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Bacterial biofilm formation inhibitory activity revealed for plant derived natural compounds

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ABSTRACT

Use of herbal plant remedies to treat infectious diseases is a common practice in many countries in traditional and alternative medicine. However to date there are only few antimicrobial agents derived from botanics. Based on microbiological screening tests of crude plant extracts we identified four compounds derived from *Krameria*, *Aesculus hippocastanum* and *Chelidonium majus* that showed a potentially interesting antimicrobial activity. In this work we present an in depth characterization of the inhibition activity of these pure compounds on the formation of biofilm of *Staphylococcus aureus* as well as of *Staphylococcus epidermidis* strains. We show that two of these compounds possess interesting potential to become active principles of new drugs.

1. Introduction

Alarm is being increasingly raised by several international organizations dealing with public health, for the emergency of bacterial resistance as a health and social problem.[1–3] In fact multi-resistant bacteria endanger with increasing frequency the successful outcome of antibiotic therapy. Among the Gram positive bacterial pathogens *Staphylococcus aureus* (*S. aureus*) is clinically significant and the emergence of methicillin resistant variants represents a major clinical concern. The ability of staphylococci to adhere on both eukaryotic cells and abiotic surfaces and to form biofilm are important virulence factors in chronic infections associated with implanted biomaterials, which are particularly difficult to eradicate.[4] At the core of the problem there is the fact that the spread of drug resistant organisms has largely outpaced antibiotic research and development. In fact, in comparing the periods 1983–1987 and 2003–2008, FDA approval of new antibiotics decreased of 70%, with 16 drugs approved in the former and only 5 in the latter period. This trend in the last 30 years has been constantly decreasing.[3] The strategies to search for new antibiotics have been the subject of a number of excellent recent reviews.[5–7] In the quest for new molecules showing activity against bacterial infections, screening for new lead compounds is a common step. Compounds from natural origin still provide a high number of interesting structures, even in this era of combinatorial chemistry. These are used as lead compounds in pharmaceutical industry, that is, anticancer treatment.[8]

Considering that plants have already yielded compounds with inhibiting activities against Gram positive bacteria, acting as efflux pumps inhibitors (EPI),[9] and that the use of medicinal and herbal remedies to treat infectious diseases is common in many countries,[10] we are aiming at the discovery of new leads from plants, that can be used to develop drugs for human therapy in persistent infections. Our rationale is to look for new antimicrobials inhibiting virulence rather than bacterial growth; in fact this choice may impose a weaker selective pressure for the development of antibiotic resistance relative to current antibiotics.

With this objective we started a program exploring several plant extracts searching for specific antibacterial activity from fractionated pools. We considered extracts from *Krameria*, *Aesculus hippocastanum* and *Chelidonium majus* plants; these plants have proved to possess a plethora of active principles in diverse pathologies. *Krameria* plant is well known in medicine for its bronchodilator, antiviral, antioxidant and photoprotective properties due to the high production of lignans and neolignans.[11] *A. hippocastanum* is employed in clinical practice for the treatment of peripheral chronic venous insufficiency,[12] and its extracts have shown a protective effect against UV damage due to the strong antioxidant properties of proanthocyanidin.[12] *C. majus* L. is known as being useful for the treatment of many diseases in European countries and in Chinese herbal medicine. Several alkaloids and flavonoids components present both in its aerial and root parts exhibit multiple biological actions such as antiviral, antitumor, antibacterial/antifungal and anti-inflammatory effects.[13] From active fractions we purified and

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identified the compounds responsible for the antimicrobial activity. We tested their bacteriostatic and bactericidal action on the planktonic form and quantified their efficacy at inhibition of biofilm formation and growth on *S. aureus*, and *Staphylococcus epidermidis*. We also present preliminary proteomic evidence that correlates the peculiarities of the inhibition mechanism with the alteration of the surface bacterial proteome.

2. Materials and methods

2.1. Bacterial strains

Bacterial strains used in this work are: *S. aureus* ATCC 6538P (DSMZ 346), reference strain for antimicrobial testing, and *S. epidermidis* ATCC 35984 (DSMZ RP62A), reference strain isolated from infected catheter.

Brain Heart Infusion broth (BHI, Oxoid, UK) was used for *S. aureus* liquid cultures and Tryptic Soy Broth (TSB, Oxoid, UK) was used for *S. epidermidis* liquid cultures, respectively. Tryptic Soy Agar (TSA, Difco Laboratories, Detroit, MI, USA) was used for cultures on solid support.

Bacteria were stored at −80 °C in BHI supplemented with 15% glycerol.

2.2. Chemicals

Natural products used in this study are: Chelerythrine (CH, MW 383.82) and Sanguinarine (SA, MW 367.8) collectively termed QBAs, DiHydroxyBenzoFuran (DHBF, MW 226.2), proAnthocyanidin A2-phosphatidylCholine (proAc, MW 592.6) (Indena Spa., Milan, Italy); their chemical formulas are shown in Figure 1.

ProAc was isolated from *A. hippocastanum* following procedures previously described.[12] DHBF was ex novo synthesized using as model a molecule isolated from *Krameria lappacea*, the synthesis and purification has been described elsewhere.[14] CH and SA were purified from *Macleya cordata* using standard procedures for alkaloids.

CH was suspended in deionized water at 1.0 mg/ml (2.6 mM), while SA, DHBF and proAc were suspended in 100% ethanol (Sigma–Aldrich) at 1.0 mg/ml (respectively at 2.7, 4.4, 1.7 mM).

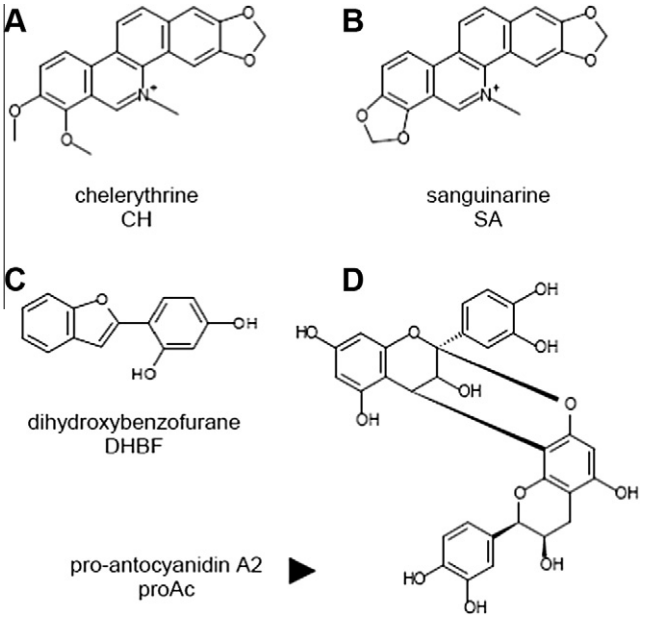

Figure 1. Structural formulas of the inhibitors used in this study, the positive charges in CH and SA are neutralized by chloride ion.

Commercial reagents: TPCK-treated trypsin, dithiothreitol (DTT), iodoacetamide (IAA) and alfa-cyano-4-hydroxycinnamic acid (Sigma); ammonium bicarbonate (Fluka); trifluoroacetic acid (TFA)-HPLC grade (Carlo Erba). All other reagents and solvents were from Baker.

2.3. Determination of minimal inhibitory concentration (MIC), and minimal bactericidal concentration (MBC)

MIC was determined following the guidelines of Clinical Laboratory Standards Institute. Natural compounds were added directly from mother stocks and solutions prepared by twofold serial dilutions. A total of 11 concentrations were used within the 100–0.1 μg/ml range. Experiments were performed in quadruplicate. The MIC was determined as the lowest concentration at which observable bacterial growth was inhibited. The MBC was determined by spreading 50 μl on TSA plate from the sample showing no visible growth and it was further incubated for 18 h at 37 °C.

2.4. Biofilm formation inhibition

Before biofilm formation experiments, bacterial growth curves were assessed at sub-MIC concentration for each compound to evidence their effects on the growth rate of *S. aureus* and *S. epidermidis*. We used SA at 8 μM, CH at 4 μM, DHBF at 14 μM, and proAc at 42 μM, both for *S. aureus* and *S. epidermidis* strains.

For all strains then, biofilm formation was assessed in static chamber system at 37 °C and planktonic cultures were grown at 37 °C under vigorous agitation (180 rpm).

Quantification of in vitro biofilm production was based on a previously described method,[15] where a sterile 24-well flat-bottomed polystyrene plate (Falcon) was filled with 900 μl/well of the appropriate medium, using only the four central body column wells. Hundred microliter of overnight bacterial cultures were added per well. The plates were incubated aerobically for 24 h at 37 °C. Growth was monitored by measuring the OD_{600}, and after 24 h incubation the ability of *S. aureus* and *S. epidermidis* strains to adhere the polystyrene plates was tested. The plates were then washed with sterile distilled water. The plates were stained with crystal violet for 5 min, excess stain rinsed off. The plates were air dried, the dye bound to adherent cells was resolubilized with 10% (v/v) glacial acetic acid and 10% (v/v) ethanol per well. The OD of each well was measured at 590 nm.

The inhibition data were interpreted using a Hill-type equation,[16] Figure 2, where EC50 (the effective concentration at which 50% inhibition of biofilm formation is observed) is obtained with a fitting procedure.

2.5. Surface protein extraction and processing

Cultures were grown up to 0.6 OD/ml in the presence of each compound at above defined concentration. Surface proteins were extracted following centrifugation of 50 ml of each bacterial culture (OD_{600} = 0.6). Pellets were washed with PBS and suspended in 500 μl in PBS containing 1% SDS. Samples incubated at 37 °C for 15 min and centrifuged, the supernatants collected and used for SDS–PAGE and zymogram analyses. The protein content in the samples was determined by the Bradford procedure.

2.6. SDS–PAGE and zymogram

SDS–PAGE was carried out by standard methods with SDS–polyacrylamide separating gel (10% acrylamide, pH 8.8). Following electrophoresis proteins were stained with Coomassie Brilliant Blue (BioRad). Denaturing SDS–PAGE was performed

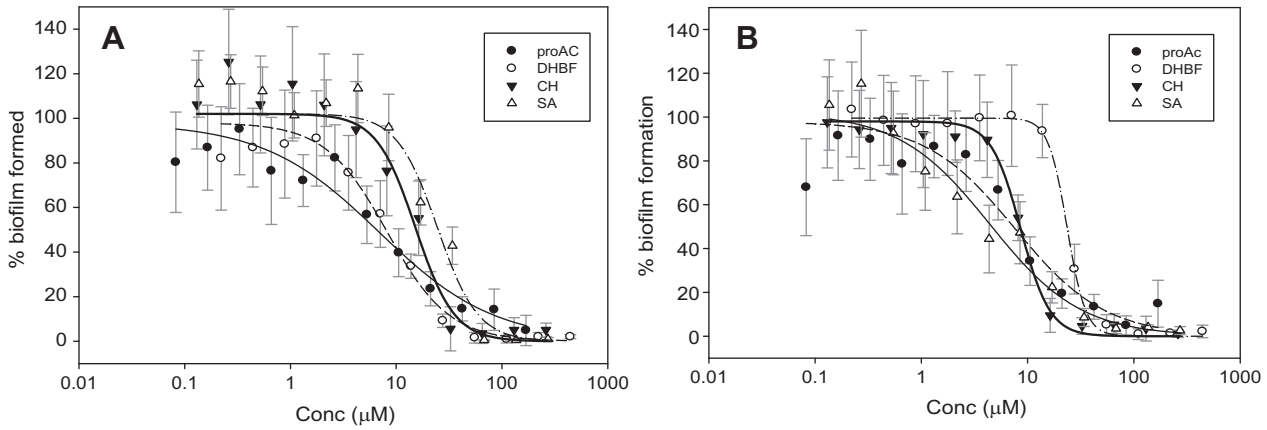

Figure 2. Inhibitory action of proAc, DHBF, CH and SA, exerted on the ex novo biofilm formation on *S. aureus* 6538P (panel A) and on *S. epidermidis* RP62A (panel B). Standard deviations from 4 replicates are represented as error bars in dark gray. The fitting curves shown on the curves were obtained with a single step inhibition model and fitted with the equation "*f* = {%biofilm formation/[1 + (C/EC50)^*slope*]}"; where *C* is the actual concentration (μM) of the inhibitor present in the well. In a Hill-type equation *slope* value empirically accounts for the cooperativity of the process. The parameters resulting from the fitting, EC50 and *slope*, are reported in Table 1.

according to Lauderdale,[17] with some modifications. SDS–polyacrylamide separating gel containing 0.2% (wt/v) lyophilized *Micrococcus luteus* cells provided by Sigma, used to detect the lytic activities. After electrophoresis, the gels were soaked (2 times, 15 min) in distilled water at room temperature. The gels were then transferred into renaturing buffer (50 mM Tris–HCl pH 8.0 containing 1% Triton X-100) and shaken at 60 rpm for 2 h at 37 °C to allow renaturation. The renatured autolysins appeared as clear translucent bands on opaque background. For each experiment, two gels were prepared from the same stock solution and electrophoresed in the same apparatus at the same time. No differences in the migration of the standards due to the presence of *M. luteus* cells in the gel were noted.

2.7. In situ digestion

Comassie blue-stained protein bands were excised from the gel and washed with bidistilled MilliQ water, acetonitrile (ACN) and then 0.1 M ammonium bicarbonate. Protein samples were reduced with 10 mM DTT for 45 min at 56 °C, free cysteines were alkylated by treatment with 55 mM iodoacetamide for 30 min at room temperature in dark. Gel particles were washed with ACN and ammonium bicarbonate. Tryptic digestion was carried out using 12.5 ng/μl of enzyme in 10 mM ammonium bicarbonate pH 7.8 at 4 °C for 2 h. Excess of trypsin solution was then removed and samples were incubated with 50 μL of 10 mM ammonium bicarbonate pH 7.8 for 18 h al 37 °C. Peptides were extracted washing the gel particles with 10 mM ammonium bicarbonate and 0.1% formic acid in 50% ACN at room temperature.

2.8. Protein identification

The peptide mixtures were analyzed by LC–MS/MS using a CHIP-QTOF 6520 equipped with a capillary 1200 HPLC system and a chip cube (Agilent Technologies, Palo Alto, CA). After loading, each peptide mixture (8 μl in 0.2% formic acid) was first concentrated and washed at 4 μl/min in 40 nl enrichment column (Agilent Technologies chip), with 0.2% formic acid in 2% acetonitrile as eluent. Each sample was fractionated on a C18 reverse-phase capillary column (75 × 43 mm in the Agilent Technologies chip) at flow rate of 400 nl/min, with a linear gradient of eluent B (0.1% formic acid in 95% acetonitrile) in A (0.1% formic acid in 2% acetonitrile) from 7% to 60% in 50 min. Peptide analysis was performed using data-dependent acquisition of one MS scan (mass range from 300 to 2000 m/z) followed by MS/MS of the 3 most abundant ions. Raw data from nano LC–MS/MS analyses were employed to search non-redundant protein databases (NCBI, with *Other Firmicutes* as appropriate taxonomy restriction) using in-house MASCOT software (Matrix Science, Boston, USA).

3. Results

3.1. MIC and MBC determination

In vitro bacteriostatic and bactericidal activities of the four selected natural compounds were evaluated on bacterial strains by broth microdilution methods.

Results are schematized in Table 1. The activity profile of each single compound was similar on both strains, with a slight variation on the actual dosage at which the effect was found. ProAc showed no bacteriostatic and no bactericidal activity within the range of concentrations explored. CH and DHBF exhibit similar bacteriostatic activity on both strains, within the range 16.3–32.6 and 55.3–110.6 μM, respectively. SA was fourfold more effective on *S. epidermidis* (8.5–17 μM) than on *S. aureus* (34.0–68.0 μM). Both SA and DHBF showed a marked bactericidal activity regardless of the strain and considering the actual molar concentrations, SA action results roughly twofold more effective than DHBF (34.0–68.0 vs 55.0–110.0 μM).

3.2. Biofilm inhibition

Preliminary experiments were carried out to assess the effects of molecules at sub-MIC concentration, on the planktonic growth rate of *S. aureus* and *S. epidermidis*. Results obtained showed that these molecules did not affect staphylococci duplication rate. Bacterial growth curves were superimposable both in the presence and in the absence of natural compounds (see Supplementary data).

The dose–response experiments of biofilm formation inhibition (Fig. 2) and mature biofilm disruption (Fig. 3) were evaluated for each compound on *S. aureus* (A) and *S. epidermidis* (B). The EC50 value is taken as the concentration at which 50% of the biofilm formation is inhibited, while the *slope* is related to the smoothness of the response around the transition from 100 to 0% of biofilm formation, Table 1.

All four compounds affected biofilm formation, with comparable efficacy, in fact the inhibition ranged between 1.3 and 5.5-fold of the strongest inhibitory effect (SA on *S. epidermidis*). On *S. aureus*, SA and CH show a similar inhibitory action with EC50 values of 24.5 and 15.2 μM, respectively, while DHBF and proAc were more

Table 1
Antibacterial activity of Indena compounds

		S. aureus 6538P				*S. epidermidis* RP62A			
MW	Compound	MIC [μM][a]	MBC [μM][a]	EC50 (μM)[b]	*Slope*	MIC [μM][a]	MBC [μM][a]	EC50 (μM)[b]	*Slope*
383.8	CH	16.3; 32.6	>260	15.2 ± 2.3	2.4 ± 0.7	16.3; 32.6	>260	8.6 ± 0.4	3.1 ± 0.5
367.8	SA	34.0; 68.0	34.0; 68.0	24.5 ± 3.6	2.3 ± 0.7	8.5; 17.0	34.0; 68.0	4.4 ± 1.3	1.0 ± 0.2
226.2	DHBF	55.3; 110.6	55.3; 110.6	8.2 ± 1.2	1.5 ± 0.3	55.3; 110.6	55.3; 110.6	23.5 ± 0.6	4.9 ± 0.6
592.6	proAC	>168.7	>168.7	6.9 ± 2.4	0.8 ± 0.2	>168.7	>168.7	7.6 ± 2.7	1.0 ± 0.3

[a] MIC and MBC were determined using CLSI guidelines.
[b] EC50 is referred to biofilm formation.

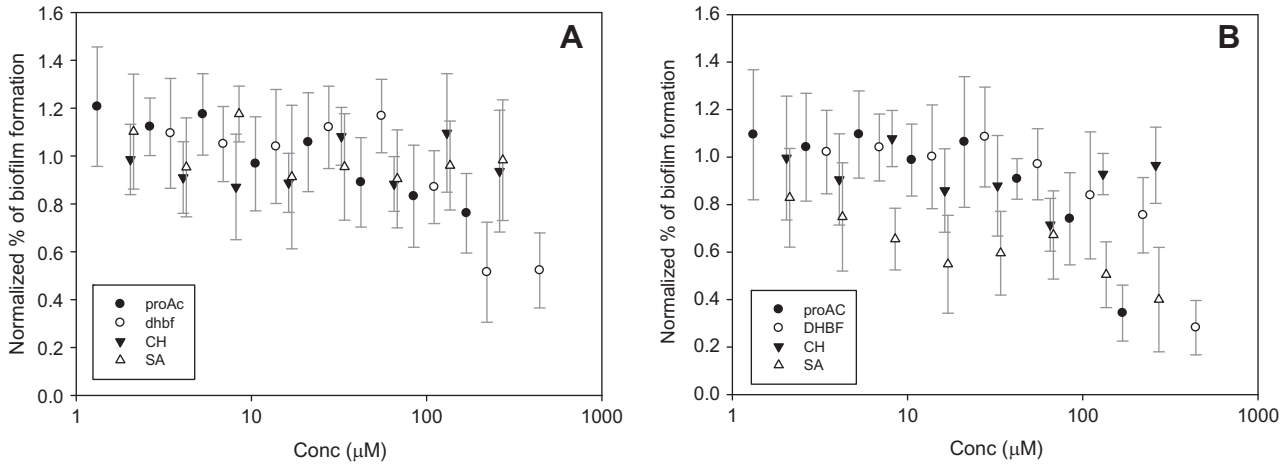

Figure 3. Inhibitory action of proAc, DHBF, CH and SA, exerted on the *24 h pre-formed* biofilm on *S. aureus* 6538P (panel A) and on *S. epidermidis* RP62A (panel B). Standard deviations from 4 replicates are represented as error bars in dark gray. Data have been normalized with untreated control readouts.

effective, EC50 = 8.2 and EC50 = 6.9 μM, respectively. *S. epidermidis* RP62A was more sensitive to the compounds, with CH, SA and proAc inhibiting at EC50 = 8.6, 4.4 and 7.6 μM, respectively; while the DHBF was less effective with EC50 = 23.5 μM.

The *slope* parameter denotes that only proAc exerts a noncooperative inhibitory action on both strains examined with a value of 0.8 and 1.0 (and, taking into account the SD, the difference is not meaningful). On the contrary CH and SA show a similar highly cooperative effect on *S. aureus* with *slope* value of 2.4 and 2.3, respectively. DHBF shows only a very weak cooperative effect on *S. aureus* with a *slope* value of 1.5. *S. epidermidis* was much more sensitive to the cooperative effect, with CH and DHBF *slope* values of 3.1 and 4.9, respectively; while both the SA and the proAc inhibition effects were noncooperative.

3.3. Surface protein pattern following the treatment with inhibitors

Cell surface protein samples from treated and untreated cultures of both strains were simultaneously analyzed by SDS–PAGE and zymogram assays. The SDS–PAGE profiles of the protein mixtures obtained from *S. aureus* (Fig. 4) and *S. epidermidis* (Fig. 5) following colloidal Coomassie blue staining (A) and after the autolytic pattern analysis (B) are shown.

S. aureus protein profiles showed slight modifications after inhibitor treatment but for DHBF treatment were no evident modification was observed, Figure 4A. Conversely, *S. epidermidis* protein profiles showed a dramatic disappearance of most of protein bands after incubation with SA and proAc, Figure 5A.

The autolytic profiles of treated and untreated surface proteins extracted from both bacterial strains, shown in the zymograms of Figures 4 and 5(B panels), were in accordance with the results observed from SDS–PAGE analysis. Treatment with SA, CH, DHBF and proAc produced no significant variation in the zymograms of *S. aureus* and *S. epidermidis* except for proAC treated *S. epidermidis* cells, where a complete disappearance of the bands in the zymogram was observed. To exclude artefacts this result has been confirmed three times.

3.4. Protein identification

Since the inhibitors affected several but still discrete number of proteins in *S. aureus*, while the effect on *S. epidermidis* appeared broader and more unspecific, we performed the proteomic analysis only on the former. In Figure 6 are evidenced the bands corresponding to the proteins extracted from untreated *S. aureus* (lane 1) and from inhibitor treatment (lanes 2–5). Black dots at the right of the corresponding band evidence variations.

The bands showing variations were excised from the gel and submitted to identification by mass spectrometry.

Treatment with SA and CH alters the levels of proteins belonging to different bacterial pathways. In particular the chaperone protein HchA involved in heat shock response (A8YZP8), the formate acetyltransferase (Q7A1W9), and malonyl CoA-acyl carrier protein transacylase (Q7A124) involved in lipids metabolism, disappeared. Furthermore some proteins involved in protein synthesis were impaired, such as threonine dehydratase (Q7A5L8) and branched-chain-amino-acid aminotransferase (P63513) after SA treatment and D-alanine aminotransferase (P99090) and cysteine synthase (P63872) after CH treatment, respectively.

Other proteins influenced by treatment with QBAs were related to bacterial cytoskeleton: cell-division protein (Q7A1R9) and DNA topoisomerase 4 subunit B (P66939) after SA treatment, and cell-division protein FtsA (P63765) after CH treatment.

SA treatment also impairs the thioredoxin system (thioredoxin reductases Q9KX07 and Q99U10).

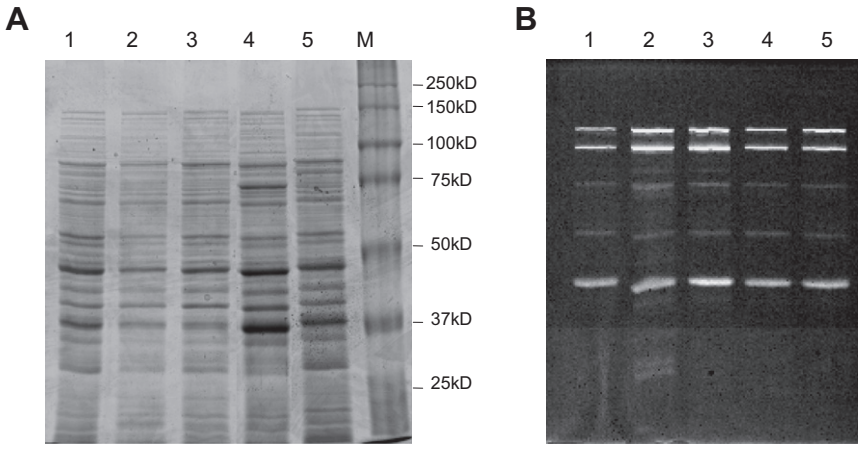

Figure 4. SDS–PAGE and zymogram analyses of *S. aureus* 6538P surface proteins. Crude cell envelope SDS extracts from treated and untreated (control) *S. aureus* 6538P cells analyzed by SDS–PAGE (left panel) and zymogram assay (right panel). The apparent molecular masses of standard indicated are in kDa. Autolysins formed translucent areas in the zymogram. 1: untreated sample; 2: SA-treated sample; 3: CH-treated sample; 4: proAc-treated sample; 5: DHBF-treated sample; *M*: Molecular weight marker (BioRad).

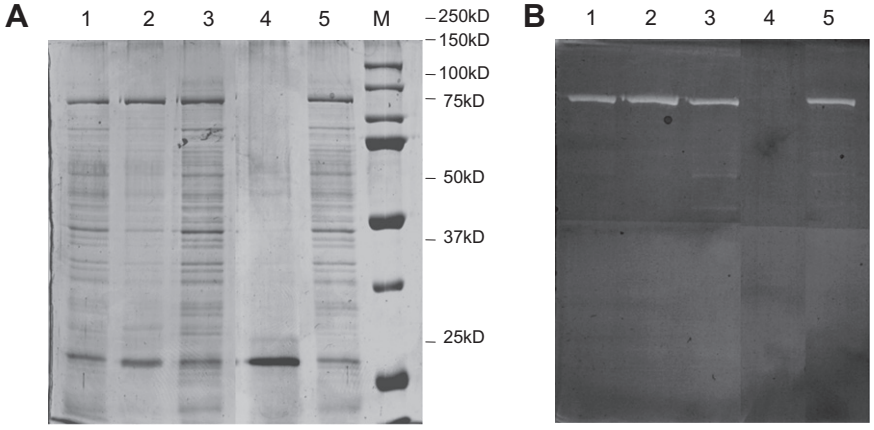

Figure 5. SDS–PAGE and zymogram analyses of *S. epidermidis* RP62A surface proteins. Crude cell envelope SDS extracts from treated and untreated (control) *S. aureus* 6538P cells analyzed by SDS-PAGE (left panel) and zymogram assay (right panel). The apparent molecular masses of standard indicated are in kDa. Autolysins formed translucent areas in the zymogram. 1: untreated sample; 2: SA-treated sample; 3: CH-treated sample; 4: proAc-treated sample; 5: DHBF-treated sample; *M*: Molecular weight marker (BioRad).

QBAs molecules determine the disappearance of a large number of enzymes implicated in carbohydrate metabolism and ATP synthesis (ATP synthase gamma chain Q7A0C5). Proteins implicated are: pyruvate kinase (Q7A0N4), phosphoenolpyruvate carboxykinase (P0C1S4), phosphoglucomutase (Q8NUV4), 6-phosphofructokinase (P65695), glucokinase (Q99TU7), glycerophosphoryl diester phosphodiesterase (Q99UY3) after SA treatment; glyceraldehyde-3-phosphate dehydrogenase 1 (P09A37), phosphoenolpyruvate-protein phosphotransferase (Q99V14), pyruvate kinase (Q7A0N4), glucokinase (Q99TU7), 6-phosphogluconate dehydrogenase (P63335), alcohol dehydrogenase (Q8NXU1) after CH treatment.

ProAc treatment impairs in addition to the chaperone protein HchA (A8YZP8) also cysteine synthase (P63872), an iron-binding protein (Q99W03) and the penicillin binding protein 2 (Q53724).

Following DHBF treatment a single protein band disappeared, identified as the serine proteinase Do (Q99TD6).

4. Discussion

Despite many conventional drugs originate from natural products, only few antimicrobial agents derive from plants. Since most of the conventional drugs of natural origin derive from microorganisms.[7] Thus, searching for new antibacterial agents derived from plants appears a promising approach as shown by an increasing number of reports in this field.[18–20]

Sparse prior evidence exists that the plants used in our study may contain principles active against microbial pathogens, that is, a botanically derived medicament consisting of the dried root of three species of *Krameria* was used as antimicrobial remedy, moreover Peruvian rhatany root was anciently used as toothbrush/toothpaste by peruvian women, suggesting some benefit respect to microbial plaque. These facts prompted us to analyze the molecular principles that could be responsible for this activity. From preliminary microbiological assays probing fractions of compounds extracted from the *K. lappacea* (Dombey) Burdet (para or brazilian rhatany), we isolated a dihydroxybenzofuran (DHBF) derivative, as a possible active compound.

C. majus is listed as one of the most active antimicrobial plants in a screening study,[21] it has been shown that crude extracts *C. majus* exhibited antibacterial activity against *S. aureus* ATCC 25923.[22] From the preliminary microbiological assays probing fractions of compounds extracted from *A. hippocastanum* and *C. majus* we

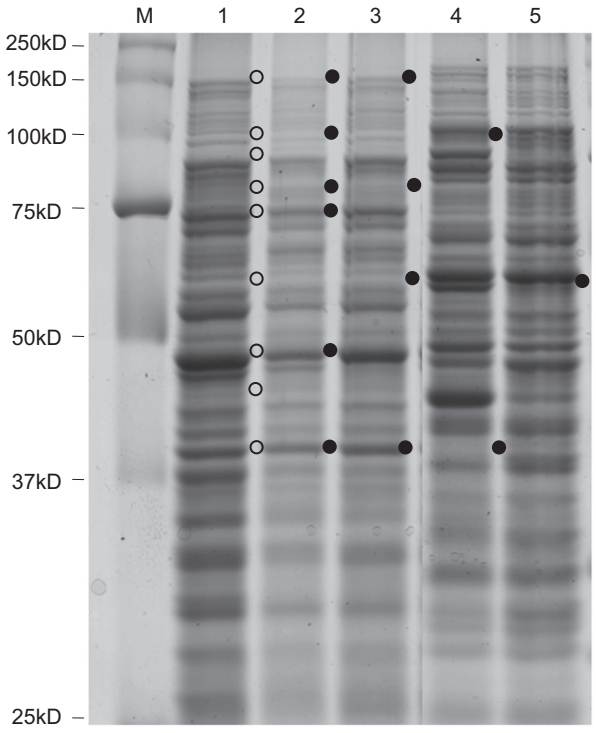

Figure 6. SDS–PAGE of *S. aureus* 6538P surface proteins. *M*: Molecular weight marker (BioRad); 1: untreated sample; 2: SA-treated sample; 3: CH-treated sample; 4: proAc-treated sample; 5: DHBF-treated sample. Protein bands indicated with dots correspond to the bands identified by mass spectrometry analysis. We identified protein bands present in untreated samples that completely disappeared following the treatment (empty dots). As control, the treated samples were cut in correspondence of the disappeared bands to confirm the absence of the identified proteins (black dots).

identified and successively isolated the proAc from the former plant and the CH and the SA alkaloids from the latter plant to study their antimicrobial activity.

Both DHBF and SA had a similar marked bactericidal effect. Interestingly both DHBF and SA were able to inhibit biofilm accumulation in *S. aureus* at concentrations between 1.4 and sixfold lower than those corresponding to MIC/MBC, Figure 2A and Table 1. Moreover growth curves in the presence and in the absence of sub-MIC concentration of both compounds did not show any difference. Thus, it can be inferred that these compounds are able to inhibit the biofilm formation through a mechanism different from mere killing bacterial cells in the planktonic form. They would rather prevent *S. aureus* transition to the sessile phenotype. On mature biofilm both DHBF and SA are not effective, Figure 3. Both SA and DHBF exhibit an inhibitory activity of de novo biofilm formation in *S. epidermidis* at a concentration about twofold lower than the MIC and MBC range. SA also shows an inhibitory activity on the mature biofilm, DHBF shows no such activity at all (Fig. 3B).

A different behavior was noticed for CH and proAc: both displayed no appreciable bactericidal activity. CH has an inhibitory activity on planktonic growth (with a MIC 16–32 μM) both on *S. aureus and S. epidermidis*, no such activity was found for proAc (Table 1). However, both compounds are quite effective at inhibiting the sessile growth of *S. aureus* and *S. epidermidis*. The proAc seems to be the best inhibitor performing similarly on both strains, CH inhibits biofilm formation better in *S. epidermidis* than in *S. aureus*. While CH and proAc are good inhibitors of the de novo biofilm formation they display no meaningful inhibitory activity on the mature biofilm (Fig. 3A and B).

In Table 1 is also reported the value of the *slope* of the fittings shown in Figure 2. The steepest *slope* is presented by DHBF inhibiting *S. epidermidis* (*slope* 4.9), while only a modest cooperative effect is shown on *S. aureus* (*slope* 1.5). CH also displays a cooperative attitude in inhibiting biofilm formation in *S. epidermidis* (*slope* 3.1) and in *S. aureus* (*slope* 2.4). On the contrary SA shows no cooperativity on *S. epidermidis* (*slope* 1.0) and an elevated cooperativity at inhibiting *S. aureus* biofilm (*slope* 2.3). ProAc is the only compound that shows non cooperative effect on both bacterial species. The value of the *slope* is likely related to the mechanism through which inhibition is achieved. To give a first glimpse into which pathway could be affected by the inhibitory action of these natural compounds we performed proteomic experiments. We find that treatment of *S. aureus* with SA, CH and proAc downregulates some important proteins belonging to different pathways. Of note is that even if there are several cell surface proteins affected, the vast majority are cytoplasmic proteins. Thus hinting that these compounds are cell penetrating and that they are likely to affect intracellular processes.

In particular following the treatment with SA and CH, some proteins involved in heat shock response (chaperone protein HchA), surface exposed lipids, methoxy–mycolic acid synthase and protein synthesis disappeared. These data are in agreement with recent results obtained from transcriptome analysis of *Mycobacterium tuberculosis* after the treatment with CH[23] the inflammatory response already described for benzophenanthridine alkaloid molecules.[24]

Both SA and CH also act on some elements of the bacterial cytoskeleton. This structural compart has been recognized as a potential target for antimicrobial therapy and inhibitors of cytoskeletal proteins may function as lead compounds for the development of novel antimicrobials.[25] SA, for example, has been found inhibiting the assembly of FtsZ in both *Escherichia coli* and *Bacillus subtilis*.[26]

Following the treatment with either one benzophenanthridine alkaloid molecule, a large number of enzymes implicated in carbohydrate metabolism (see Section 3) and ATP synthesis (ATP synthase gamma chain) were affected. Currently it is in phase two clinical trial a molecule (TMC207) which blocks the enzyme rotational motor inhibiting proton translocation and the coupled ATP biosynthesis.[27] It is tempting to speculate that both CH and SA could have a similar effect, since this would impair all energy consuming processes of bacterial cells, blocking growth both in planktonic and sessile phases, thus accounting for the observed inhibition response.

Furthermore, ATP synthase is controlled by a complex mechanism of allosteric feedback; our finding that CH and SA have a significant cooperative effect (*slope* >2) during biofilm inhibition, suits well with a possible impact on the regulation of this enzyme.

Besides the similarities, the main difference between SA and CH action has been found in the thioredoxin reductase enzyme downregulation. The disappearance of this enzyme within SA treated bacteria, accounts for its bactericidal effect. In fact, thioredoxin system, together with glutathione/glutaredoxin system, are deputed to the preservation of the balance of the reducing conditions into the bacterial cytoplasm.[28] Mediator molecules of the inflammatory response have notoriously an effect on this latter and SA is reported to have a strong oral anti-inflammatory effect in human therapy.[24]

Similarly, proAc treatment impaired some proteins involved in inflammatory response, such as chaperone protein HchA, cysteine synthase and an iron-binding protein. The downregulation of the iron binding protein could result into the impairment of iron uptake. Iron is an essential micronutrient for bacterial growth, in particular depletion of iron blocks the switch process from vegetative (planktonic) to sessile,[29] accounting for the strong biofilm inhibition observed.

Treatment with proAc also acts on a penicillin-binding protein by determining its complete disappearance. Penicillin-binding proteins (PBPs) are involved in the synthesis of peptidoglycan. These

enzymes are known targets of the β-lactam antibiotics.[30] For proAc the downregulation of PBP could induce a similar but not entirely identical effect, which could act in a synergic way with the iron blockage as a deterrent to biofilm formation.

Zymogram analysis showed that none of the compounds had any major effect on autolysin pattern, except for proAc on *S. epidermidis*. In fact, in this case the total disappearance of autolytic bands was observed, suggesting that proAc biofilm inhibition mechanism correlate with the autolysins ablation.

Following DHBF treatment, bacteria were effectively killed, and since no clear mechanistic evidence is deduced from proteomic results we did not further pursue this compound.

In conclusion we have shown that both proAc and, if at appropriate dosing, CH are molecules which fulfill the requirements for inhibition of de novo biofilm formation without bactericidal activity. They represent thus promising scaffolds to use for further development of antibacterial drugs which may overcome the insurgence of resistance.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.11.052.

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