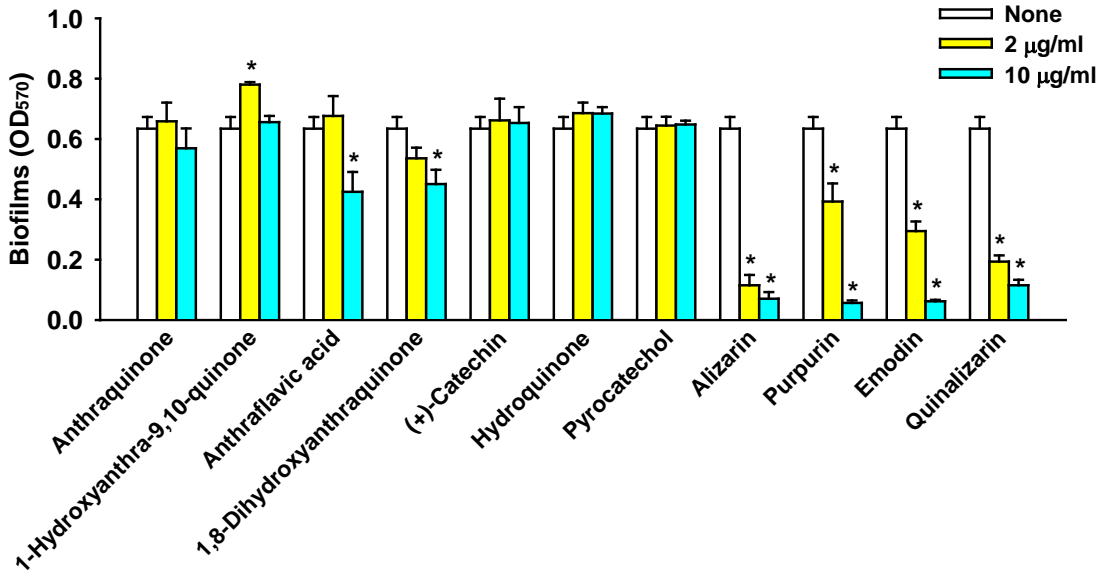


Supplementary Figure S3. Cell growth of *S. aureus* in the presence of alizarin. Planktonic cell growth of *S. aureus* MSSA 6538 was measured by counting CFUs in 250 ml-flasks agitated at 250 rpm.

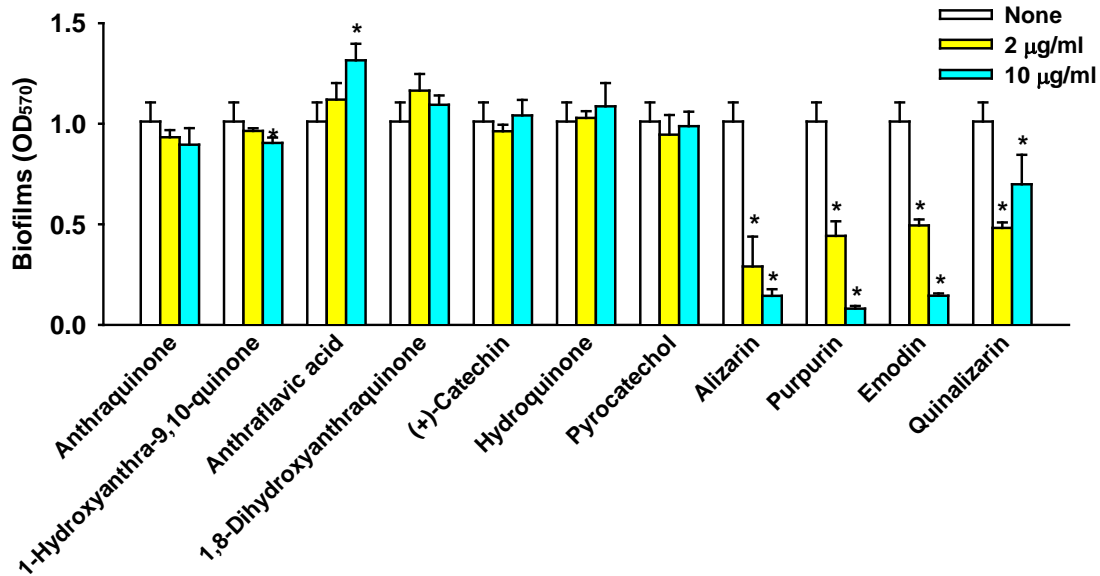


Supplementary Figure S4. Inhibitory effects of alizarin-related compounds on biofilm formation. Biofilm formations by MSSA 25923 (a) and MRSA MW2 (b) were quantified in the presence of the selected compounds at 0, 2, or 10 $\mu\text{g/ml}$ after incubation for 24 h in 96-well plates without shaking. At least two independent experiments were conducted (total 12 wells). Error bars indicate standard deviations. *, $P < 0.05$ versus non-treated controls.

a MSSA 25923



b MRSA MW2



Supplementary Table S1. COMSTAT results for biofilm inhibition by alizarin against three *S. aureus* strains

| Strain | Alizarin | Volume/Area ($\mu\text{m}^3 \mu\text{m}^{-2}$) | Mean thickness (μm) | Substratum coverage (%) |
|---------------|---------------------|---|-------------------------------------|----------------------------|
| MSSA 6538 | None | 12 \pm 1 | 11 \pm 1 | 99 \pm 1 |
| | 2 $\mu\text{g/ml}$ | 7.3 \pm 0.9 | 6.7 \pm 0.7 | 84 \pm 9 |
| | 10 $\mu\text{g/ml}$ | 0.9 \pm 0.2 | 0.7 \pm 0.1 | 32 \pm 9 |
| MSSA 25923 | None | 4.0 \pm 0.5 | 3.8 \pm 0.6 | 88 \pm 10 |
| | 2 $\mu\text{g/ml}$ | 2.5 \pm 0.3 | 2.7 \pm 0.3 | 66 \pm 4 |
| | 10 $\mu\text{g/ml}$ | 0.8 \pm 0.3 | 0.9 \pm 0.3 | 24 \pm 9 |
| MRSA MW2 | None | 5.5 \pm 0.6 | 5.8 \pm 0.9 | 91 \pm 7 |
| | 2 $\mu\text{g/ml}$ | 1.7 \pm 0.3 | 2.8 \pm 0.5 | 35 \pm 5 |
| | 10 $\mu\text{g/ml}$ | 1.6 \pm 0.2 | 1.9 \pm 0.2 | 37 \pm 7 |

Supplementary Table S2. Cell counting in the biofilms affected by alizarin. Biofilms were produced in 96-well polystyrene plates over 24 h with or without alizarin (0, 2, 10, or 100 $\mu\text{g/ml}$) and then washed three times with PBS. Biofilms were then resuspended in 300 μl PBS, disrupted by pipetting vigorously for 60 sec and vortexing for 30 sec, and CFUs were measured. Three independent experiments were conducted.

| Strains | None | Alizarin 2 $\mu\text{g/ml}$ | Alizarin 10 $\mu\text{g/ml}$ | Alizarin 100 $\mu\text{g/ml}$ |
|-----------------------|-------------------------------|--------------------------------|---------------------------------|----------------------------------|
| MSSA 6538 | 159 ± 10 $\times 10^6$ | 86 ± 13 $\times 10^6$ | 21 ± 2 $\times 10^6$ | N/A |
| MSSA 25923 | 167 ± 11 $\times 10^6$ | 102 ± 15 $\times 10^6$ | 49 ± 5 $\times 10^6$ | N/A |
| MRSA MW2 | 41 ± 6 $\times 10^6$ | 10 ± 4 $\times 10^6$ | 4 ± 1 $\times 10^6$ | N/A |
| <i>S. epidermidis</i> | 40 ± 6 $\times 10^6$ | N/A | 12 ± 2 $\times 10^6$ | 5 ± 1 $\times 10^6$ |

Supplementary Table S3. Sequences of the primers used for quantitative RT-PCR.

| Gene | Name | Primer |
|-----------------|-----------------------------------|--|
| <i>16S rRNA</i> | A component of ribosomes | Forward 5'-TGT TTG ACG ATG TTT GAG CA-3' Reverse 5'-CCT TCC TCC AGT TCA GAT GC -3' |
| <i>agrA</i> | Quorum-sensing regulator A | Forward 5'-TGA TAA TCC TTA TGA GGT GCT T-3' Reverse 5'-CAC TGT GAC TCG TAA CGA AAA-3' |
| <i>aur</i> | Zinc metalloproteinase aureolysin | Forward 5'-ACC GTG TGT TAA TTC GTG TGC TA-3' Reverse 5'-ATG GTC GCA CAT TCA CAA GTT T-3' |
| <i>cidA</i> | Holin-like protein | Forward 5'-AGC GTA ATT TCG GAA GCA ACA TCC-3' Reverse 5'-TAC CGC TAA CTT GGG TAG AAG ACG-3' |
| <i>cidB</i> | Holin-like protein | Forward 5'-TGT TTT TGT TGA CTG TCG TT-3' Reverse 5'-TCA TGT GAC ACT TCG ATA CC-3' |
| <i>cidR</i> | LysR-type regulator | Forward 5'-TGG TGC ATT CCA TCA ACA AT-3' Reverse 5'-ATT TTG CGA GTC GAT GCT CT-3' |
| <i>clfB</i> | Clumping factor B | Forward 5'-TGC AAG ATC AAA CTG TTC CT-3' Reverse 5'-TCG GTC TGT AAA TAA AGG TA-3' |
| <i>clp9</i> | Serine protease | Forward 5'-CAG GTA CCA TCA CTT CAT C-3' Reverse 5'-GGT TCA CAA ATT GAT GAC AAC G-3' |
| <i>coa</i> | Coagulase | Forward 5'-CAC GGA AAT GGC CAA GTA TC-3' Reverse 5'-TCG GAC GAG CTC CAT ATG AT-3' |
| <i>hla</i> | α -Hemolysin | Forward 5'-CGG CAC ATT TGC ACC AAT AAG GC-3' Reverse 5'-GGT TTA GCC TGG CCT TCA GC-3' |
| <i>icaA</i> | Intercellular adhesion A | Forward 5'-TGA ACC GCT TGC CAT GTG-3' Reverse 5'-CAC GCG TTG CTT CCA AAG A-3' |
| <i>icaD</i> | Intercellular adhesion D | Forward 5'-ACC CAA CGC TAA AAT CAT CG-3' Reverse 5'-GCG AAA ATG CCC ATA GTT TC-3' |
| <i>icaR</i> | Intercellular locus regulator | Forward 5'-TCG AAC TAT TCA ATT GAT GCT TTA-3' Reverse 5'-CAG AAA ATT CCT CAG GCG TA-3' |
| <i>isaA</i> | Transglycosylase | Forward 5'-GCT CAA ATC ATG GCT CAA CGT-3' Reverse 5'-TTG ATT CAC GAG CGA TGA TTG-3' |
| <i>lrgA</i> | Anti-hollin-like | Forward 5'-TGA AAC AAC AAA AAG ACG CAT CAA AAC CAG-3' |

| | | |
|-------------|----------------------------------|---|
| | protein | Reverse 5'-ACT TCG CCT AAC TTA ACA GCA CCA G-3' |
| <i>lrgB</i> | Anti-hollin-like protein | Forward 5'-TCG GAG GTA TTG GTA TCG-3' Reverse 5'-CTG CTT GAG GTA ACA TTG A-3' |
| <i>nuc1</i> | Nuclease | Forward 5'-CAC CTG AAA CAA AGC ATC CTA A-3' Reverse 5'-TAT ACG CTA AGC CAC GTC CAT-3' |
| <i>nuc2</i> | Nuclease | Forward 5'-ATG GAC GTG GCT TAG CGT AT-3' Reverse 5'-TGA CCT GAA TCA GCG TTG TC-3' |
| <i>psma</i> | Phenol soluble modulins α | Forward 5'-ACC CAT GTG AAA GAC CTC CTT TGT-3' Reverse 5'-ATG GGT ATC ATC GCT GGC ATC-3' |
| <i>rbf</i> | Regulator of biofilm formation | Forward 5'-TTA GAA GGA ATC TTT AAA ACC TTA TTG AAT AA-3' Reverse 5'-TTG TGA ATT TTT CTT CTT CGG ACA-3' |
| <i>sarA</i> | Transcriptional regulator | Forward 5'-GAG TTG TTA TCA ATG GTC-3' Reverse 5'-GTT TGC TTC AGT GAT TCG-3' |
| <i>sigB</i> | RNA Polymerase sigma factor | Forward 5'-AAG TGA TTC GTA AGG ACG TCT-3' Reverse 5'-TCG ATA ACT ATA ACC AAA GCC T-3' |
| <i>spa</i> | Protein A | Forward 5'-ACC AGA AAC TGG TGA AGA AAA TCC-3' Reverse 5'-TAA CGC TGC ACC TAA GGC TAA TG-3' |