UNIVERSITY OF HELSINKI

https://helda.helsinki.fi

Screening of natural compounds identifies ferutinin as an antibacterial and anti-biofilm compound

Gilbert-Girard, Shella; Reigada, Inés; Savijoki, Kirsi; Yli-Kauhaluoma, Jari; Fallarero, Adyary

2021-08-30

Taylor and Francis Ltd.

http://hdl.handle.net/10138/347551

Gilbert-Girard, S, Reigada, I, Savijoki, K, Yli-Kauhaluoma, J & Fallarero, A 2021, 'Screening of natural compounds identifies ferutinin as an antibacterial and anti-biofilm compound', Biofouling, vol. 37, no. 7, pp. 791-807. https://doi.org/10.1080/08927014.2021.1971655

Downloaded from Helda, University of Helsinki institutional repository. https://helda.helsinki.fi

This is an electronic reprint of the original article.

This reprint may differ from the original in pagination and typographic detail.

Please cite the original version.

Screening of natural compounds identifies ferutinin as an antibacterial and anti-biofilm compound

Shella Gilbert-Girard[a] Inés Reigada[a] Kirsi Savijoki[a] Jari Yli-Kauhaluoma[b] Adyary Fallarero[a]

[a]*Drug Research Program, Division of Pharmaceutical Biosciences, Faculty of Pharmacy, University of Helsinki, Helsinki, Finland;*

[b]*Drug Research Program, Division of Pharmaceutical Chemistry and Technology, Faculty of Pharmacy, University of Helsinki, Helsinki, Finland*

Supplemental data for this article is available online at https://doi.org/10.1080/08927014.2021.1971655

CONTACT: Shella Gilbert-Girard, shella.gilbert-girard@helsinki.fi

This is an Accepted Manuscript of an article published by Taylor & Francis in Biofouling on August 30th 2021, available online: https://www.tandfonline.com/doi/full/10.1080/08927014.2021.1971655.

Abstract

Antibacterial screenings are most commonly targeted at planktonic bacteria but less effort is dedicated to the exploration of agents acting on biofilms. Here, a natural compounds library was screened against *Staphylococcus aureus* using a 384-well plate platform to identify compounds preventing biofilm formation. Five structurally diverse hits were selected for follow-up studies: honokiol, tschimganidin, ferutinin, oridonin and deoxyshikonin. The compounds were evaluated against different bacterial species for their capacity to prevent and disrupt biofilms. The development of resistance and cytotoxicity were also investigated. Ferutinin displayed the best antibacterial activity, with a minimum inhibitory, bactericidal and biofilm preventive concentration of 25 µM against *S. aureus*. It efficiently disrupted pre-formed biofilms (over 5-log reduction of viable cells) and reduced biofilm formation on a catheter in presence of neutrophils. This work provided new information on the antibacterial activity of five natural compounds and identified ferutinin as a promising candidate against *S. aureus* biofilms.

Keywords: antibacterial screening; anti-biofilm; ferutinin; natural products; *Staphylococcus aureus*

Introduction

One of the most important challenges of modern medicine is the increasing number of pathogenic multi-drug resistant bacteria, which are becoming a major threat to patients in healthcare worldwide (Ferri et al. 2017; Mantravadi et al. 2019; Breijyeh et al. 2020). The remarkable capacity of some bacterial species to develop resistance, the overuse of antibiotics and the lack of research for new antibiotics by large pharmaceutical companies in the last decades have led to an urgent need of new antibacterial drugs and therapeutic options (Hug et al. 2018; Mantravadi et al. 2019; Wang et al. 2019). In addition to this multifaceted problem, bacterial infections can be intrinsically very difficult to treat once a biofilm is formed (Uruén et al. 2020). Biofilms are estimated to be responsible for over 80% of all microbial infections and are particularly prevalent in chronic wound infections and medical device-related infections that are tolerant to antimicrobial therapy (Omar et al. 2017; Del Pozo 2018; Penesyan et al. 2019). A biofilm-related infection of a medical device can typically only be treated by the surgical replacement of the device and recurrence of infection tends to be high (Arciola et al. 2012; Zimmerli and Moser 2012; Davidson et al. 2019). Despite the high prevalence of biofilms in infections, antibiotics

have conventionally been developed to treat planktonic cells and susceptibility of bacteria in discovery programs for new antibacterial compounds is still often measured using only planktonic cells (Omar et al. 2017). The exploration of antimicrobials from natural sources has been a traditionally successful discovery strategy, given the obvious role that secondary metabolites play in the natural defence of organisms, especially in the case of microorganisms. Natural compounds are regarded as a significant source of unique and diverse chemical structures with untapped biological activities. Many of the antibiotics currently in clinical use were derived or originally discovered from natural sources (Silver 2011). In hope to discover new ones, the last 20 years have seen a great increase in antibacterial research on natural compounds as technological improvements have been made in culture and screening methods (Mantravadi et al. 2019; Castronovo et al. 2021). Given the widespread presence of bacterial biofilms in nature, it can also be expected that anti-biofilm compounds can be successfully found from natural sources. To that end, efforts on the field of anti-biofilm screening utilizing chemical libraries of natural compounds can hold real promise.

Here, in order to identify compounds not only with antibacterial activity, but also anti-biofilm effects, a chemical library containing 500 natural compounds was screened against *Staphylococcus aureus* using a biofilm-based platform previously optimized in 384-well plates (Gilbert-Girard et al. 2020a). Five structurally different natural compounds: honokiol, tschimganidin, ferutinin, oridonin and deoxyshikonin, were initially identified as hits, and follow-up studies were carried out to characterize the antibacterial and anti-biofilm effects of these compounds.

Material and Methods

Bacterial strains and culture conditions

S. aureus ATCC 25923 and *Staphylococcus epidermidis* RP62A (ATCC 35984) were provided by the Faculty of Pharmacy of the University of Helsinki (Helsinki, Finland) and *Escherichia coli* K12 by the Faculty of Agriculture and Forestry (HAMBI collection) of the University of Helsinki (Helsinki, Finland). *Acinetobacter baumannii* NCTC 13423 was bought from the National Collection of Type Culture (NCTC) (Salisbury, UK). Both *Pseudomonas aeruginosa* strains (ATCC 15442 and ATCC 9027) were purchased from the American Type Culture Collection (ATCC; Wesel, Germany). *P. aeruginosa* was

grown in Lennox broth (LB; Lab M Ltd., Heywood, UK) while the other species were grown in tryptic soy broth (TSB; Lab M Ltd., Heywood, UK). Prior to all experiments, bacteria were plated on tryptic soy agar (TSA; Lab M Ltd., Heywood, UK), or LB agar (Lab M Ltd., Heywood, UK) for *P. aeruginosa*, from a frozen glycerol stock and grown overnight at 37 °C. Colonies were added to 5 ml of liquid media and grown to exponential phase at 37 °C with shaking (220 rpm). *S. aureus* was diluted to a concentration of 7×10^4 CFU ml^{-1} for the screening and prior to all other experiments, bacteria cultures were diluted to 1×10^6 CFU ml^{-1}.

Chemical library and compounds

The chemical library screened was the Screen-Well Natural Product Library Version 7.1 (ENZO Life Sciences, Helsinki, Finland), which contains 500 isolated natural compounds and derivatives, belonging to different structurally diverse classes such as alkaloids, coumarins, flavones, macrolides, peptolides and terpenoids. According to the manufacturer claims, all compounds have a purity of at least 85% (above 90% for most compounds). Prior to screening, all compounds (provided in 2 mg ml^{-1} DMSO stock solutions) were diluted in DMSO ≥ 99.9% (34943, Sigma–Aldrich, St. Louis, MO, USA) to a concentration of 2 mM. The antibiotics used for cell culture (penicillin and streptomycin) were bought from Sigma-Aldrich (St. Louis, MO, USA). For follow-up studies, honokiol was purchased from Sigma-Aldrich (St. Louis, MO, USA), oridonin and deoxyshikonin were acquired from Carbosynth (Compton, UK) while tschimganidin and ferutinin were bought from Latoxan (Portes-lès-Valence, France). All compounds used in follow-up studies had a purity of at least 95%.

Screening of the chemical library

The screening was performed against *S. aureus* in flat-bottom transparent 384-well plates (242757, Thermo Scientific, Waltham, MA, USA) in a volume of 40 µl per well using a platform based on the combined viability and biomass measurements of the biofilms within one same plate that has been optimized in our previous study (Gilbert-Girard et al. 2020a). Planktonic cells were separated from the biofilms and their turbidity and viability were measured as well. At the start of the experiment, 1 µl of each compound (2 mM) was added per well for a final concentration of 50 µM (2.5% DMSO final concentration), along with 39 µl of the bacterial suspension at 7×10^4 CFU ml^{-1}. The plates were

incubated for 18 h at 37 °C with shaking (220 rpm). The planktonic cells were then transferred to a new plate and their optical density (OD) was measured at 595 nm using a Varioskan LUX Multimode microplate reader (Thermo Scientific, Waltham, MA, USA). After this, 2 µl of a 400 µM resazurin solution (Sigma-Aldrich, St. Louis, MO, USA), diluted in phosphate-buffered saline (PBS) (Thermo Scientific, Waltham, MA, USA), was added to the wells (final concentration of 20 µM). The planktonic plate was incubated for a maximum of 5 min at room temperature (RT) in darkness. Fluorescence of the plates was measured $\lambda_{excitation}$ = 560 nm and $\lambda_{emission}$ = 590 nm using the top optics of the Varioskan LUX Multimode microplate reader. The planktonic plate was then discarded. The biofilms in the original plates were washed once in PBS and 40 µl of a 20 µM resazurin solution (in PBS) were added to the wells. After a 40-min incubation in darkness at RT with shaking (220 rpm), fluorescence was measured as with the planktonic cells. The resazurin solution was then removed and the biofilms were fixed with ethanol 100% for 15 min. After removing the ethanol, the biofilms were left to completely air-dry and were then stained for 5 min with 40 µl of crystal violet 0.023% (commercial stock solution diluted 1:100 in deionized water; Sigma-Aldrich, St. Louis, MO, USA). Next, the biofilms were washed twice with 50 µl of deionized water and left to dry for 10 min before the crystal violet dye was solubilized with 40 µl of ethanol 100% for 1 h. Absorbance was measured at 595 nm using a Multiskan Sky microplate spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Pre-exposure experiments (antibacterial and biofilm inhibitory activity)

The following pre-exposure experiments were carried out to investigate the inhibitory activity of the compounds against planktonic cells and biofilms of staphylococci and Gram-negative bacteria (*E. coli*, *A. baumannii* and *P. aeruginosa*) using the same platform as for the screening, with a few variations. Like previously, compounds were added directly with the bacteria and their activity was measured after 18 h. However, the bacterial suspension was adjusted to a concentration of 1×10^6 CFU ml^{-1} and added to flat-bottom transparent 96-well plates (161093, Thermo Scientific, Waltham, MA, USA) in a volume of 198 µl per well with 2 µl of compounds solubilized in DMSO at concentrations ranging from 12.5-200 µM (final concentration of 1% DMSO in the wells). The activity of the compounds was measured using the same procedures as for the screening, but in a working volume of 200 µl, rather than 40 µl. The conditions of the

resazurin staining of the biofilms were adapted to each species as the Gram-negative bacteria used here require longer incubation times and a different temperature to reach an optimal fluorescence signal, as previously described (Gilbert-Girard et al. 2020a, 2020b). The incubation time of the resazurin with the biofilms was 30 min with the staphylococci at RT, 1 h with *A. baumannii* at 37 °C or 1 h 30 min with *E. coli* and *P. aeruginosa* at 37 °C. The minimum inhibitory concentration (MIC) of each compound was determined from these experiments as the lowest concentration where no growth was visible. Of note, throughout all experiments, an inhibitory or disruptive activity lower or equal to 20% was not considered of consequence as the solvent (DMSO) control often reached that value.

The minimum bactericidal concentration (MBC) and the biofilm prevention concentration (BPC) were assessed using viable colony-forming-units (CFUs) counts, after following the same protocol (bacteria incubated with compounds for 18 h). MBC and BPC are defined here as the lowest concentration where a reduction of at least 99.9% of the number of CFU was observed in either the planktonic solution (MBC) or on the bacteria attached to the wells (BPC). For the MBC, in wells where no growth (planktonic or adherent) was visible, 50 µl of planktonic solution was transferred onto TSA. After removal of the 50 µl for MBC assessment, the wells were emptied and washed once with PBS. A volume of 50 µl of PBS was added to the wells and the cells potentially adhered to the bottom of the wells were scrapped with a pipette tip, after what the 50 µl were transferred onto TSA. Viable cells were counted after an overnight incubation at 37 °C.

Post-exposure experiments (biofilm-eradication)

For post-exposure experiments, a similar procedure was used as for the pre-exposure experiments, but with the compounds being tested on pre-formed biofilms. The bacteria (1×10^6 CFU ml^{-1} in 200 µl per well) were first incubated at 37 °C in 96-well plates with shaking (220 rpm) without compounds for 18 h to allow the formation of biofilms. Afterwards, the media was carefully refreshed (198 µl), and 2 µl of compounds (for 1% DMSO in the wells) were added to the biofilms for an additional incubation period of 24 h in the same conditions. To assess the compounds' capacity to eradicate the pre-formed biofilms, the planktonic solution was removed and the same combination of assays (resazurin and crystal violet staining) was used on the biofilms, as described for the inhibition experiments.

The effect of the compounds on pre-formed biofilms was further quantified using viable plate count (CFU counts). The experiments were started in the same way as the previous post-exposure experiments. After the 24-h incubation of the 18 h-old biofilms with the compounds, the planktonic solution was removed from the wells and the biofilms were washed once with 200 µl of PBS before being scraped in 100 µl of PBS. The bacteria were then diluted serially and 10 µl drops were plated on TSA. Colonies were counted after an overnight incubation at 37 °C.

Resistance development study

Resistance development was investigated as previously described (de Breij et al. 2018; Gilbert-Girard et al. 2020b). *S. aureus* was grown in TSB overnight and then diluted to 1 × 10^6 CFU ml^{-1} and 200 µl were added per well in a 96-well plate containing the compounds at their MIC or 0.5 × MIC. After a 24-h incubation at 37 °C with shaking (220 rpm), bacterial growth was assessed visually. A volume of 10 µl was transferred from the well with visible growth with the highest concentration of each compound (either 0.5 × MIC or the MIC, if resistance was developed) into two wells containing 190 µl of fresh TSB. Fresh compounds were also added at the same concentrations as previously or 2 folds higher if the MIC had increased. The procedure was repeated sequentially in the same manner until 20 days were reached.

Cell lines and culture conditions

All cell lines were maintained in a humidified incubator at 37 °C in 5% CO_2. The human lung adenocarcinoma epithelial cells A549 (CCL-185, ATCC, Wesel, Germany) were grown in Dulbecco's Modified Eagle Medium (DMEM; Lonza, Basel, Switzerland) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), 2 mM glutamine (Sigma-Aldrich, St. Louis, MO, USA) and 20 µg ml^{-1} gentamycin (Sigma-Aldrich, St. Louis, MO, USA). The cervical adenocarcinoma HeLa-derived human epithelial type 2 (HEp-2) cells (CCL-223, ATCC, Wesel, Germany) were grown in DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10 % (v/v) heat-inactivated FBS and 20 µg ml^{-1} gentamycin. HL-60 (ATCC CCL-240) cells were grown and maintained in Roswell Park Memorial Institute (RPMI) 1640 Medium (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 20% (v/v) heat-inactivated FBS and 1% (v/v) penicillin/streptomycin. Before each experiment, HL-60 cells were

differentiated into neutrophil-like cells by 6 days of incubation in the maintenance medium supplemented with 100 mM of *N*,*N*–dimethylformamide (DMF; Sigma-Aldrich, St. Louis, MO, USA) (Collins et al. 1978). The success of the differentiation was assessed visually after Giemsa staining using a Leica DMLS microscope (Leica, Wetzlar, Germany).

Cytotoxicity studies

For the cytotoxicity assessment of the identified hit compounds, the two epithelial cell lines (A549 and HEp-2) were first seeded in 96-well plates at a density of 1×10^4 cells per well in a volume of 200 µL per well and were allowed to attach to the wells for 24 h. The media was changed and the compounds were added at final concentrations of 3.125 to 50 µM (final concentration of 0.25% DMSO). Simultaneously, the HL-60 were also seeded in the 96-well plates at a density of 1×10^4 cells per well in 200 µl with the compounds. Untreated cells were used as negative controls and cells treated with 1% TritonX-100 (Sigma-Aldrich, St. Louis, MO, USA) were used as positive controls for cytotoxicity. All cells were then incubated with the compounds for 22 h. For the adherent cells, the media was removed, and the cells were washed once with PBS. Then, 200 µl of 20 µM resazurin were added per well and cells were incubated for 2 h more before measurement of the fluorescence as mentioned earlier. For the HL-60, 10 µl of 400 µM resazurin were added directly to the wells (final concentration of 20 µM resazurin) and the cells were incubated for 4 h more before measurement of the fluorescence. The half-maximal inhibitory concentration (IC_{50}), corresponding to a 50% inhibition of the viability, was calculated for the A549 and HEp-2 cells from the results of 4 experiments, each with 2 replicates, and for and HL-60 from 2 experiments, each with 2 replicates.

Biofilm prevention in a co-culture of bacteria and neutrophils

The compounds were tested in a *S. aureus* and HL-60 cells co-culture system as previously described (Reigada et al. 2020). Briefly, the media of the differentiated HL-60 cells was first refreshed with RPMI 1640 (10% FBS) the day before the experiment to remove possible traces of antibiotics. The next day, the bacteria were prepared as described above in TSB before being centrifuged at 4500 × g for 10 min, washed twice in PBS and diluted to a concentration of 2×10^7 CFU ml^{-1} in RPMI 1640. The HL-60 cells were counted and adjusted to a concentration of 2×10^5 cells ml^{-1} in RPMI 1640

(10% FBS). The bacteria culture and the HL-60 cells (500 µl of each) were added on sections of 1 cm of a sterilized fine bore low-density polyethylene (LDPE) tubing (Smiths Medical ASD, Minneapolis, MN, USA) in flat-bottom transparent 24-well plates (Thermo Scientific, Waltham, MA, USA). A volume of 2.5 µl of the compounds (tschimganidin and ferutinin), diluted in DMSO, was added to the wells–for final concentrations of 25 and 50 µM (0.25% DMSO). Co-culture controls and bacteria mono-culture controls with no added compound were included as well. After a 24-h incubation in the same conditions as for cell maintenance, the media was removed and the LDPE tubes were gently washed with 1 ml of TSB and transferred to Eppendorf tubes containing 1 ml of 0.5% (w/v) Tween 20 - TSB solution. The tubes were vortexed for 20 s, sonicated at RT for 5 min using a Branson 3800 ultrasonic bath (Cleanosonic, Richmond, VA, USA) at 35 kHz and vortexed again. The bacterial suspensions from each tube were serially diluted in PBS and plated on TSA plates for CFU count after an overnight incubation at 37 °C.

Statistical analysis

The performance of the screening assay was monitored by the calculation of the screening window coefficient (Z') as described in (Zhang JH et al. 1999). For multiple comparisons, one-way ANOVA with the Welch correction (in case of unequal variance) and a post-hoc Dunnet test were used. In other cases, unpaired Student's t-tests were used. The IC_{50} on human cell lines were calculated as the concentration inhibiting 50% of the cell's viability using GraphPad Prism v. 8.00 with a 95% confidence interval.

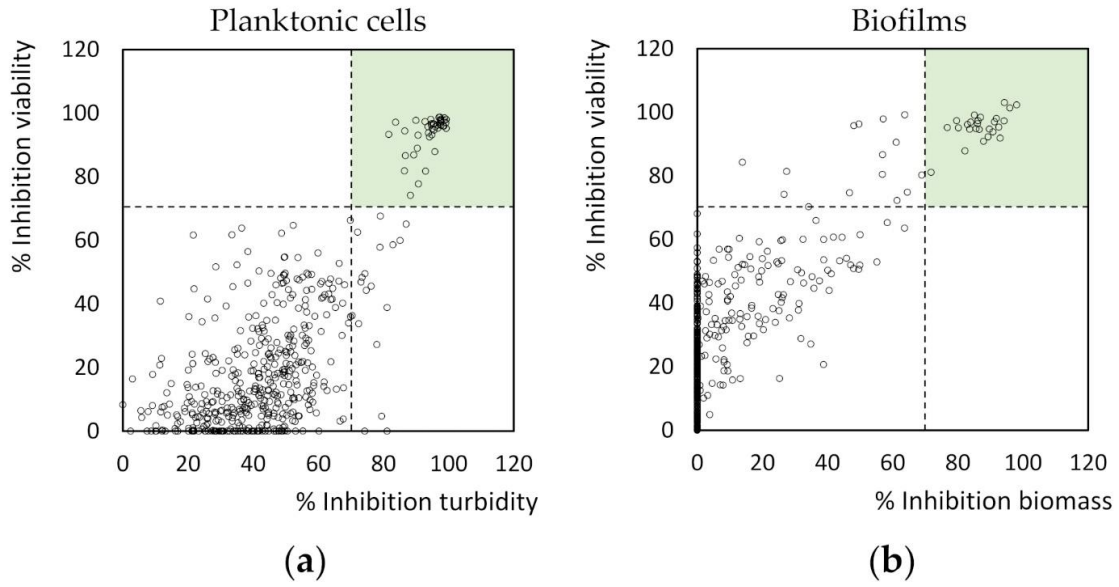
Results

Screening of a natural compounds library

The 500 natural compounds were screened at 50 µM for their capacity to prevent *S. aureus* planktonic growth and biofilm formation using a 384-well plate platform optimized in our previous work (Gilbert-Girard et al. 2020a). Two different parameters were measured for both planktonic cells (turbidity and viability) and biofilms (viability and total biomass). All compounds were tested twice with a single replicate in each case. The natural compounds that inhibited by at least 70% the growth of planktonic cells (both turbidity and viability) and the biofilm formation (both viability and biomass) were

considered active hits. Compounds displaying conflicting replicate results, as well as the identified positive hits were retested a third time. Figure 1 shows the averaged inhibition results of the two or three independent experiments. Screening results for all compounds are available as supplementary information (Supplementary Table 1).

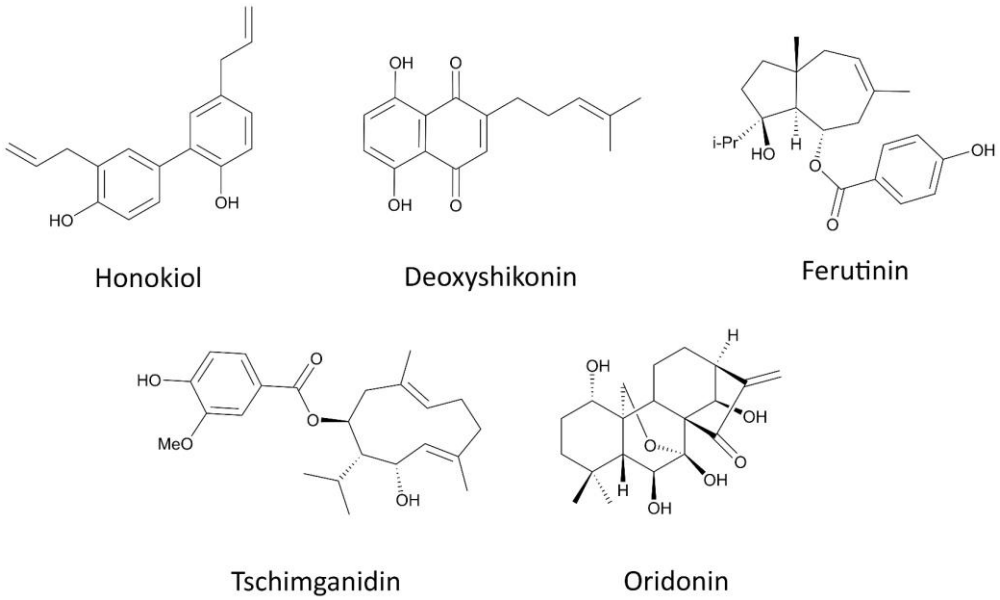
Figure 1. Screening of a natural compounds library (500 compounds) at 50 µM against *S. aureus* ATCC 25923. (a) Inhibition percentage (%) of the planktonic cells turbidity and viability (metabolic activity); (b) inhibition percentage (%) of the biofilm's total biomass formation and viability (metabolic activity). Compounds inhibiting > 70% of each parameter measured (green areas) were considered positive hits. Each compound was tested two to three times.



In total, 45 compounds were identified as active against planktonic cells and 26 of them were also active against biofilms. Of those 26 hits, 15 were compounds with a known antibiotic activity (actinomycin D, antibiotic A-23187, chelerythrine, chromomycin A3, coumermycin A1, lincomycin, mithramycin A, neomycin, nigericin, rifampicin, troleandomycin, narasin, streptonigrin, echinomycin and lasalocid A) and were therefore discarded. Five compounds (doxorubicin, ellipticine, etoposide, gliotoxin and gossypol) with well-reported cytotoxic effects, or low biocompatibility indices, were also discarded. Six hit compounds remained: honokiol, tschimganin, tschimganidin, ferutinin, oridonin and deoxyshikonin. Tschimganin (bornyl vanillate) has limited supply options and it did not seem to be cost-friendly (prices can be up to 100€ per mg, although they can of course vary between regions), which made it a less interesting option for further developments as a potential antibacterial compound. Moreover, a more affordable and structurally

related compound was identified (tschimganidin), and thus tschimganin was not studied further. The structures of the five selected hit compounds are presented in Figure 2.

Figure 2. Structures of the five selected hits.



Antibacterial and biofilm-inhibitory activity against staphylococci

The compounds were first tested at various concentrations within the range 12.5-100 µM against staphylococci (*S. aureus* and *S. epidermidis*) in pre-exposure. Supplementary Figure S1 shows the inhibitory activity of the five compounds against planktonic cells (turbidity and viability) and biofilms (viability and total biomass). The MIC values obtained against *S. aureus* are presented in Table 1.

Table 1. MIC, MBC and BPC (µM) of the natural compounds against *S. aureus* ATCC 25923 (from two experiments with two replicates each).

	MIC	MBC	BPC
Honokiol	75	75	75
Tschimganidin	25	50	> 100
Ferutinin	25	25	25
Oridonin	75	75	> 200
Deoxyshikonin	25	100	> 100

MBC and BPC determined as a reduction of at least 99.9% of the number of CFU in the planktonic solution and on the surface of the wells respectively.

All five compounds showed a clear activity against staphylococci. Honokiol had a MIC of 75 µM (20 µg ml^{-1}) against *S. aureus* and 50 µM (13.3 µg ml^{-1}) against *S. epidermidis*,

at which concentrations it also prevented biofilm growth. Oridonin had an MIC of 75 µM (27.3 µg ml^{-1}) against both strains but did not fully prevent biofilm formation even at 100 µM (below 90% inhibition). Unlike the other compounds, deoxyshikonin was slightly more effective against *S. aureus* than against *S. epidermidis*, with an MIC of 25 µM (6.8 µg ml^{-1}) against the former and 50 µM (13.6 µg ml^{-1}) against the latter. Additionally, deoxyshikonin inhibited biofilm formation of both staphylococci. Both tschimganidin and ferutinin performed better than the other compounds, with an MIC of 25 µM (9.7 and 9.0 µg ml^{-1} respectively) and they prevented biofilm formation at their MIC against both staphylococci.

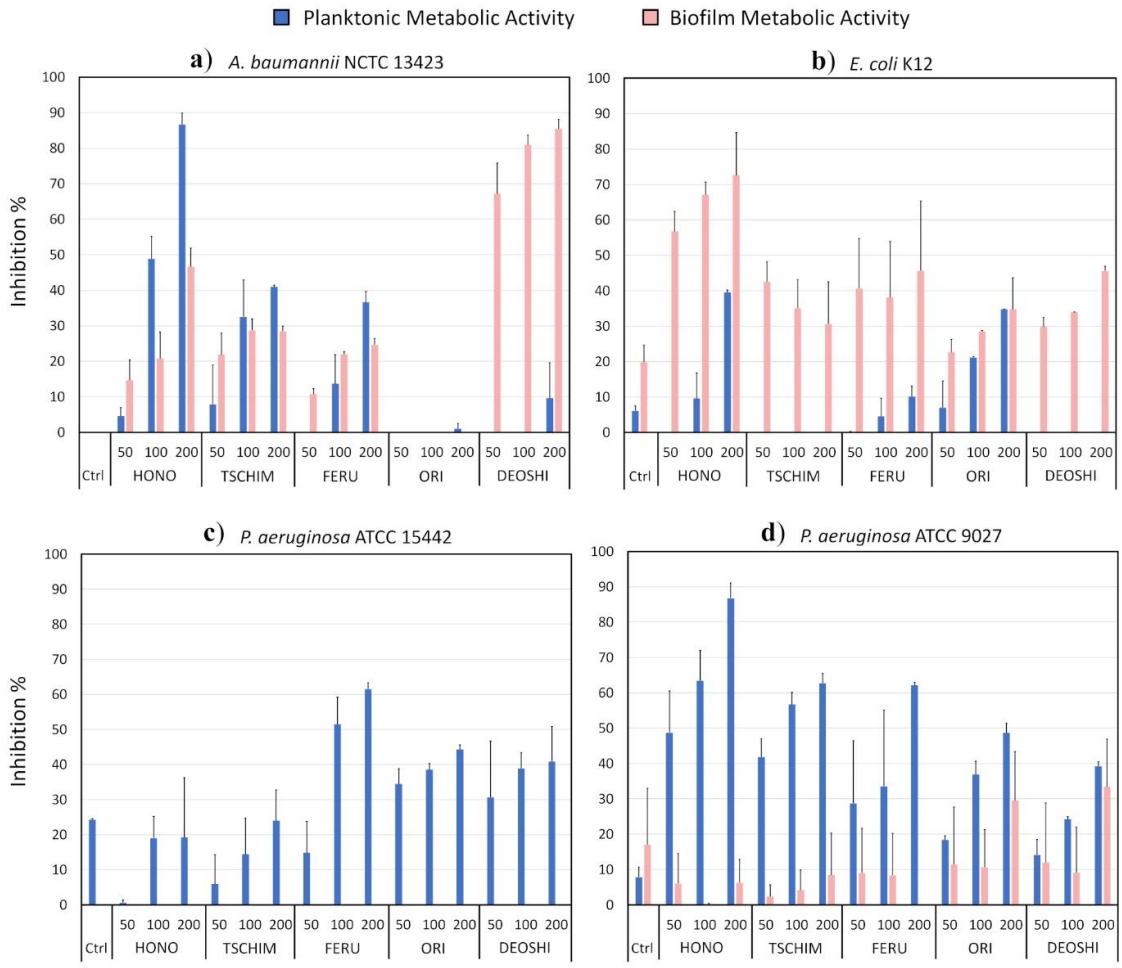
To confirm whether or not the observed anti-staphylococcal activity of the compounds was bactericidal, the planktonic solution and the scraped adhered *S. aureus* cells were plated on agar after the 18-h exposure to the compounds, from all wells where no growth (planktonic or adherent) was visible. Honokiol and oridonin, having a higher MIC than the other compounds, were tested at concentrations up to 200 µM. Table 1 shows the minimal bactericidal concentration (MBC, killing > 99.9% cells in the planktonic solution) and the biofilm preventing concentration (BPC, killing > 99.9% cells adhered to the bottom of the wells) obtained against *S. aureus*.

Honokiol's activity was confirmed to be bactericidal and to prevent adhesion and survival of cells on the surface of the wells at its MIC. Oridonin had an MBC equal to its MIC as well, but it did not kill adhered cells at any of the tested concentrations. Deoxyshikonin seemed to be bacteriostatic at its MIC as its MBC was only reached at 100 µM (27.2 µg ml^{-1}), and this compound also did not kill all adhered cells at any of the tested concentrations. Of the two compounds isolated from *Ferula* genus plants (ferutinin and tschimganidin), the first one showed the most potent bactericidal activity, killing over 99.9% of the planktonic and adhered cells at its MIC and leaving no viable cells in the well from 50 µM (17.9 µg ml^{-1}). On the other hand, tschimganidin also had a bactericidal activity with an MBC of 50 µM (19.4 µg ml^{-1}) but did not kill adhered cells at any concentration. According to Mogana et al. (Mogana et al. 2020), compounds with an MBC/MIC ratio ≤ 4 are considered bactericidal, which is the case for all our selected compounds, although it is important to note that deoxyshikonin was bactericidal at a higher concentration than its MIC.

Antibacterial and biofilm-inhibitory activity against Gram-negative species

The five selected hit compounds were tested in the pre-exposure assay for their antibacterial and biofilm-inhibitory activities, at concentrations within the range of 12.5-200 µM, against *A. baumannii*, *E. coli* and two strains of *P. aeruginosa*. Figure 3 shows the inhibition of the viability (metabolic activity) of the planktonic cells and biofilms, measured by resazurin staining, of each bacterial species by the compounds at the highest concentrations tested. Inhibition of the turbidity of the planktonic cells and total biomass of the biofilms is presented in Supplementary Figure S2.

Figure 3. Inhibiton of bacterial viability (metabolic activity) by the five natural compounds: honokiol (HONO), tschimganidin (TSCHIM), ferutinin (FERU), oridonin (ORI) and deoxyshikonin (DEOSHI) at 50, 100 and 200 µM, after a 18 h-long exposure on (a) *A . baumannii* NCTC 13423, (b) *E. coli* K12, (c) *P. aeruginosa* ATCC 15442 and (d) *P. aeruginosa* ATCC 9027. In the control (Ctrl) wells, bacteria were exposed to the solvent (DMSO 1%). The results are expressed as averages of the percentage of inhibition ± SD. The experiment was repeated twice.

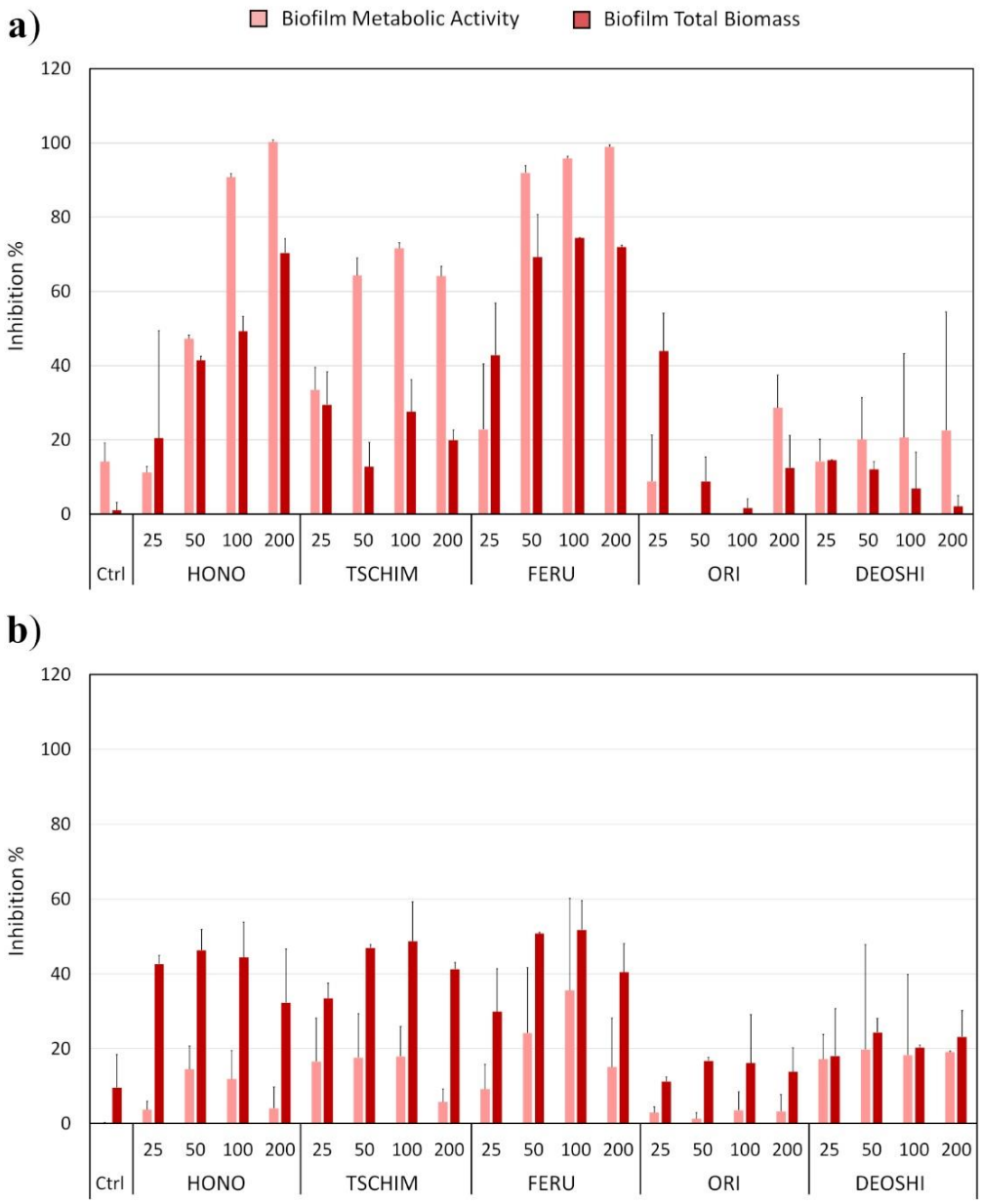


Honokiol had limited effects on all tested species (MIC > 200 µM; 53 µg ml^{-1}). It reduced the planktonic and biofilm growth of *A. baumannii* by almost 50% at 200 µM. Although it did not highly inhibit the planktonic growth of *E. coli*, it did reduce its biofilm formation by over 50% from 50 µM. Honokiol had a better activity against planktonic *P. aeruginosa* ATCC 9027, but it did not affect the biofilm formation of either strains of *P. aeruginosa*. Oridonin showed no activity against *A. baumannii* and a limited activity against *E. coli* and *P. aeruginosa* (mostly ATCC 9027) planktonic cells and biofilms. Deoxyshikonin inhibited the biofilm formation of *A. baumannii* much more so than its planktonic growth and was by far the most effective compound at inhibiting this species' biofilms. It was however one of the least effective against *E. coli* and it did not display a particularly noteworthy activity against either strains of *P. aeruginosa*, with a maximal 40% inhibition of the planktonic cells. Tschimganidin and ferutinin both displayed a limited activity against *A. baumannii* (tschimganidin being slightly better than ferutinin) and a biofilm-specific inhibition of *E. coli*. Beside honokiol, they were the most effective at inhibiting *E. coli* biofilm biomass formation (50-60% inhibition). While both compounds similarly inhibited planktonic *P. aeruginosa* ATCC 9027, ferutinin was the most effective compound against ATCC 15442, with over 50% reduction of the planktonic cells from 100 µM.

Biofilm-eradication activity on pre-formed biofilms

The ability of the compounds to disrupt pre-formed biofilms ('post-exposure' assay), was next investigated. The five compounds were tested against *S. aureus*, *S. epidermidis*, *A. baumannii* and *E. coli* as they had biofilm prevention activities against those species. Figure 4 shows the activity of the compounds against *S. aureus* and *E. coli*. The activity of the compounds against *S. epidermidis* and *A. baumannii* is available as supplementary material (Figure S3).

Figure 4. Disruption of biofilm viability (metabolic activity) and total biomass by the five natural compounds: (honokiol (HONO), tschimganidin (TSCHIM), ferutinin (FERU), oridonin (ORI) and deoxyshikonin (DEOSHI) at 25, 50, 100 and 200 µM after a 24 h-long exposure of 18 h-old preformed biofilms of (a) *S. aureus* ATCC 25923 and (b) *E. coli* K12. In the control (Ctrl) wells, bacteria were exposed to the solvent (DMSO 1%). The results are expressed as averages of the percentage of inhibition ± SD. The experiment was repeated twice.

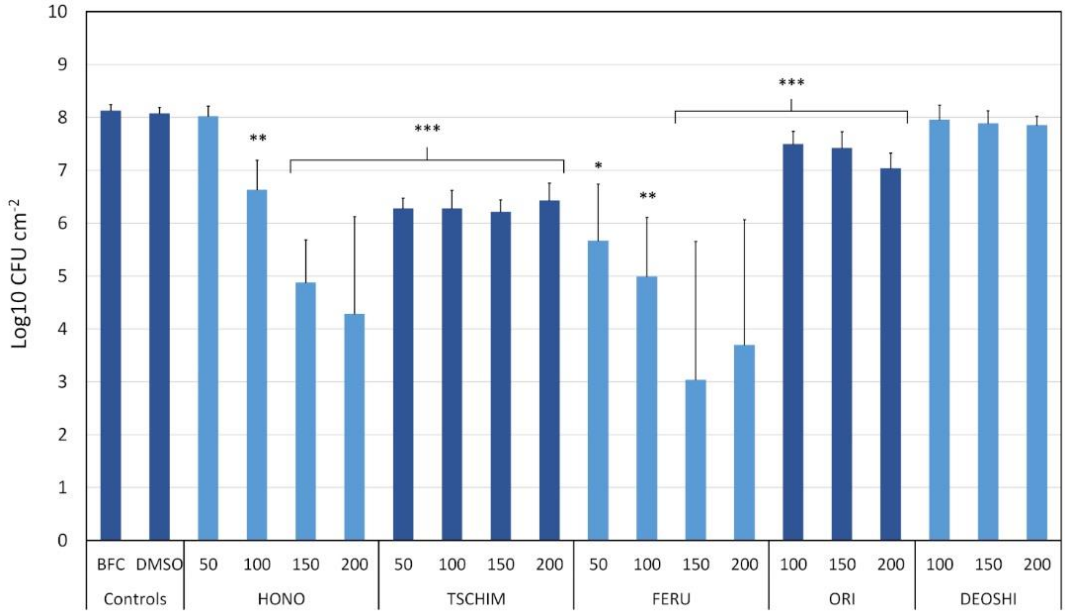


No compound disrupted *A. baumannii* pre-formed biofilms (inhibition mostly below 20-30%). Honokiol reduced the total biomass of *E. coli* biofilms by at least 40%. It was more active against the staphylococci however and reduced the viability (metabolic activity) of their biofilms by 100% and their total biomass by 60% at 200 µM (53 µg ml^{-1}). On the other hand, oridonin and deoxyshikonin did not disrupt the pre-formed biofilms of any species (inhibition mostly below 20%). Tschimganidin and ferutinin, like honokiol, slightly reduced the total biomass of pre-formed *E. coli* biofilms (over 40% at 50 µM), but they also performed best against staphylococci. Tschimganidin reduced the viability of the biofilm cells by over 60% from 50 µM (*S. aureus*) or 25 µM (*S. epidermidis*), although it did not strongly reduce the total biomass. The compound with the best biofilm-

disrupting activity was ferutinin, which reduced both the pre-formed biofilms' viability and biomass by at least 80% and 60% respectively from concentrations of 50 µM. While it had a more potent activity at 25 µM against *S. epidermidis*, it reached a higher disruptive effect against *S. aureus* with over 70% reduction of the total biomass.

Resazurin staining allows the measurement of the metabolic activity of the cells, therefore when measuring the effects on pre-grown biofilms, it is possible that viable but dormant cells are not detected using this method (Sandberg et al. 2009). On the other hand, crystal violet also stains dead cells and matrix and does not inform on the potential viability of the remaining cells in the biofilm. To confirm whether the disruption observed in *S. aureus* pre-formed biofilms translated into a reduced number of viable cells within the pre-formed biofilms, the biofilms were scraped after the 24-h treatment and colony-forming-units (CFUs) were counted (Figure 5).

Figure 5. Viable cells expressed as a function of the well area (Log10 CFU cm^{-2} + SD) of 18 h-old *S. aureus* ATCC 25923 biofilms exposed for 24 h to 50, 100, 150 and 200 µM honokiol (HONO), tschimganidin (TSCHIM), ferutinin (FERU), oridonin (ORI) and deoxyshikonin (DEOSHI), in comparison with untreated biofilm controls (BFC) and solvent controls where bacteria were exposed to 1% DMSO. The experiment was repeated four times, with two biofilm replicates each time. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.



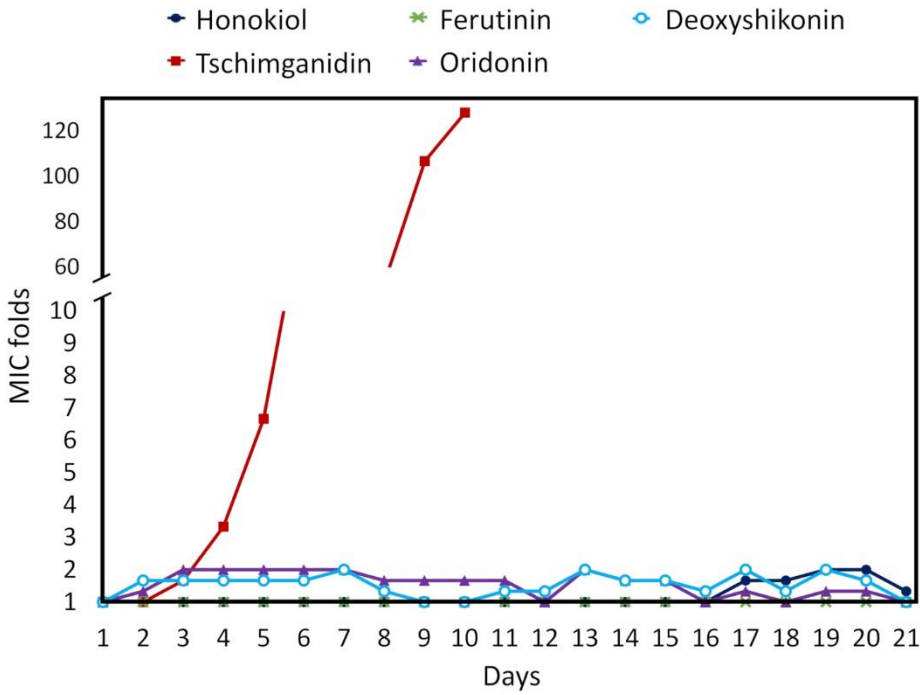
Honokiol displayed a significant concentration-dependent reduction of the viable cells consistent with the viability and biomass staining. The highest effect at 200 µM was a 4-

log reduction of the viable counts. As expected, deoxyshikonin did not reduce the number of live cells within the biofilms. On the other hand, despite its limited disruptive effect observed in Figure 4a, oridonin did reduce the number of CFUs in the pre-formed biofilms by 1 log. Tschimganidin significantly reduced the number of viable cells in the mature biofilms from a concentration as low as 50 µM, but by no more than 1.5-2 logs, regardless of the concentration used. Again, the most effective compound at eradicating biofilms was ferutinin, causing more than a 5-log reduction in average at 150 and 200 µM. Of note, among the biofilms treated with 150-200 µM, 38-50% were found to have no remaining viable cells (8-log reduction), while the other replicates displayed a 3-log reduction compared to the controls (for an average of a 5-log reduction).

Resistance development

To evaluate whether resistance could be rapidly built against the five compounds, *S. aureus* was exposed to a sub-inhibitory concentration of each compound for 20 days (Figure 6). In a previous study, using this resistance development protocol, resistance was shown to develop against a few model antibiotics, with an increase of the MIC by 4-fold for doxycycline, almost 100-fold for oxacillin and over 1000-fold for penicillin G after 20 days (Gilbert-Girard et al. 2020b).

Figure 6. Evolution of MIC changes measured at 0.5 x MIC for each compound (honokiol, tschimganidin ferutinin, oridonin and deoxyshikonin) during exposure for 20 days on *S. aureus* ATCC 25923. The experiment was repeated with three biological replicates.



Interestingly, no resistance was developed to any of the compounds, except tschimganidin, over the course of the experiment duration. Resistance was very quickly built against tschimganidin and the experiment was brought to an end for this compound after 10 days as it was clear that *S. aureus* cultures were fully resistant, and the concentration used was too high to allow full solubilisation of the compound. To the best of our knowledge, this is the first report investigating any possible resistance development for all five compounds.

Cytotoxicity

A key aspect to consider when evaluating drug candidates is their potential cytotoxicity. Three human cell lines were exposed to the compounds at concentrations ranging from 3.125 to 50 µM. The IC_{50} for each compound were calculated from viability measurements after a 24-h incubation (Table 2).

Table 2. Cytotoxic effects of the compounds tested (IC_{50}) measured on the three different human cell lines A549, HEp-2 and HL-60.

	IC_{50} (µM) and (confidence interval)		
	A549	**HEp-2**	**HL-60**
Honokiol	32.0 (24.5 – 47.7)	> 50	> 50
Tschimganidin	> 50	> 50	> 50
Ferutinin	14.6 (10.6 – 21.2)	45.6 (40.3 – 50.3)	40.7 (30.1 – 66.3)
Oridonin	> 50	> 50	14.7 (10.5 – 23.3)
Deoxyshikonin	14.3 (9.9 – 22.0)	24.2 (19.2 – 31.7)	< 3.125

Honokiol expressed some cytotoxicity against all cell lines with an IC_{50} of 32 µM (8.52 µg ml^{-1}) and > 50 µM (> 13.3 µg ml^{-1}) against A549 and HEp-2 respectively. Deoxyshikonin was the most cytotoxic compound with an IC_{50} of 14.3-24.2 µM (3.9 – 6.6 µg ml^{-1}) on the epithelial cells and < 3.125 µM (< 0.85 µg ml^{-1}) on the HL-60 cells. Oridonin did not show important cytotoxic effects against the epithelial cell lines (IC_{50} > 50 µM; 18.2 µg ml^{-1}) but did affect the HL-60 cells with an IC_{50} of 14.7 µM (5.4 µg ml^{-1}). Tschimganidin was the least cytotoxic compound with IC_{50} consistently above 50 µM (19.4 µg ml^{-1}), a higher concentration than its MIC against *S. aureus* (25µM). Ferutinin was slightly more cytotoxic, especially above 50 µM and particularly affected the A549 cells with an IC_{50} as low as 14.6 µM (5.2 µg ml^{-1}), which is below its MIC against *S. aureus* (25 µM).

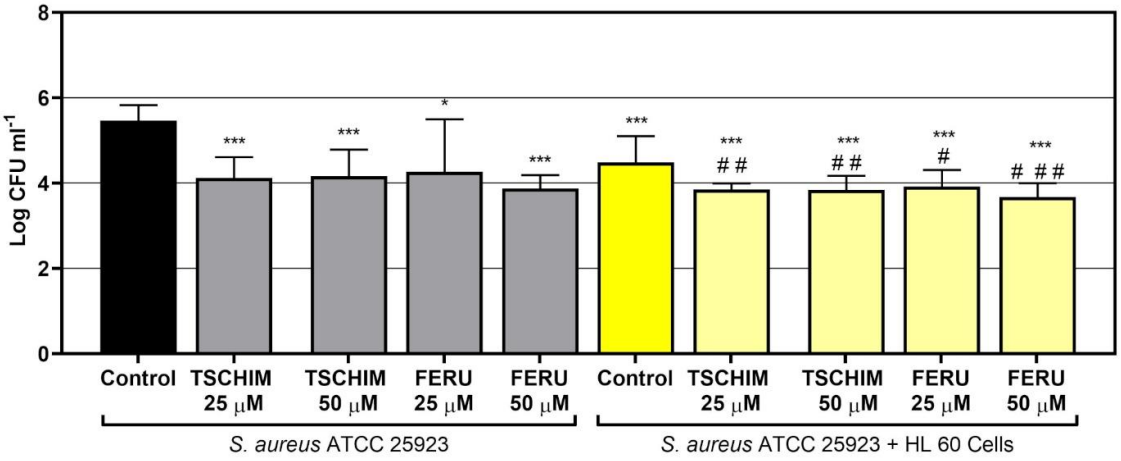
Biofilm prevention on an LDPE catheter model, in presence and absence of immune cells

As some of the compounds showed a good capacity to prevent biofilm formation, they were tested in a co-culture model that resembles more closely an infection occurring in a medical device *in vivo*. Factors that are not found *in vitro*, such as cellular products and proteins, affect the dynamic of a potential infection *in vivo* (Del Pozo 2018). In addition, insertion of a device produces an immune response and, following tissue injury caused by intubation with an endotracheal catheter for instance, neutrophils are recruited to the device (Puyo and Dahms 2012; Puyo et al. 2017). To simulate that, a model system has been previously developed in our group involving the co-culture of *S. aureus* and differentiated HL-60 cells (neutrophils-like) on the surface of LDPE tubes (Reigada et al. 2020).

Tschimganidin and ferutinin were selected for this study, as they displayed the best MIC and had limited cytotoxicity against HL-60 cells. They were added in the co-

culture media for 24 h, and their effects on the growth of *S. aureus* on the surface of the tubes were measured in presence and absence of HL-60 cells (Figure 7).

Figure 7. Biofilm formation by *S. aureus* ATCC 25923 on LDPE tubes exposed to different compounds (tested at 50 and 25 µM). The first half of the histogram shows S. aureus biofilm formation when exposed to the compounds alone, while the second half shows the results when the bacteria were co-cultured with HL-60 cells differentiated with DMF. The results are expressed as means ± SD of three technical replicates in experiments repeated three times. '*' indicates statistical differences with the untreated bacteria control in monoculture, while '#' represents statistical differences with the untreated control in co-culture (* p < 0.05; ** p < 0.01; ***p < 0.001), (## p < 0.01; ### p < 0.001).



In this model, both tschimganidin and ferutinin significantly reduced *S. aureus* biofilm formation on the catheters either in the absence or in the presence of the HL-60 cells. When *S. aureus* was cultured alone, the compounds reduced the amount of viable bacterial cells colonizing the surface of the tubes by ~ 1.5 log. When *S. aureus* was cultured with HL-60 cells, the immune cells on their own reduced the number of viable bacteria by nearly as much, and the compounds both caused a significant additional reduction of the biofilm formation. No difference in the antibacterial activity was visible for tschimganidin between 25 and 50 µM and, in the case of ferutinin, only a little difference was observed. Both compounds reduced *S. aureus* biofilm formation slightly more so (albeit not significantly) in presence of the human cells than alone, suggesting that cells were still alive and having some activity against the bacteria.

Discussion

The screening of the natural compounds library led to the identification of 15 known

antibiotics as active hits. The identification of these agents was a reliable indication that the screening campaign was successful at identifying compounds with an antibacterial activity. Most other known antibacterial compounds present in the library also displayed some activity in the screening, but they either inhibited only planktonic cells or had a lower activity below the limit threshold, so that they were not selected as hits. No antibacterial known to be highly active against staphylococci gave a negative result in this screening, further supporting the ability of the screening platform to accurately discriminate active from non-active molecules. The five compounds selected for follow-up studies (honokiol, tschimganidin, ferutinin, oridonin and deoxyshikonin) displayed a potent inhibitory activity (over 70% inhibition) of the planktonic cells and biofilm formation of *S. aureus* during the screening. With the exception of honokiol, the effects of these selected hit compounds on biofilms have not been studied, and only limited information has been gathered on their antibacterial activity. It was decided to continue performing follow-up studies with all five compounds, including honokiol, as the results with honokiol could be used for reference and comparison purposes.

Honokiol is a polyphenolic compound (lignan) found in *Magnolia* plants, which are known to contain bioactive compounds with antibacterial activity (Zeb et al. 2017; Lee et al. 2018; Lovecká et al. 2020). Honokiol, as well as its regioisomer magnolol, are not novel antibacterial compounds. Their potential has been explored in very recent years and many derivatives have been synthesized to improve their activity against various strains (Solinski et al. 2018; Wu et al. 2018; Ochoa et al. 2020). Honokiol and magnolol have been tested against a large variety of strains, including *Staphylococcus* spp., *Streptococcus mutans*, *Bacillus subtilis*, *E. coli*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *P. aeruginosa*, and generally showed to display a greater efficacy against Gram-positive bacteria (Kim YS et al. 2010; Choi et al. 2015; Kim SY et al. 2015; Li WL et al. 2016; Sakaue et al. 2016; Lee et al. 2018; Wu et al. 2018; Chiu et al. 2020). The MIC and MBC values (75 µM; 20 µg ml^{-1}) and biofilm preventive activity reported here for honokiol against *S. aureus* are consistent with the literature (MIC: 10-30 µg ml^{-1}, MBC: 16-32 µg ml^{-1}) (Syu et al. 2004; Kim YS et al. 2010; Choi et al. 2015; Zuo et al. 2015; Li WL et al. 2016; Lee et al. 2018; Wu et al. 2018; Chiu et al. 2020). Honokiol has been found to be inactive against Gram-negative bacteria such as *E. coli* and *P. aeruginosa* (Ho et al. 2001; Kim YS et al. 2010; Lee et al. 2018; Wu et al. 2018). Accordingly, no MIC was found here for honokiol against these species, nor against *A. baumannii*. The compound did not affect the biofilm formation of either strains of *P.*

aeruginosa or *A. baumannii* but did inhibit the biofilm formation of *E. coli*. This could suggest a biofilm-specific mechanism of action against this species. Honokiol was also shown to causes a significant eradication of pre-formed *S. aureus* biofilms from 100 to 200 µM, which also is aligned with the literature as honokiol has been reported to display an important disruptive activity against *S. aureus* and *S. mutans* mature biofilms (Li WL et al. 2016; Sakaue et al. 2016; Chiu et al. 2020). The same is true for our cytotoxicity results as previous observations of cytotoxic effects by honokiol have obtained similar IC_{50} values against A549 cells (IC_{50} 33-35.4 µM) as well as HeLa and HEp-G2 cells (IC_{50} 45 µM) (Syu et al. 2004; Luo et al. 2009; Lin et al. 2019; Ren et al. 2020). Despite this *in vitro* cytotoxicity, *in vivo* studies have indicated that honokiol is safe and does not cause toxicity (Banik et al. 2019), suggesting that honokiol could potentially be used as an antibacterial compound despite its IC_{50} being lower than its MIC against *S. aureus*. Honokiol was selected here as reference as it was extensively studied and, overall, our results are consistent with previous findings and further contributes to suggest honokiol as a potential antibacterial compound active against biofilms, although only at high concentrations, which limits its potential.

On the other hand, oridonin is a diterpenoid found in the plants of the Labiatae family (*Isodon* species) which have been used in Chinese folk medicine. Numerous derivatives of oridonin have been synthesized and mainly evaluated as anticancer agents in the last decades (reviewed in (Xu J et al. 2018)), but very little attention has been given to oridonin's antibacterial activity. It has been found active against mycobacteria, *S. aureus* (including methicillin-resistance *S. aureus*; MRSA) and *B. subtilis*, but it was inactive against *E. coli* (Xu S, Li, et al. 2014; Xu S, Pei, et al. 2014; Li D et al. 2016; Yuan et al. 2019). Oridonin's activity has not been reported on *S. epidermidis* before, but our results against *S. aureus* are consistent with the reported MIC of 31.2 µg ml^{-1} (Li D et al. 2016). While oridonin inhibited planktonic staphylococcal strains (MIC and MBC of 75 µM; 27.3 µg ml^{-1}), its inability to fully prevent biofilm formation could indicate a slower mechanism of action or a lower activity against adhered cells. Additionally, oridonin did not disrupt pre-formed *S. aureus* biofilms. In agreement with a previous report (Li D et al. 2016), oridonin displayed a very limited activity against *E. coli* and it was further shown to be inactive or poorly active against *A. baumannii* and *P. aeruginosa*. In our study, oridonin only showed important cytotoxic effects against the HL-60 cells, but previous studies have also reported a wide range of IC_{50} values ranging from 5.9 to 50.3 µM (2.2 to 18.3 µg ml^{-1}) on different cell lines (Bai et al. 2010; Li et al. 2019; He et

al. 2020). Since oridonin has a limited antibacterial activity, and as this compound showed toxicity *in vivo* at high concentrations (Xu L et al. 2020), its potential as an antibacterial compound is limited.

Deoxyshikonin has not been extensively studied as an antibacterial compound and even less as an anti-biofilm. Shikonins are naturally occurring naphthoquinones found in plants of the Boraginaceae family and are known to possess various biological activities, including antimicrobial functions (Haghbeen et al. 2011; Andújar et al. 2013; Zhang S et al. 2015; Durán et al. 2017). Like many other natural products, shikonin derivatives have gathered more interest in the last decades and a few studies have explored their antibacterial potential against Gram-negative and especially against Gram-positive strains (Brigham et al. 1999; Shen et al. 2002; Rajbhandari et al. 2007; Vukic et al. 2017). Of note, shikonin was also part of the screened library, but it fell below the activity threshold established for this study, and thus it was not considered a hit. Deoxyshikonin itself, and its *S* enantiomer deoxyalkannin, have been tested in a few studies against various strains such as *S. aureus*, streptococci, *B. subtilis*, *K. pneumoniae*, *P. aeruginosa and E. coli* (Brigham et al. 1999; Rajbhandari et al. 2007; Damianakos et al. 2012; Zhang S et al. 2015; Tufa et al. 2017). Here, it was shown to inhibit planktonic staphylococcal growth at 25-50 µM (7-14 µg ml^{-1}), but it only completely killed *S. aureus* at a higher concentration and did not kill all adherent cells at 100 µM. Deoxyshikonin did not affect pre-formed *S. aureus* biofilms either. When tested against Gram-negative species, the compound was not very effective against *E. coli* nor *P. aeruginosa*, but it inhibited the biofilm formation of *A. baumannii* more than its planktonic growth. While this could suggest an anti-biofilm activity specific to *A. baumannii*, it did not translate into disruption of pre-existing biofilms of that species and might rather reflect a bacteriostatic activity. Overall, our results are in agreement with studies that have found deoxyshikonin (and other shikonins) to be generally more active against Gram-positive than Gram-negative species (Brigham et al. 1999; Naz et al. 2006; Rajbhandari et al. 2007; Andújar et al. 2013; Zhang S et al. 2015). Deoxyshikonin was the most cytotoxic compound tested here, which is in line with various other reports of deoxyshikonin's cytotoxic effects (Rajbhandari et al. 2007; Damianakos et al. 2012; Rajasekar et al. 2012; Zhu et al. 2019). Similarly to oridonin, deoxyshikonin did not display a significant antibacterial or anti-biofilm activity and its cytotoxicity reduces its applicability for treatment of bacterial infections.

Tschimganidin, is a sesquiterpene found in *Ferula* plants. Although the antibacterial activity of similar compounds has been studied (Trusheva et al. 2010), tschimganidin has not been specifically investigated and, to our knowledge, no report exists on its antimicrobial potential. It has been only reported, along with ferutinin, as an agonist of the estrogen receptors (Ikeda et al. 2002). Tschimganidin was confirmed here to be active against *S. aureus* and *S. epidermidis* (MIC 25 µM: 9.7 µg ml^{-1}) but it had a limited activity against *A. baumannii* and planktonic *P. aeruginosa*. It slightly inhibited the biofilm formation of *E. coli* as well. Tschimganidin showed some activity against the pre-formed biofilms of *S. aureus* and, to a lesser extent, *E. coli*. Interestingly, in the case of *S. aureus* biofilms, tschimganidin caused a constant reduction (nearly 70% inhibition of the viability and a 2-log reduction of the CFUs) at all concentrations from 50 to 200 µM. That could be due to the compound only killing the outer population of the biofilm regardless of the concentration, either because of a reduced penetration into the biofilm or because of a mechanism of action targeting metabolically active cells. Additionally, tschimganidin did not display cytotoxic effects and reduced *S. aureus* biofilm formation in presence of serum and neutrophils. However, it was no more effective in doing so at 50 µM than at 25 µM. Cytotoxicity probably did not affect the dynamic of the co-culture system, but this could be due to the poor solubility of the compound or a strong binding to proteins in the media, trumping the effect of an increased concentration. While tschimganidin was quite effective against staphylococci and does not appear cytotoxic, *S. aureus* quickly developed resistance against it. Taken together, our results do not suggest this compound as a good anti-biofilm candidate and should not be used alone as an antibacterial drug, but it could be interesting to explore its potential in a combination therapy.

Ferutinin (jaeschkeanadiol *p*-hydroxybenzoate) is a daucane sesquiterpene ester extracted from *Ferula* plants. The Apiaceae (or Umbelliferae) family, and especially the genus *Ferula*, contain over 150 species of plants rich in bioactive compounds, including antibacterial ones (Nazari and Iranshahi 2011; Liu et al. 2015; Dastan et al. 2016; Zhou et al. 2017; Znati et al. 2017; Utegenova et al. 2018). Ferutinin has been reported to be active against Gram-positive strains such as *S. aureus*, *S. epidermidis*, *E. faecalis* and *B. subtilis*, while no activity against Gram-negative bacteria has been detected with this compound (Galal et al. 2001; Tamemoto et al. 2001; Trusheva et al. 2010; Ibraheim et al. 2012; Kuete et al. 2012). Of the five hit compounds identified here, ferutinin displayed the highest activity against staphylococci, with an MIC, MBC and BPC of 25 µM (9 µg

ml^{-1}) against *S. aureus*. This is consistent with previously reported MIC values between 6.25-16 µg ml^{-1} against various *S. aureus* strains (Galal et al. 2001; Tamemoto et al. 2001). Ferutinin was slightly more effective than tschimganidin against *P. aeruginosa* but otherwise expressed a similarly limited activity against Gram-negative species, which is aligned with earlier reports (Tamemoto et al. 2001; Trusheva et al. 2010; Ibraheim et al. 2012; Kuete et al. 2012). Ferutinin was also the most effective compound against pre-formed *S. aureus* biofilms, causing a reduction of the viable cells from over 2 to 5-log between 50 to 200 µM. The compound displayed cytotoxic effects against one of the tested cell lines at a concentration below its MIC. The literature has reported a very wide and variable range of IC_{50} (6.9 to 138 µM; 2.5 to 49.5 µg ml^{-1}) on different cell lines, making it challenging to draw clear conclusions on the cytotoxicity of ferutinin (Kuete et al. 2012; Matin et al. 2014; Iranshahi et al. 2018; Soltani et al. 2019; Macrì et al. 2020). However, ferutinin has been reported to be selectively cytotoxic against cancer cell lines and to cause no toxicity *in vivo* (Arghiani et al. 2014; Matin et al. 2014; Naji Reyhani Garmroudi et al. 2021). Considering its high potency on bacteria, particularly against pre-formed biofilms, more research, particularly *in vivo* studies, would be worthwhile in order to evaluate the safety and potential of this compound. Using a co-culture model, ferutinin was shown to still possess an antibacterial activity against *S. aureus* in presence of human cells and serum. It was not significantly more effective at 50 µM, in comparison to 25 µM, and its cytotoxic effects, reducing the neutrophils' activity against bacteria, could be responsible for this. Similar to the case of tschimganidin, this could also result from low solubility or binding to proteins in the serum. Overall, our results indicate a good potential for ferutinin for the treatment of biofilm-related infections. No resistance was observed at any point against ferutinin by *S. aureus*, further supporting its potential as a promising antibacterial compound.

Conclusions

Research around new natural antibacterial compounds is more intense than ever, but anti-biofilm activity is often overlooked despite the high prevalence of biofilms in infections. Here, bacterial biofilms were not eliminated by compounds at concentrations that are bactericidal to planktonic cells, which highlights the urgent need for investigating the activity of new antibacterial compounds against biofilms. Five natural compounds were identified, one with a well-reported antibacterial potential (honokiol) and four with

previously unknown anti-biofilm activity (tschimganidin, ferutinin, oridonin and deoxyshikonin), with potent antibacterial activity against *S. aureus* using a biofilm-based screening platform as well as follow-up assays to estimate their MIC, MBC and BPC. This is the first report of tschimganidin's antibacterial activity (MIC and MBC of 25 and 50 µM). The compounds were also evaluated against other clinically relevant bacterial species and their activity on mature biofilms was additionally investigated. The potential resistance development in *S. aureus* to each of the compounds was assessed over 20 days and they were tested on human cell lines to measure their cytotoxicity. In addition, the most potent compounds, tshimganidin and ferutinin, were evaluated for their capacity to prevent biofilm formation on a catheter in a more complex *S. aureus* and human cells co-culture model. Ferutinin displayed a potent bactericidal activity against staphylococci (MIC/MBC/BPC = 25 µM) with a notable ability to disrupt also pre-formed biofilms. Our results indicate that ferutinin is a promising antibacterial and anti-biofilm compound on its own and provides scaffolds for potential medicinal chemistry optimization efforts to reduce their cytotoxicity.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement number 722467; the Finnish Pharmaceutical Society provided a personal grant to S.G-G.

References

Andújar I, Ríos JL, Giner RM, Recio MC. 2013. Pharmacological properties of shikonin - a review of literature since 2002. Planta Med. 79(18):1685-1697.

Arciola CR, Campoccia D, Speziale P, Montanaro L, Costerton JW. 2012. Biofilm formation in Staphylococcus implant infections. A review of molecular mechanisms and implications for biofilm-resistant materials. Biomaterials. 33(26):5967-5982.

Arghiani N, Matin MM, Bahrami AR, Iranshahi M, Sazgarnia A, Rassouli FB. 2014. Investigating anticancer properties of the sesquiterpene ferutinin on colon carcinoma cells, in vitro and in vivo. Life Sci. 109(2):87-94.

Bai N, He K, Zhou Z, Tsai ML, Zhang L, Quan Z, Shao X, Pan MH, Ho CT. 2010. Ent-kaurane diterpenoids from Rabdosia rubescens and their cytotoxic effects on human cancer cell lines. Planta Med. 76(2):140-145.

Banik K, Ranaware AM, Deshpande V, Nalawade SP, Padmavathi G, Bordoloi D, Sailo BL, Shanmugam MK, Fan L, Arfuso F et al. 2019. Honokiol for cancer therapeutics: A traditional medicine that can modulate multiple oncogenic targets. Pharmacol Res. 144:192-209.

Breijyeh Z, Jubeh B, Karaman R. 2020. Resistance of Gram-Negative Bacteria to Current Antibacterial Agents and Approaches to Resolve It. Molecules. 25(6):1340.

Brigham LA, Michaels PJ, Flores HE. 1999. Cell-specific production and antimicrobial activity of naphthoquinones in roots of lithospermum erythrorhizon. Plant Physiol. 119(2):417-428.

Castronovo LM, Vassallo A, Mengoni A, Miceli E, Bogani P, Firenzuoli F, Fani R, Maggini V. 2021. Medicinal Plants and Their Bacterial Microbiota: A Review on Antimicrobial Compounds Production for Plant and Human Health. Pathogens. 10(2):106.

Chiu KC, Shih YH, Wang TH, Lan WC, Li PJ, Jhuang HS, Hsia SM, Shen YW, Yuan-Chien Chen M, Shieh TM. 2020. In vitro antimicrobial and antipro-inflammation potential of honokiol and magnolol against oral pathogens and macrophages. J Formos Med Assoc. 102(2):827-837.

Choi EJ, Kim HI, Kim JA, Jun SY, Kang SH, Park DJ, Son SJ, Kim Y, Shin OS. 2015. The herbal-derived honokiol and magnolol enhances immune response to infection with methicillin-sensitive Staphylococcus aureus (MSSA) and methicillin-resistant S. aureus (MRSA). Appl Microbiol Biotechnol. 99(10):4387-4396.

Collins SJ, Ruscetti FW, Gallagher RE, Gallo RC. 1978. Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds. Proc Natl Acad Sci. 75(5):2458-2462.

Damianakos H, Kretschmer N, Sykłowska-Baranek K, Pietrosiuk A, Bauer R, Chinou I. 2012. Antimicrobial and cytotoxic isohexenylnaphthazarins from Arnebia euchroma (Royle) Jonst. (Boraginaceae) callus and cell suspension culture. Molecules. 17(12):14310-14322.

Dastan D, Salehi P, Aliahmadi A, Gohari AR, Maroofi H, Ardalan A. 2016. New coumarin derivatives from Ferula pseudalliacea with antibacterial activity. Nat Prod Res. 30(24):2747-2753.

Davidson DJ, Spratt D, Liddle AD. 2019. Implant materials and prosthetic joint infection: the battle with the biofilm. EFORT Open Rev. 4(11):633-639.

de Breij A, Riool M, Cordfunke RA, Malanovic N, de Boer L, Koning RI, Ravensbergen E, Franken M, van der Heijde T, Boekema BK et al. 2018. The antimicrobial peptide SAAP-148 combats drug-resistant bacteria and biofilms. Sci Transl Med. 10(423).

Del Pozo JL. 2018. Biofilm-related disease. Expert Rev Anti Infect Ther. 16(1):51-65.

Durán AG, Gutiérrez MT, Rial C, Torres A, Varela RM, Valdivia MM, Molinillo JMG, Skoneczny D, Weston LA, Macías FA. 2017. Bioactivity and quantitative analysis of isohexenylnaphthazarins in root periderm of two Echium spp.: E. plantagineum and E. gaditanum. Phytochemistry. 141:162-170.

Ferri M, Ranucci E, Romagnoli P, Giaccone V. 2017. Antimicrobial resistance: A global emerging threat to public health systems. Crit Rev Food Sci Nutr. 57(13):2857-2876.

Galal AM, Abourashed EA, Ross SA, ElSohly MA, Al-Said MS, El-Feraly FS. 2001. Daucane sesquiterpenes from Ferula hermonis. J Nat Prod. 64(3):399-400.

Gilbert-Girard S, Savijoki K, Yli-Kauhaluoma J, Fallarero A. 2020a. Optimization of a High-Throughput 384-Well Plate-Based Screening Platform with Staphylococcus aureus ATCC 25923 and Pseudomonas aeruginosa ATCC 15442 Biofilms. Int J Mol Sci. 21(9):3034.

Gilbert-Girard S, Savijoki K, Yli-Kauhaluoma J, Fallarero A. 2020b. Screening of FDA-Approved Drugs Using a 384-Well Plate-Based Biofilm Platform: The Case of Fingolimod. Microorganisms. 8(11):1834.

Haghbeen K, Pourmolaei S, Mareftjo MJ, Mousavi A, Akbari Noghabi K, Hosseini Shirazi F, Meshkat A. 2011. Detailed investigations on the solid cell culture and antimicrobial activities of the Iranian Arnebia euchroma. J Biomed Biotechnol. 2011:165852.

He SD, Huang SG, Zhu HJ, Luo XG, Liao KH, Zhang JY, Tan N, Li DY. 2020. Oridonin suppresses autophagy and survival in rheumatoid arthritis fibroblast-like synoviocytes. Pharm Biol. 58(1):146-151.

Ho KY, Tsai CC, Chen CP, Huang JS, Lin CC. 2001. Antimicrobial activity of honokiol and magnolol isolated from Magnolia officinalis. Phytother Res. 15(2):139-141.

Hug JJ, Bader CD, Remškar M, Cirnski K, Müller R. 2018. Concepts and Methods to Access Novel Antibiotics from Actinomycetes. Antibiotics (Basel). 7(2):44.

Ibraheim ZZ, Abdel-Mageed WM, Dai H, Guo H, Zhang L, Jaspars M. 2012. Antimicrobial antioxidant daucane sesquiterpenes from Ferula hermonis Boiss. Phytother Res. 26(4):579-586.

Ikeda K, Arao Y, Otsuka H, Nomoto S, Horiguchi H, Kato S, Kayama F. 2002. Terpenoids found in the umbelliferae family act as agonists/antagonists for ER(alpha) and ERbeta: differential transcription activity between ferutinine-liganded ER(alpha) and ERbeta. Biochem Biophys Res Commun. 291(2):354-360.

Iranshahi M, Rezaee R, Najaf Najafi M, Haghbin A, Kasaian J. 2018. Cytotoxic activity of the genus Ferula (Apiaceae) and its bioactive constituents. Avicenna J Phytomed. 8(4):296-312.

Kim SY, Kim J, Jeong SI, Jahng KY, Yu KY. 2015. Antimicrobial Effects and Resistant Regulation of Magnolol and Honokiol on Methicillin-Resistant Staphylococcus aureus. Biomed Res Int. 2015:283630.

Kim YS, Lee JY, Park J, Hwang W, Lee J, Park D. 2010. Synthesis and microbiological evaluation of honokiol derivatives as new antimicrobial agents. Arch Pharm Res. 33(1):61-65.

Kuete V, Wiench B, Hegazy ME, Mohamed TA, Fankam AG, Shahat AA, Efferth T. 2012. Antibacterial activity and cytotoxicity of selected Egyptian medicinal plants. Planta Med. 78(2):193-199.

Lee YS, Lee YJ, Park SN. 2018. Synergistic Antimicrobial Effect of Lonicera japonica and Magnolia obovata Extracts and Potential as a Plant-Derived Natural Preservative. J Microbiol Biotechnol. 28(11):1814-1822.

Li D, Han T, Xu S, Zhou T, Tian K, Hu X, Cheng K, Li Z, Hua H, Xu J. 2016. Antitumor and Antibacterial Derivatives of Oridonin: A Main Composition of Dong-Ling-Cao. Molecules. 21(5):575.

Li WL, Zhao XC, Zhao ZW, Huang YJ, Zhu XZ, Meng RZ, Shi C, Yu L, Guo N. 2016. In vitro antimicrobial activity of honokiol against Staphylococcus aureus in biofilm mode. J Asian Nat Prod Res. 18(12):1178-1185.

Li Y, Li N, Shi J, Ahmed T, Liu H, Guo J, Tang W, Guo Y, Zhang Q. 2019. Involvement of Glutathione Depletion in Selective Cytotoxicity of Oridonin to p53-Mutant Esophageal Squamous Carcinoma Cells. Front Oncol. 9:1525.

Lin D, Yan Z, Chen A, Ye J, Hu A, Liu J, Peng J, Wu X. 2019. Anti-proliferative activity and structure-activity relationship of honokiol derivatives. Bioorg Med Chem. 27(16):3729-3734.

Liu T, Wang S, Xu L, Fu W, Gibbons S, Mu Q. 2015. Sesquiterpenoids with Anti-MDR Staphylococcus aureus activities from Ferula ferulioides. Chem Biodivers. 12(4):599-614.

Lovecká P, Svobodová A, Macůrková A, Vrchotová B, Demnerová K, Wimmer Z. 2020. Decorative Magnolia Plants: A Comparison of the Content of Their Biologically Active Components Showing Antimicrobial Effects. Plants (Basel). 9(7):879.

Luo Y, Xu Y, Chen L, Hu J, Peng C, Xie D, Shi J, Huang W, Xu G, Peng M et al. 2009. Semi-synthesis and anti-proliferative activity evaluation of novel analogues of Honokiol. Bioorg Med Chem Lett. 19(16):4702-4705.

Macrì R, Musolino V, Gliozzi M, Carresi C, Maiuolo J, Nucera S, Scicchitano M, Bosco F, Scarano F, Ruga S et al. 2020. Ferula L. Plant Extracts and Dose-Dependent Activity of Natural Sesquiterpene Ferutinin: From Antioxidant Potential to Cytotoxic Effects. Molecules. 25(23):5768.

Mantravadi PK, Kalesh KA, Dobson RCJ, Hudson AO, Parthasarathy A. 2019. The Quest for Novel Antimicrobial Compounds: Emerging Trends in Research, Development, and Technologies. Antibiotics (Basel). 8(1):8.

Matin MM, Nakhaeizadeh H, Bahrami AR, Iranshahi M, Arghiani N, Rassouli FB. 2014. Ferutinin, an apoptosis inducing terpenoid from Ferula ovina. Asian Pac J Cancer Prev. 15(5):2123-2128.

Mogana R, Adhikari A, Tzar MN, Ramliza R, Wiart C. 2020. Antibacterial activities of the extracts, fractions and isolated compounds from Canarium patentinervium Miq. against bacterial clinical isolates. BMC Complement Med Ther. 20(1):55.

Naji Reyhani Garmroudi S, Karimi E, Oskoueian E, Homayouni-Tabrizi M, Iranshahi M. 2021. Ferutinin: A phytoestrogen from ferula and its anticancer, antioxidant, and toxicity properties. J Biochem Mol Toxicol.e22713.

Naz S, Ahmad S, Ajaz Rasool S, Asad Sayeed S, Siddiqi R. 2006. Antibacterial activity directed isolation of compounds from Onosma hispidum. Microbiol Res. 161(1):43-48.

Nazari ZE, Iranshahi M. 2011. Biologically active sesquiterpene coumarins from Ferula species. Phytother Res. 25(3):315-323.

Ochoa C, Solinski AE, Nowlan M, Dekarske MM, Wuest WM, Kozlowski MC. 2020. A Bisphenolic Honokiol Analog Outcompetes Oral Antimicrobial Agent Cetylpyridinium Chloride via a Membrane-Associated Mechanism. ACS Infect Dis. 6(1):74-79.

Omar A, Wright JB, Schultz G, Burrell R, Nadworny P. 2017. Microbial Biofilms and Chronic Wounds. Microorganisms. 5(1):9.

Penesyan A, Nagy SS, Kjelleberg S, Gillings MR, Paulsen IT. 2019. Rapid microevolution of biofilm cells in response to antibiotics. NPJ Biofilms Microbiomes. 5(1):34.

Puyo CA, Dahms TE. 2012. Innate immunity mediating inflammation secondary to endotracheal intubation. Arch Otolaryngol Head Neck Surg. 138(9):854-858.

Puyo CA, Peruzzi D, Earhart A, Roller E, Karanikolas M, Kollef MH, Krupnick AS, Kreisel D, Ibrahim M, Gelman AE. 2017. Endotracheal tube-induced sore throat pain and inflammation is coupled to the release of mitochondrial DNA. Mol Pain. 13:1744806917731696.

Rajasekar S, Park DJ, Park C, Park S, Park YH, Kim ST, Choi YH, Choi YW. 2012. In vitro and in vivo anticancer effects of Lithospermum erythrorhizon extract on B16F10 murine melanoma. J Ethnopharmacol. 144(2):335-345.

Rajbhandari M, Schoepke TH, Mentel R, Lindequist U. 2007. Antibacterial and antiviral naphthazarins from Maharanga bicolor. Pharmazie. 62(8):633-635.

Reigada I, Guarch-Pérez C, Patel JZ, Riool M, Savijoki K, Yli-Kauhaluoma J, Zaat SAJ, Fallarero A. 2020. Combined Effect of Naturally-Derived Biofilm Inhibitors and Differentiated HL-60 Cells in the Prevention of Staphylococcus aureus Biofilm Formation. Microorganisms. 8(11):1757.

Ren C, Wang J, Tan Y, Guo M, Guo J, Liu Y, Wu X, Feng Y. 2020. Synthesis, Characterization and Biological Evaluation of Magnolol and Honokiol Derivatives with 1,3,5-Triazine of Metformin Cyclization. Molecules. 25(24):5779.

Sakaue Y, Domon H, Oda M, Takenaka S, Kubo M, Fukuyama Y, Okiji T, Terao Y. 2016. Anti-biofilm and bactericidal effects of magnolia bark-derived magnolol and honokiol on Streptococcus mutans. Microbiol Immunol. 60(1):10-16.

Sandberg ME, Schellmann D, Brunhofer G, Erker T, Busygin I, Leino R, Vuorela PM, Fallarero A. 2009. Pros and cons of using resazurin staining for quantification of viable Staphylococcus aureus biofilms in a screening assay. J Microbiol Methods. 78(1):104-106.

Shen CC, Syu WJ, Li SY, Lin CH, Lee GH, Sun CM. 2002. Antimicrobial activities of naphthazarins from Arnebia euchroma. J Nat Prod. 65(12):1857-1862.

Silver LL. 2011. Challenges of antibacterial discovery. Clin Microbiol Rev. 24(1):71-109.

Solinski AE, Ochoa C, Lee YE, Paniak T, Kozlowski MC, Wuest WM. 2018. Honokiol-Inspired Analogs as Inhibitors of Oral Bacteria. ACS Infect Dis. 4(2):118-122.

Soltani S, Amin G, Salehi-Sourmaghi MH, Iranshahi M. 2019. Histone deacetylase inhibitory and cytotoxic activities of the constituents from the roots of three species of Ferula. Iran J Basic Med Sci. 22(1):93-98.

Syu WJ, Shen CC, Lu JJ, Lee GH, Sun CM. 2004. Antimicrobial and cytotoxic activities of neolignans from Magnolia officinalis. Chem Biodivers. 1(3):530-537.

Tamemoto K, Takaishi Y, Chen B, Kawazoe K, Shibata H, Higuti T, Honda G, Ito M, Takeda Y, Kodzhimatov OK et al. 2001. Sesquiterpenoids from the fruits of Ferula

kuhistanica and antibacterial activity of the constituents of F. kuhistanica. Phytochemistry. 58(5):763-767.

Trusheva B, Todorov I, Ninova M, Najdenski H, Daneshmand A, Bankova V. 2010. Antibacterial mono- and sesquiterpene esters of benzoic acids from Iranian propolis. Chem Cent J. 4:8.

Tufa T, Damianakos H, Graikou K, Chinou L. 2017. Comparative Study of Naphthoquinone Contents of Selected Greek Endemic Boraginaceae Plants - Antimicrobial Activities. Nat Prod Commun. 12(2):179-180.

Uruén C, Chopo-Escuin G, Tommassen J, Mainar-Jaime RC, Arenas J. 2020. Biofilms as Promoters of Bacterial Antibiotic Resistance and Tolerance. Antibiotics (Basel). 10(1).

Utegenova GA, Pallister KB, Kushnarenko SV, Özek G, Özek T, Abidkulova KT, Kirpotina LN, Schepetkin IA, Quinn MT, Voyich JM. 2018. Chemical Composition and Antibacterial Activity of Essential Oils from Ferula L. Species against Methicillin-Resistant Staphylococcus aureus. Molecules. 23(7):1679.

Vukic MD, Vukovic NL, Djelic GT, Popovic SL, Zaric MM, Baskic DD, Krstic GB, Tesevic VV, Kacaniova MM. 2017. Antibacterial and cytotoxic activities of naphthoquinone pigments from Onosma visianii Clem. Excli J. 16:73-88.

Wang DY, van der Mei HC, Ren Y, Busscher HJ, Shi L. 2019. Lipid-Based Antimicrobial Delivery-Systems for the Treatment of Bacterial Infections. Front Chem. 7:872.

Wu B, Fu SH, Tang H, Chen K, Zhang Q, Peng AH, Ye HY, Cheng XJ, Lian M, Wang ZL et al. 2018. Design, synthesis and antibacterial evaluation of honokiol derivatives. Bioorg Med Chem Lett. 28(4):834-838.

Xu J, Wold EA, Ding Y, Shen Q, Zhou J. 2018. Therapeutic Potential of Oridonin and Its Analogs: From Anticancer and Antiinflammation to Neuroprotection. Molecules. 23(2):474.

Xu L, Bi Y, Xu Y, Zhang Z, Xu W, Zhang S, Chen J. 2020. Oridonin inhibits the migration and epithelial-to-mesenchymal transition of small cell lung cancer cells by suppressing FAK-ERK1/2 signalling pathway. J Cell Mol Med. 24(8):4480-4493.

Xu S, Li D, Pei L, Yao H, Wang C, Cai H, Wu X, Xu J. 2014. Design, synthesis and antimycobacterial activity evaluation of natural oridonin derivatives. Bioorg Med Chem Lett. 24(13):2811-2814.

Xu S, Pei L, Li D, Yao H, Cai H, Wu X, Xu J. 2014. Synthesis and antimycobacterial evaluation of natural oridonin and its enmein-type derivatives. Fitoterapia. 99:300-306.

Yuan Z, Ouyang P, Gu K, Rehman T, Zhang T, Yin Z, Fu H, Lin J, He C, Shu G et al. 2019. The antibacterial mechanism of oridonin against methicillin-resistant Staphylococcus aureus (MRSA). Pharm Biol. 57(1):710-716.

Zeb A, Ullah F, Ayaz M, Ahmad S, Sadiq A. 2017. Demonstration of biological activities of extracts from Isodon rugosus Wall. Ex Benth: Separation and identification of bioactive phytoconstituents by GC-MS analysis in the ethyl acetate extract. BMC Complement Altern Med. 17(1):284.

Zhang JH, Chung TD, Oldenburg KR. 1999. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J Biomol Screen. 4(2):67-73.

Zhang S, Wang J, Xu W, Liu Y, Wang W, Wu K, Wang Z, Zhang X. 2015. Antibacterial effects of Traditional Chinese Medicine monomers against Streptococcus pneumoniae via inhibiting pneumococcal histidine kinase (VicK). Front Microbiol. 6:479.

Zhou Y, Xin F, Zhang G, Qu H, Yang D, Han X. 2017. Recent Advances on Bioactive Constituents in Ferula. Drug Dev Res. 78(7):321-331.

Zhu Y, Zhong Y, Long X, Zhu Z, Zhou Y, Ye H, Zeng X, Zheng X. 2019. Deoxyshikonin isolated from Arnebia euchroma inhibits colorectal cancer by down-regulating the PI3K/Akt/mTOR pathway. Pharm Biol. 57(1):412-423.

Zimmerli W, Moser C. 2012. Pathogenesis and treatment concepts of orthopaedic biofilm infections. FEMS Immunol Med Microbiol. 65(2):158-168.

Znati M, Filali I, Jabrane A, Casanova J, Bouajila J, Ben Jannet H. 2017. Chemical Composition and In Vitro Evaluation of Antimicrobial, Antioxidant and Antigerminative Properties of the Seed Oil from the Tunisian Endemic Ferula tunetana Pomel ex Batt. Chem Biodivers. 14(1).

Zuo GY, Zhang XJ, Han J, Li YQ, Wang GC. 2015. In vitro synergism of magnolol and honokiol in combination with antibacterial agents against clinical isolates of methicillin-resistant Staphylococcus aureus (MRSA). BMC Complement Altern Med. 15:425.