

BACKGROUND AND OBJECTIVES

Biofilms, structured communities of bacteria embedded in a matrix of extracellular polymeric substances (EPS), are involved in 80% of all bacterial infections and responsible for majority of recalcitrant, antibiotic-resistant infections. However, there is only a very limited selection of compounds that can selectively act on existing biofilms and effectively eradicate them at low concentrations. Furthermore, no antibiotic and only one biocide have been accepted by a regulatory agency to be used specifically against biofilms. Hence, the need for new anti-biofilm compounds is immense [1, 2].

Flavonoids, plant secondary metabolites, are one of the most extensively studied classes of natural products with various biological activities. The antibacterial properties of flavonoids are widely reported while fewer studies focus on anti-biofilm activity. Moreover, the data from previously published studies is often conflicting due to differences in experimental conditions [3]. Therefore, the objective of this work was to systematically screen a commercial collection of 500 natural and synthetic flavonoids for inhibitory activity against *Staphylococcus aureus* biofilms. Additionally, an improved methodological workflow for anti-biofilm screens taking into account connections between anti-biofilm and antibacterial properties was developed and applied here.

EXPERIMENTAL SECTION

WORKFLOW AND SELECTION CRITERIA

Anti-biofilm activity of flavonoids collection was explored utilizing a two-phased phenotypic high throughput screening (HTS) platform, previously optimized for screening of natural compound libraries [4-6]. Thereafter, a selection process combining activity and selectivity was applied and two synthetic flavans were identified as the most promising flavonoids (leads) [7] (Figure 1).

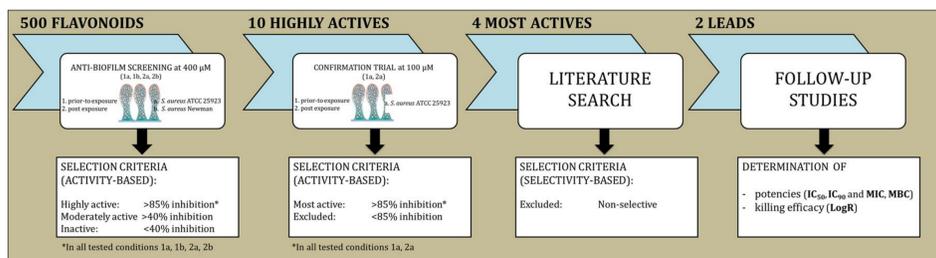


Figure 1. Schematic representation of the anti-biofilm screening process and selection criteria applied to this work.

METHODS

Inhibitory activity based on biofilm viability and total biomass was determined with resazurin staining and crystal violet staining assay, respectively (a, b). Literature search was performed using PubMed search engine and PubChem Bioassays (c). Anti-biofilm and antibacterial potencies (IC₅₀, IC₉₀ and MIC values, respectively) were defined on the basis of resazurin assay (a), while MIC values were estimated by turbidity measurements (d). Killing efficacy was measured with LogR (reduction): difference of log₁₀ density (CFU/ml) in control and treated wells utilizing plate count method (e).



Figure 2. Methods used in this work. From left to right resazurin assay (a), crystal violet staining assay (b), literature search (c), turbidity measurements (d) and plate count method (e).

CONCLUSIONS

- ✓ This work provides a large amount of new bioactivity data of flavonoids and identifies several new flavonoids with anti-biofilm activity
- ✓ Two synthetic flavans were identified and characterized as the most potent antimicrobials combining anti-biofilm and antibacterial properties
- ✓ Because connections between antibacterial and anti-biofilm effects were taken into account, this work could serve as a model for future studies of natural compounds known to be antibacterial

REFERENCES

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[2] Donlan RM & Costerton JW. 2002 *Clin Microbiol*, 15:167-93. [6] Skogman ME, et al. 2012 *J Antibiot (Tokyo)*, 65:453-459.
[3] Cushnie TP & Lamb AJ. 2011 *Int J Antimicrob Agents*, 38:99-107. [7] Manner S, et al. 2013 *Int J Mol Sci*, 14:19434-51.
[4] Sandberg M, et al. 2008 *Int J Antimicrob Agents*, 32:233-240.

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RESULTS

1. ANTI-BIOFILM SCREENING

On the basis of results obtained in the first screening (Figure 3), flavonoids were classified as inactive (443), moderately active (47) and highly active (10) (Figure 4). Results from the primary screening and reconfirmation trial of the highly active flavonoids are shown in Table 1.

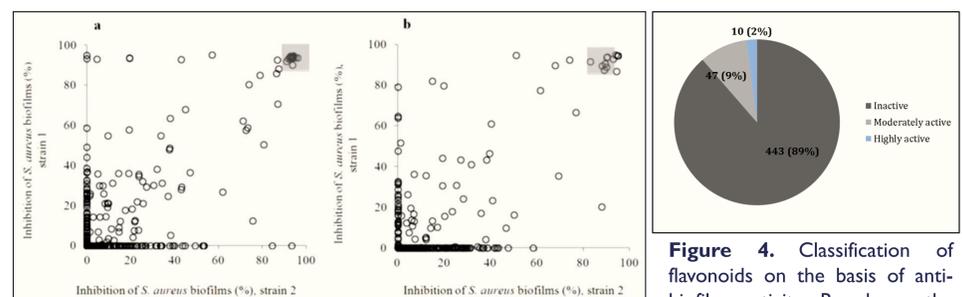


Figure 3. Inhibitory activity (based on biofilm viability) of the entire collection when added prior-to (a) and post-biofilm formation (b) against two strains of *S. aureus*. Strains 1 and 2 refer to *S. aureus* ATCC 25923 and Newman, respectively. Highly active flavonoids are presented in both shadowed areas.

Figure 4. Classification of flavonoids on the basis of anti-biofilm activity. Based on the amount of compounds identified as highly active, the calculated overall hit rate was 2%.

| Code | Company ID | Class | Inhibition of biofilm formation (%) | | | | | |
|------|------------|------------|-------------------------------------|------------------------|----------------------------|------------------------|--------------------------------|------------------------|
| | | | Primary screening at 400 µM | | | | Reconfirmation trial at 100 µM | |
| | | | Prior-to-exposure strain 1 | Post-exposure strain 1 | Prior-to-exposure strain 2 | Post-exposure strain 2 | Prior-to-exposure strain 1 | Post-exposure strain 1 |
| 33 | ST014848 | isoflavone | 93.8 | 93.8 | 94.5 | 94.9 | 19.7 ± 5.6 | 0 |
| 59 | ST024709 | flavanone | 94.1 | 92.6 | 94.8 | 94.0 | 95.5 ± 0.1 | 92.5 ± 3.2 |
| 139 | ST056204 | flavanone | 93.8 | 95.1 | 86.9 | 94.3 | 95.0 ± 0.1 | 93.6 ± 1.1 |
| 291 | ST075672 | flavan | 93.2 | 92.6 | 94.6 | 94.7 | 95.4 ± 0.1 | 94.1 ± 1.2 |
| 369 | ST081006 | flavan | 93.6 | 96.1 | 93.9 | 90.3 | 95.3 ± 0.2 | 93.3 ± 1.7 |
| 424 | ST092293 | chalcone | 93.8 | 94.3 | 93.7 | 90.2 | 15.9 ± 5.1 | 0 |
| 432 | ST093738 | flavan | 94.1 | 94.0 | 89.4 | 88.1 | 0 | 0 |
| 446 | ST098360 | flavanone | 92.0 | 91.2 | 92.9 | 92.9 | 0 | 0 |
| 464 | ST095411 | chalcone | 93.7 | 93.7 | 90.5 | 89.4 | 0 | 0 |
| 469 | ST095417 | chalcone | 94.8 | 94.1 | 88.8 | 90.0 | 0 | 0 |

Strains 1 and 2 refer to *S. aureus* ATCC 25923 and Newman clinical strains, respectively.

Table 1. List of the highly active flavonoids selected based on the initial screening results and results from the reconfirmation test performed for further selection of the most potent leads. Strains 1 and 2 correspond to *S. aureus* ATCC 25923 and Newman, respectively. The most actives are located within the red box.

2. FOLLOW UP-STUDIES OF THE LEAD FLAVONOIDS

Upon the literature search two synthetic flavans (Figure 5) were identified as the most selective and follow-up inhibition and efficacy studies were conducted (Tables 2 & 3).

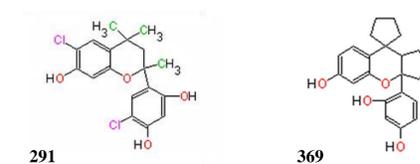


Figure 5. Structures of the selected lead compounds of flavan class.

Table 2. Anti-biofilm potencies and effects on suspended bacteria of the lead compounds.

| Compound | Effects on biofilms µM (µg/ml) | | | Effects on suspended bacteria µM (µg/ml) | |
|----------|--------------------------------|---------------|-------------------|--|-----------|
| | IC ₅₀ | | IC ₉₀ | MIC | MBC |
| | Prior-to-exposure | Post-exposure | Prior-to-exposure | | |
| 291 | 10.2 (3.8) | 27.9 (10.3) | 13 (4.6) | 20 (7.4) | 15 (5.5) |
| 369 | 17.7 (6.2) | 60.5 (21.3) | 40 (14.1) | 40 (14.1) | 40 (14.1) |

Anti-biofilm and antibacterial potencies (Table 2) were defined. They indicate that both compounds prevent biofilm formation likely by killing planktonic bacteria. Additionally, both compounds can affect specific biofilm processes because they were found to be active also in post-exposure mode of assay.

Table 3. Results from LogR assay.

| Compound | Concentration µM | Log Reduction (Biofilms) | Log Reduction (Planktonic) |
|----------|------------------|--------------------------|----------------------------|
| 291 | 20 | 0.6 | 0.1 |
| | 80 | 1.5 | 0.7 |
| | 200 | 3.5 | 3.5 |
| | 400 | 4.6 | 4.7 |
| 369 | 50 | 1.5 | 1.2 |
| | 100 | 3.9 | 4.1 |
| | 250 | 3.1 | 9.0 |
| | 400 | 3.9 | 9.0 |

Further, the killing efficacy was quantified in both lifestyles (Table 3). Compound 291 was confirmed to display equal antibacterial and anti-biofilm activity, whereas compound 369 was found to be more effective against bacteria in planktonic phase with lesser activity against biofilm bacteria.