Ellagic Acid Derivatives from *Rubus ulmifolius* Inhibit *Staphylococcus aureus* Biofilm Formation and Improve Response to Antibiotics

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Abstract

**Background:** Biofilms contribute to the pathogenesis of many forms of *Staphylococcus aureus* infection. Treatment of these infections is complicated by intrinsic resistance to conventional antibiotics, thus creating an urgent need for strategies that can be used for the prevention and treatment of biofilm-associated infections.

**Methodology/Principal Findings:** This study demonstrates that a botanical natural product composition (220D-F2) rich in ellagic acid and its derivatives can limit *S. aureus* biofilm formation to a degree that can be correlated with increased antibiotic susceptibility. The source of this composition is *Rubus ulmifolius* Schott. (Rosaceae), a plant used in complementary and alternative medicine in southern Italy for the treatment of skin and soft tissue infections. All *S. aureus* clonal lineages tested exhibited a reduced capacity to form a biofilm at 220D-F2 concentrations ranging from 50–200 μg/mL, which were well below the concentrations required to limit bacterial growth (530–1040 μg/mL). This limitation was therapeutically relevant in that inclusion of 220D-F2 resulted in enhanced susceptibility to the functionally-distinct antibiotics daptomycin, clindamycin and oxacillin. Testing with kidney and liver cell lines also demonstrated a lack of host cell cytotoxicity at concentrations of 220D-F2 required to achieve these effects.

**Conclusions/Significance:** These results demonstrate that extract 220D-F2 from the root of *Rubus ulmifolius* can be used to inhibit *S. aureus* biofilm formation to a degree that can be correlated with increased antibiotic susceptibility without toxic effects on normal mammalian cells. Hence, 220D-F2 is a strong candidate for development as a botanical drug for use in the prevention and treatment of *S. aureus* biofilm-associated infections.

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Introduction

*Staphylococcus aureus* is arguably the most problematic of all bacterial pathogens owing in large part to the persistent emergence of antibiotic resistant strains. This is evident in the recent appearance of methicillin-resistant strains even among isolates causing community-acquired infections [1,2,3]. While this has created an urgent need for new antimicrobial agents, many *S. aureus* infections are recalcitrant to antimicrobials even in the absence of issues related to acquired-antibiotic resistance. A primary contributing factor to this recalcitrance is the formation of a biofilm. Indeed, the National Institutes of Health estimates that 80% of all bacterial infections are biofilm related [4]. In addition to their impact on antimicrobial therapy, bacteria within biofilms reach a much higher density (10¹¹ CFU/mL) than their planktonic counterparts (10⁹ CFU/mL) [5], thus increasing the opportunity for gene transfer and the emergence of strains with new resistance and/or virulence profiles. The presence of a foreign body decreases the minimal infecting dose of *S. aureus* >100,000-fold [6], thus significantly increasing the chances of a biofilm-associated infection by comparison to individuals without such devices. In most cases, treatment of such biofilm-associated infections requires both aggressive antimicrobial treatment and surgical debridement to remove all infected tissues and/or indwelling medical devices [7,8].

The paradigm of biofilm resistance presents a major hurdle for the treatment of many types of infectious disease, including dental
caries, mastitis, otitis media, endocarditis, chronic wounds, and osteomyelitis [5]. The economic burden of these infections is tremendous with biofilm-associated infections leading to longer hospital stays, recurrent infection, and increased fatalities in the most recalcitrant cases. In total, up to 17 million new biofilm infections occur each year in the US, resulting in up to 550,000 fatalities annually [9,10]. These statistics emphasize the fact that, while there is indeed a pressing need for new antibiotics, there is an equally urgent need to develop agents that could be used to limit biofilm formation to a therapeutically relevant degree. Such agents could be used to prevent colonization or as adjunct therapy to enhance the therapeutic efficacy of conventional antibiotics [10,11].

Taking an ethnobotanical approach to drug discovery [12] offers considerable promise in many clinical contexts including infectious disease. Indeed, studies on botanical complementary and alternative (CAM) therapies have led to the discovery of novel anti-virulence agents. For instance, proanthocyanidins from Fazcinum macrocarpon (cranberry) disrupt adhesion of F-imbrillated E. coli to uroepithelial cells [13,14], accounting for efficacy in preventing recurrent urinary tract infections [15]. Garlic extract (Allium sativum) attenuates virulence by inhibiting hyphae formation in Candida albicans [16] and by blocking quorum sensing in Pseudomonas aeruginosa [17,18,19]. In S. aureus, inhibitors of quorum sensing, biofilm formation, and the NorA efflux pump have all been isolated from botanical sources [20,21,22,23,24,25,26,27,28,29]. Natural products offer a distinct advantage over their synthetic counterparts due to their rich structural diversity, chirality, and extensive functional group chemistry [24]. Thus, plants are a likely source of the next generation of anti-infectives [30].

Rubus ulmifolius Schott., Rosaceae (Elmleaf blackberry) is a wild shrub native to the Mediterranean. A limited number of published studies that have examined the antibacterial properties of R. ulmifolius. For example, Flamini et al. [31] identified an anthrone (Rubanthrone A) in the leaves, branches and flowering tops of R. ulmifolius that had an MIC of 4,500 µg/mL against S. aureus (ATCC 25923). Panizzi et al. [32] examined a crude extract of R. ulmifolius leaves, flowering tops and branches as well as subsequent extracts of increasing polarity for antibacterial activity using the same S. aureus strain and found that the greatest activity was present in the crude extract. The importance of R. ulmifolius as a traditional medicine was highlighted in an ethnobotanical field study conducted in rural communities of southern Italy [33]. In particular, the fresh leaves are topically applied with pork fat in the treatment of skin and soft tissue infections (SSTI) and a decoction of the roots is used as a wash to prevent hair loss. In a screening study that followed this fieldwork, ethanolic extracts from 104 Italian plants were assessed for their anti-biofilm potential and extracts from R. ulmifolius and nine other species were found to show some promise [28]. Here, we expanded on this work to determine whether bioassay-guided fractionation could be used to isolate a more potent extract of R. ulmifolius and whether this extract could limit biofilm formation in genotypically and phenotypically-diverse strains of S. aureus to a degree that can be correlated with increased antibiotic susceptibility.

Results

Bioassay-guided fractionation of the active constituents

Fractionation of the crude ethanolic root extract was done as illustrated in Fig. 1. The activity of each fraction was assessed based on the ability to limit biofilm formation in a microtiter plate assay using the methicillin-sensitive (MSSA) strain UAMS-1 and the methicillin-resistant (MRSA) USA300 isolate FPR3757 (UAMS-1782). The greatest degree of inhibition was observed in the butanol partition of the aqueous phase obtained after successive extraction with hexane and ethyl acetate (Fig. 2). Further partitioning of the butanol extract with MeOH:CH2Cl2 demonstrated that the greatest activity was observed with fractions ranging from 40:60 to 60:40 MeOH:CH2Cl2 (220D-F2, 220D-F3 and 220D-F4). In fact, all three of these fractions limited biofilm formation in this assay in a dose dependent manner and to a degree that exceeded that observed with mutation of sarA (Fig. 2). Moreover, biofilm assays confirmed that the activity in these extracts was increased by comparison to the crude extract while activity in other MeOH:CH2Cl2 extracts was decreased. This was true with both UAMS-1 and UAMS-1782 although at the lowest concentration tested (50 µg/mL inhibition was slightly greater with the former by comparison to the latter (Fig. 2). While comparable inhibition was observed with the 220D-F2, 220D-F3 and 220D-F4 partitions, overall yield was greatest with 220D-F2 with a percent yield of 0.329% of dry root weight (3.29 g per kg of dry root weight). For this reason, 220D-F2 was selected for subsequent experiments.

A preparative reversed-phase high-performance liquid chromatography (RP-HPLC) method was developed to further separate 220D-F2 into four fractions. Bioassays for biofilm inhibition with these fractions revealed that no single fraction was more effective than 220D-F2 itself, suggesting that more than one fraction was necessary for the anti-biofilm activity (Fig. 3). Thus, additional experiments were conducted in which fractions were combined in all possible permutations and tested for activity (data not shown). The only combination in which activity was restored was when all four fractions were recombined (Fig. 3). These results were also compared with 220D-F2 before and after running through the HPLC system to determine if the separation protocol itself had any effect on the activity. No significant difference between samples could be determined. These data suggest that the anti-biofilm effect of 220D-F2 is due to the synergistic activity of multiple compounds, and that the relative proportions of this mixture is important to the activity.

Identification of Active Constituents

LC-UV/MS/MS (liquid chromatography-ultraviolet absorption-tandem mass spectrometry) studies on 220D-F2 revealed the presence of ellagic acid (EA) and several ellagic acid derivatives (EADs) or sapogenin-related compounds. Molecular formulas of C14H6O8, C20H16O12, C19H14O12 were identified and confirmed with accurate mass measurements (<3 ppm). Possible structures for three of these components (C14H6O8, C20H16O12, C19H14O12) are shown in Fig. 4. Neutral loss of a pentose group (m/z 132) was observed for peaks 1 (m/z 435) and 3 (m/z 449). A product ion of m/z 303 was observed for both of these peaks suggesting that both components contain an ellagic acid core. A similar product ion was not observed for peaks corresponding to molecular formulae C20H16O7, and C19H14O7 suggesting that these compounds do not contain an ellagic acid core. Likely structures of minor constituents in 220D-F2 could not be determined from these studies.

Prophylactic efficacy of 220D-F2 in limiting S. aureus biofilm formation

The ability of 220D-F2 to limit biofilm formation was assessed against 15 genotypically-diverse clinical isolates of S. aureus. The MBIC50 for these strains ranged from 50–200 µg/mL (Fig. 5). The MIC and MBC was also determined for all 15 strains, with the MIC50 ranging from 330–1040 µg/mL and the MBIC50 from 530–2040 µg/mL (Table 2). The observation that the MBIC50 was at
least 2-fold lower than the MIC\textsubscript{90} and at least 4-fold lower than the MBC\textsubscript{90} indicates that the inhibition of biofilm formation observed with 220D-F2 was due to something other than growth limitation. This was confirmed by demonstrating that, while the presence of the extract did delay entry into the exponential growth phase by \( \sim \)2 hours, cultures with and without 220D-F2 reached the same density within 12 hours and maintained the same cell counts throughout the stationary growth phase (Fig. 6).

The ability of 220D-F2 to inhibit biofilm formation was also confirmed by confocal microscopy (Fig. 7). At concentrations as low as 50 \( \mu \)g/mL, the degree of inhibition observed with 220D-F2 was comparable to that associated with mutation of \textit{sarA} in both UAMS-1 and the USA300 isolate UAMS-1782 at concentrations as low as 50 \( \mu \)g/mL. More specifically, the untreated wild-type strains formed uniform biofilms with a thickness ranging from 88–92 \( \mu \)m while the isogenic \textit{sarA} mutants and parent strains exposed to 220D-F2 formed very patchy biofilms with a few isolated clumps of adherent cells.

Therapeutic efficacy of 220D-F2 in removal of established \textit{S. aureus} biofilms

The microtiter plate assay used in the experiments discussed above does not lend itself easily to studies assessing relative antibiotic susceptibility. To address this, we employed an \textit{in vitro} model of catheter-associated biofilm formation [34]. In order to assess the impact of 220D-F2 relative to an isogenic \textit{sarA} mutant, we first examined the ability of 220D-F2 to inhibit colonization of the catheters. The total number of viable cells adherent to catheters following exposure to 220D-F2 during colonization was significantly reduced by comparison to the untreated control (\( P<0.05 \)) and comparable to the number observed with the isogenic \textit{sarA} mutant. Although the actual reduction in the number of adherent cells was relatively modest (1.23 \( \times \)10\(^6\) CFU/catheter in the presence of 220D-F2 versus 1.34 \( \times \)10\(^7\) CFU/catheter in the untreated control), previous work comparing UAMS-1 with its isogenic \textit{sarA} mutant demonstrated that a similar level of inhibition was sufficient to improve the therapeutic response of a biofilm-associated infection to antimicrobial therapy under both \textit{in vitro} and \textit{in vivo} conditions [35].

Based on these results, we used the \textit{in vitro} catheter model to examine the therapeutic efficacy of 220D-F2 with and without concomitant antimicrobial therapy. In the absence of antibiotic, 220D-F2 had no impact on an established biofilm even after 7 consecutive days of exposure (Fig. 8). However, exposure of a UAMS-1 biofilm to 200 \( \mu \)g/mL 220D-F2 concomitantly with different antibiotics (daptomycin, clindamycin, and oxacillin) was
shown to offer a significant therapeutic benefit by comparison to antibiotic alone. For example, catheters exposed to 220D-F2 and 10 μg/mL daptomycin (10× breakpoint MIC) exhibited average colony counts of $3.57 \times 10^3$ CFU/catheter while those exposed to daptomycin alone exhibited colony counts of $1.01 \times 10^7$ (Fig. 8). Enhanced efficacy was also observed with clindamycin and oxacillin. For example, exposure to 220D-F2 and clindamycin (10×) for 7 consecutive days resulted in a ~2.5 log reduction by comparison to antibiotic alone (Fig. 8). Although we did not observe a significant difference between oxacillin and oxacillin+220D-F2 when oxacillin was used at a 10× concentration, when used at a concentration corresponding to the MIC of UAMS-1 (0.5 μg/mL), catheters exposed to 220D-F2 and oxacillin exhibited up to a 2–5 log reduction (depending on the number of days of exposure) by comparison to oxacillin alone (Fig. 8).

An experiment in which 220D-F2 was added to the growth medium during biofilm formation and then included with and without daptomycin (10×) during the treatment phase of the experiment was also undertaken. Colony counts in the group exposed to daptomycin alone fell to $3.27 \times 10^4$ CFU/catheter on day 1 while those in the group exposed to both 220D-F2 and daptomycin fell to $8.87 \times 10^1$. Moreover, ≥33% of all catheters exposed to both 220D-F2 and daptomycin were cleared of all adherent cells (lower detection limit of 10 cells/catheter) beginning on day 2 and continuing on all treatment days thereafter.

Anti-biofilm activity of select *R. ulmifolius* constituents

Ten commercially available phytochemical constituents reported in the literature [31,32,36] to have been isolated from *R.
A static crystal violet microtiter plate biofilm assay was used to assess the inhibitory activity of fractions of 220D-F2 both alone and in combination using UAMS-1. No single fraction of 220D-F2 exhibited improved biofilm-inhibiting activity over 220D-F2 as a whole. Multiple combinations of the fractions were made and the only combination which resulted in restoration of activity on the same level as 220D-F2 was when all 4 fractions (f1+f2+f3+f4) were recombined. Likewise, a single collection, in which 220D-F2 was run through the HPLC system and collected as a whole (instead of splitting into fractions) also resulted in the same level of activity as the original 220D-F2. These findings suggest that multiple components found in extract 220D-F2 are necessary for the anti-biofilm activity. Statistical significance (*, $P<0.05$; **, $P<0.001$) refers to differences observed in comparison to the untreated control.

Table 1. Compounds detected in extract 220D-F2 by accurate mass LC/UV/MS/MS.

| #  | Proposed Compound† | Molecular Formula | Retention Time (minutes) | [M+H]$^+$ m/z MS-MS Fragmentation (m/z) | MS-MS Fragmentation (m/z) |
|----|--------------------|-------------------|--------------------------|----------------------------------------|--------------------------|
| 1  | Ellagic acid xylopyranoside or Ellagic acid xylofuranoside | C$_{19}$H$_{14}$O$_{12}$ | 10.9 | 435.05594 | 303.01346 |
| 2  | Ellagic acid | C$_{14}$H$_{6}$O$_{8}$ | 11.1 | 303.01354 | 285.00281, 275.01868, 259.02338, 241.01314 |
| 3  | Ellagic acid mannopyranoside | C$_{20}$H$_{16}$O$_{12}$ | 11.1 | 449.07158 | 352.33952, 303.01366, 249.11220, 182.98514 |
| 4  | unknown | unknown | 11.5 | 437.97815 | 409.09189, 303.01351, 219.10153, 182.98507 |
| 5  | Sapogenin derivative | C$_{30}$H$_{46}$O$_{8}$ | 13.5 | 535.32637 | 517.31604, 499.30499, 481.29480, 469.29486 |
| 6  | Sapogenin derivative | C$_{30}$H$_{46}$O$_{7}$ | 13.7 | 519.31319 and 501.32097 | 501.32071, 483.31012, 473.32649, 455.31543, 437.30457, 409.30994 |
| 7  | unknown | unknown | 14.7 | 573.98540 and 396.98622 | 532.95864, 505.35274, 485.32649, 451.99400, 440.95009, 352.33958, 317.02929, 273.07586, 199.98796, 182.98517 |
| 8  | unknown | unknown | 16.9 | 1017.62898 and 999.61893 | 955.62813, 937.61896, 499.30520, 437.30489 |

*Proposed structures corresponding to this data are reported in Figure 4. Of the major UV components identified, the most abundant was EA (†2, MW 302). The second most abundant UV component (†7) did not yield a clear MS signal suggestive of a single species or reasonable molecular formula, and thus no structure is proposed. The third most abundant UV component (†1, MW 434) appears to be EA plus a C$_{14}$H$_{6}$O$_{8}$ moiety. A fourth UV component (†3, MW 448) was found to be consistent with a glycosylated derivative of EA. Investigation of the possible formulae consistent with the mass measurement of the fifth UV component (†4) did not yield sufficient data for proposal of a structure. Of the major MS components, the most abundant (†8) did not yield enough information to support the proposal of a structure, however the molecular weights and mass defects suggest that they may be dimers of MW~500 species similar to †6. The second most abundant MS component (†6) is consistent with a sapogenin. Successive loss of water (m/z 18) is consistent with a poly-hydroxylated compound. The third most abundant MS component (†5, MW 534) appears to be similar to †6 and has MS/MS losses consistent with a multiply hydroxylated compound like a sapogenin. Losses consistent with neutral loss of a sugar were not observed.

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ulmifolius (Table 3) were purchased and examined for their prophylactic efficacy in the prevention of biofilm formation using a static microtiter plate biofilm assay. The only compound exhibiting meaningful anti-biofilm activity at doses well below any growth inhibitory effects was EA (Table S1). This finding supports the hypothesis that EA and EADs present in 220D-F2 are responsible for the anti-biofilm properties of the extract.

Cytotoxicity of 220D-F2 in normal mammalian cell lines
Normal human (HK-2) and mouse (TKPTS) proximal tubular kidney cells demonstrated very good tolerance for the extract, and no IC50 could be identified even at extremely high doses of 7,000 μg/mL (Fig. 10). Rat kidney (NRK-52E) cells and mouse hepatocytes (AML-12) were slightly more sensitive and had IC50s of 4,000 and 7,000 μg/mL, respectively. Human kidney cells were

Figure 4. LC-MS/MS analysis revealed a mixture of ellagic acid and glycosylated ellagic acid derivatives in 220D-F2. Corresponding ESI(+)-MS and MS/MS data is reported in Table S1. **Compound 1.** Ellagic Acid xylopyranoside or xylofuranoside **Compound 2.** Ellagic acid. **Compound 3.** Ellagic acid mannoxyranoside. The configuration for each of the glycosylated ellagic acids could not be confirmed. Neutral loss of m/z 132 was used to confirm the presence of a pentose attached to ellagic acid.

Figure 5. Anti-biofilm activity of 220D-F2 against genotypically- and phenotypically-diverse strains of *S. aureus*. A crystal violet microtiter plate biofilm assay was used to assess the impact of 220D-F2 on biofilm formation. Strain designations are given based on both the corresponding author’s culture collection (UAMS) and the clonal lineage of each isolate (USA). Statistical significance (*, P<0.05; †, P<0.001) refers to differences between the untreated cultures and cultures exposed to the indicated concentrations. When available, the isogenic *sarA* mutant for each isolate in the absence of 220D-F2 was included as a control; results obtained with all 15 *sarA* mutants were significantly different from those obtained with the isogenic parent strain (P<0.001).
the least impacted and a significant effect in decreasing cell viability was notable only at concentrations \( \geq 500 \text{ mg/mL} \). These results are relevant as the active doses for biofilm inhibition range from 50–200 \( \text{mg/mL} \) (depending on the \( S.\ aureus \) strain) and no or very limited impact (<20%) on cell viability was notable at these concentrations in the cell lines examined.

### Table 2. Activity of 220D-F2 (\( \mu\text{g/mL} \)) against wild-type strains of \( Staphylococcus aureus \).

| USA Type | Strain I.D. of wild type* | Strain I.D. of \( sarA \) mutant | Growth | Survival | Biofilm Formation |
|----------|--------------------------|----------------------------------|--------|----------|------------------|
|          |                          |                                  | MIC\(_{50}\) | MIC\(_{90}\) | MBC\(_{50}\) | MBC\(_{90}\) | MBIC\(_{50}\) | MBIC\(_{90}\) |
| 100      | UAMS-1893                | UAMS-1941                        | 380    | 530      | 530             | 740     | 100         | 200         |
| 200      | UAMS-1                   | UAMS-929                         | 380    | 530      | 740             | 1040    | 50          | 50          |
|          | UAMS-270                 | -                                | 380    | 530      | 1460            | 2040    | 100         | 200         |
|          | UAMS-601                 | UAMS-950                         | 530    | 740      | 1460            | 2040    | 50          | 100         |
|          | UAMS-1894                | UAMS-1945                        | 380    | 530      | 740             | 1040    | 50          | 100         |
| 300      | UAMS-1625                | UAMS-1653                        | 380    | 530      | 530             | 740     | 50          | 100         |
|          | UAMS-1782                | UAMS-1804                        | 380    | 530      | 380             | 530     | 50          | 100         |
|          | UAMS-1790                | UAMS-1796                        | 380    | 530      | 380             | 530     | 50          | 100         |
| 400      | UAMS-1039                | UAMS-1938                        | 530    | 740      | 530             | 740     | 100         | 200         |
| 500      | UAMS-1895                | UAMS-1942                        | 740    | 1040     | 1040            | 1460    | 100         | 200         |
| 600      | UAMS-1896                | UAMS-1943                        | 530    | 740      | 530             | 1040    | 100         | 200         |
| 700      | UAMS-1897                | -                                | 530    | 740      | 530             | 1040    | 100         | 200         |
| 800      | UAMS-1898                | UAMS-1944                        | 530    | 740      | 1460            | 2040    | 100         | 200         |
| 1000     | UAMS-1899                | UAMS-1930                        | 530    | 740      | 1040            | 1460    | 100         | 300         |
| 1100     | UAMS-1900                | UAMS-1931                        | 380    | 530      | 740             | 1460    | 50          | 100         |

*A detailed description of the bacterial strains used in this study has been previously published [52].

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**Discussion**

In a previous study we demonstrated that an ethanolic extract from the root of \( R.\ ulmifolius \) can be used to limit \( S.\ aureus \) biofilm formation [28]. However, this work was limited to the crude extract and examined only one strain of \( S.\ aureus \). In this report, we
expanded on this work by fractionating the extract and assessing the anti-biofilm properties of the resulting fractions against genotypically and phenotypically-diverse strains of *S. aureus*. The results confirmed that an EA and EAD-rich fraction (220D-F2) obtained from the roots of *R. ulmifolius* was effective at preventing *S. aureus* biofilm formation irrespective of strain identity. While addition of the same fraction had no impact on dispersal of an established biofilm, this limitation was therapeutically relevant in that inclusion of 220D-F2 was associated with enhanced susceptibility to functionally-diverse antibiotics including daptomycin, oxacillin, and clindamycin in the specific context of an established biofilm. Moreover, when the extract was employed both prophylactically during the colonization process and therapeutically in combination with daptomycin, it was possible to completely clear infected catheters of all viable cells.

A few studies [31,32,36] have addressed the phytochemical makeup of *R. ulmifolius* and identified some compounds (Table 3), but it is unlikely that this list is comprehensive. Indeed, the EADs isolated in this study have not been previously reported for *R. ulmifolius*. Importantly, we demonstrated that EA alone has potent anti-biofilm properties at concentrations (MBIC<sub>50</sub>&lt;50 μM) well below those that impact bacterial growth (MIC<sub>90</sub>&gt;2000 μM). This is consistent with the hypothesis that the activity of 220D-F2 is likely to be related to the high content of EA and glycosylated EAs. The decline in activity when 220D-F2 is divided into four separate fractions may be related to the EAD content of the various fractions and possibly the unequal distribution of the most active forms. More work involving the isolation of substantive amounts of individual EADs for further testing and structural elucidation by NMR (nuclear magnetic resonance) is necessary to determine which compound(s) are most efficacious in preventing *S. aureus* biofilm formation and if they act in a distinctly synergistic fashion when combined.

EA is a polyphenol found at high concentrations in a number of edible fruits, such as grapes, strawberries, raspberries, blackberries, and black currants. Berries of the Rosaceae family, in particular, contain high levels of EA equivalents [37]. EA is derived from gallic acid, in which two gallic acid molecules are linked by ester bonds. EA has been the focus of many studies in recent years, primarily related to its antioxidant [38,39], anti-proliferative [40], anti-estrogenic [41] anti-inflammatory [42], anti-bacterial [37,43] and protein kinase CK-2 inhibiting [44] effects. Reports concerning the biofilm inhibiting properties of EA against *Escherichia coli* [45,46], *Streptococcus dysgalactiae* [47], *Pseudomonas putida* [46], and *Burkholderia cepacia* [46] have also recently emerged. EADs, on the other hand, have been found to have anti-plasmodial [48], anti-babesial [49], antibacterial [50] and antioxidant [50,51] effects. However, to our knowledge, this is the first report of the anti-biofilm properties of EA and EADs against *S. aureus*. This is in contrast to Durig et al. [47], who examined the impact of EA on *S. aureus* biofilm formation but did not observe any inhibitory activity. This contrast may be attributable to differences in the methodologies employed in the respective studies. Specifically, we employ in vitro assays in which the substrate, whether microtiter plate or catheter, is first coated with plasma proteins [34,52]. We do this both because indwelling medical devices are invariably coated with plasma proteins and because the results we have obtained with our *in vivo* assays have been consistent with those obtained using *in vitro* assays [35]. This was not done in the study by Durig et al. [47]. This is potentially important in that we have also demonstrated that the results obtained when examining the contribution of specific *S. aureus* factors to biofilm formation differ depending on whether the assay is done with or without plasma coating [34,52,53]. To determine whether this may account for this discrepancy, we tested both 220D-F2 and EA in a biofilm assay with and without plasma coating. Our results confirmed that, when the substrate is coated with plasma proteins, EA elicits a significant decrease in the biofilm phenotype that is not apparent in the absence of coating with plasma proteins (Fig. 11). Thus, it is likely that this difference is related to EA interactions with proteins. At the same time, the inhibitory activity of 220D-F2 was apparent under both conditions, which suggests that 220D-F2 contains components unrelated to EA that also have anti-biofilm properties or that...
R. ulmifolius Extract Inhibits S. aureus Biofilms

A

B

C

Untreated control 220D-F2
Antibiotic (1X) Antibiotic (1X) + 220D-F2
Antibiotic (10X) Antibiotic (10X) + 220D-F2

Day 1 Day 2 Day 3 Day 4 Day 5 Day 6 Day 7

Day 1 Day 2 Day 3 Day 4 Day 5 Day 6 Day 7

Day 1 Day 2 Day 3 Day 4 Day 5 Day 6 Day 7
alternative derivatives of EA exist in the extract that are not present in the commercially-available EA preparation.

The focus in this study was on further characterization of the crude *R. ulmifolius* extract and confirmation of its anti-biofilm properties in diverse strains of *S. aureus*. Nevertheless, the results discussed above potentially provide important clues about the mechanistic basis for this activity. Most notably, the fact that the inhibitory effects of 220D-F2 were apparent with and without plasma coating suggests that components within the extract are likely to impact multiple biofilm-associated processes. This also appears to be the case with *sarA* in that the negative impact of mutating *sarA* on biofilm formation is apparent, albeit to greatly varying degrees, irrespective of whether the assay is done with plasma coating [52,54]. Mutation of *sarA* results in decreased production of the polysaccharide intercellular adhesion (PIA), increased production of extracellular nucleases, and increased production of extracellular proteases [52,53,54]. The impact of *sarA* on the production of PIA appears to play a minor role by comparison to these other factors [53], and this suggests that the inhibitory effects we observed with 220D-F2 are not mediated by changes in PIA production. This is consistent with the observation that 220D-F2 did not inhibit biofilm formation in *S. epidermidis* (data not shown). We have demonstrated that the increased production of both nucleases and proteases contribute to the biofilm-deficient phenotype of *sarA* mutants [52,53,54]. However, the impact of eliminating nuclease production in a *sarA* mutant is apparent only when the assay is done without plasma coating. To the extent that 220D-F2 inhibited biofilm formation both with and without plasma coating, it is plausible to suggest that the inhibitory effects may impact both protein-dependent and protein-independent mechanisms of biofilm formation in much the same way as *sarA* itself. We included *sarA* mutants as controls based on previous work demonstrating that mutation of *sarA* limits biofilm formation to a therapeutically-relevant degree [34,35], but based on this we also examined whether the presence of 220D-F2 had any impact on expression of *sarA*, and somewhat surprisingly our results to date suggest that this is not the case (data not shown). Nevertheless, the results reported in this study provide strong support for the hypothesis that 220D-F2 or its associated components may well have therapeutic utility in the specific context of an *S. aureus* biofilm-associated infection.

**Materials and Methods**

**Acquisition of botanical materials**

Bulk samples of *Rubus ulmifolius* Schott. (Rosaceae) roots were collected from wild populations in August 2009 in the village of Ginestra, Italy. Procedures from the 2003 WHO Guidelines on

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**Figure 8. Use of 220D-F2 as adjunct therapy with conventional antibiotics.** Biofilms were formed on plasma-coated catheters for 24 hours by growth of the test strain (UAMS-1) in BM. Catheters were then placed in fresh BM containing 200 μg/mL 220D-F2 with or without the indicated amounts of antibiotic. In the case of all three antibiotics, the concentrations examined correspond to 1 × or 10 × the CLSI-defined breakpoint MIC for a sensitive strain of *S. aureus*. Statistical significance (*, P<0.05; †, P<0.001) refers to differences between the cultures treated with antibiotic alone and cultures exposed to both extract 220D-F2 and antibiotic. A. Clindamycin (1 ×: 0.5 μg/mL; 10 ×: 5 μg/mL); B. Daptomycin (1 ×: 1 μg/mL; 10 ×: 10 μg/mL); C. Oxacillin (1 ×: 0.5 μg/mL; 10 ×: 5 μg/mL).

**Figure 9. Prophylactic use of 220D-F2 prior to concomitant antibiotic therapy.** Biofilms were formed on plasma-coated catheters for 24 hours by growth of the test strain (UAMS-1) in BM containing 200 μg/mL 220D-F2. Catheters were then placed in fresh BM containing 200 μg/mL 220D-F2 with or without 10 μg/mL of daptomycin, which corresponds to 10 × the CLSI-defined breakpoint MIC for a sensitive strain of *S. aureus*. Statistical significance (*, P<0.05; †, P<0.001) refers to differences between the untreated cultures and cultures exposed to the compounds. Results observed with the isogenic *sarA* mutant with and without the same concentration of daptomycin but without 220D-F2 are shown for comparison.
A preparative HPLC method was developed to split 220D-F2 into 4 additional fractions in an effort to identify the active constituent(s). Briefly, 220D-F2 was dissolved in 2-propanol:water (2:8) at a concentration of 50 mg/mL. A C-18 column (μBondapak®-C18, 19 mm x 300 mm, 125 Å, 10 μm) was used to separate 1 mL injections (total 5 mg) with a Waters 600E system controller and pump and an isocratic mobile phase of H2O:acetonitrile:2-propanol/formic acid (74:17:8:1) at a flow rate of 7 mL/min (all solvents HPLC-grade, Fisher Chemical) and detection at a wavelength of 360 nm using a Waters 486 Tunable Absorbance Detector and Shimadzu C-R5A Chromatopac. Fractions were collected at 17.5 (220D-F2-P1), 20.5 (-2), 27 (-3) and 45 (-4) minutes. The largest single peaks were located in fractions 2 and 3, whereas fractions 1 and 4 contained multiple minor peaks. Fractions were collected for chemical analysis and bioassays described below.

**Characterization of major extract components**

Further characterization of 220D-F2 (suspended in 5% isopropanol in H2O, 1 mg/mL) was performed using accurate mass LC/UV/MS/MS to identify the major components. A MacMod HALO C18 column (3.0 × 100 mm) was used with a mobile phase (A: 0.1% formic acid in water; B: 0.1% formic acid in acetonitrile) with a gradient (hold 2% B for 2 min., 2–50% B over 10 min, 100% B for 5 min.), flow of 0.4 mL/min and PDA (photodiode array detector) detection range of 200–790 nm. MS detection was with a Thermo LTQ Orbitrap Discovery, +EIS mode, and scan range of 140–2000 Da. Searches of multiple databases (Human Metabolome Database (version 2.5), ChemSpider, SciFinder, and KEGG LIGAND Database) were performed using chemical formula queries. All mass measurements were within 0.5 mmu of the proposed formulae, well within the expected measurement tolerances of the mass spectrometer.

**Individual phytochemicals tested**

In addition to testing the chemically complex *R. ulmifolius* extracts for anti-biofilm activity, all known constituents of *R. ulmifolius* (Table 2) that were commercially available were purchased for anti-biofilm analysis using the static microtiter plate method described below. Ferulic acid, kaempferol, ursolic acid, quercetin dehydrate, caffeic acid, ellagic acid, and oleanolic acid were purchased from MP BioMedicals (Solon, OH, USA); quercetin-3-O-glucuronide and tiliroside from Chromadex (Irvine, CA, USA); and gallic acid from Acros Organics (NJ, USA). Compounds were tested for growth and biofilm inhibitory activity at doses ranging from 25–2000 μM.

**Quality control**

Extracts and drugs were suspended in either DMSO or PBS (depending on solubility), then sterile filtered (0.2 μm), and stored in sterile vials prior to use in all bioassays. Sterility controls were included in all assays (extract+media). Tests for sterility followed standard quality control methods [57] to ensure that no microbial growth was detectable prior to bioassay testing. Prior to performing bioassays, batches of extract 220D-F2 were checked for the relative ratios of constituents by RP-HPLC, and were found to be reproducible. All chemicals (including extract) were stored at −20°C.

**Bacterial strains and growth conditions**

The *Staphylococcus aureus* strains used in these experiments are listed in Table 1. When available, isogenic strains carrying a
mutation in the staphylococcal accessory regulator (sarA) were included as negative controls based on the observation that mutation of sarA results in a reduced capacity to form a biofilm that can be correlated with increased antibiotic susceptibility [35,53]. For biofilm assays, strains were grown in tryptic soy broth (TSB) supplemented with 3.0% NaCl (wt/vol) and 0.5% dextrose (biofilm medium, BM). In experiments that included daptomycin, BM was supplemented (wt/vol) with 2.5 mM CaCl2. For all assays, overnight cultures of the test strains were used to inoculate fresh medium at an initial cell density of $5 \times 10^5$ colony-forming units (CFU) per ml (confirmed with plate counts). This cell density was achieved by taking the optical density of overnight cultures and diluting to an OD560 nm of 0.05. Studies examining growth rate were done at 37°C in BM with constant shaking (200 rpm) and a volume-to-flask ratio of 0.4.

**Figure 10. Cytotoxicity of 220D-F2 against normal mammalian cell lines.** Cytotoxicity was assessed using a lactate dehydrogenase (LDH) test following 24 hours of exposure to the extract. Results are reported as the percent of cell viability after exposure to the indicated dosage of 220D-F2 in the culture growth medium. Statistical significance ($^*, P<0.05; ^{*, P<0.001}$) refers to differences observed in comparison to the untreated (excipient) control. A. Normal human kidney proximal tubular (HK-2) cells; B. Normal mouse kidney proximal tubular (TKPTS) cells; C. Normal rat kidney (NRK-52E) cells; D. Normal mouse hepatocytes (AML12). doi:10.1371/journal.pone.0028737.g010

**Figure 11. Biofilm inhibition in the presence and absence of human plasma proteins.** Previous studies have indicated that ellagic acid is not an effective biofilm inhibitor for *S. aureus* [47]. Our data suggest otherwise. UAMS-1 (USA200) and UAMS-1782 (USA300) were used to assess the efficacy of 220D-F2 and EA in experiments that included or omitted the use of plasma coating for the test wells. Treatment with 220D-F2 elicited a dose-dependent response in limiting biofilm formation under both growth conditions, whereas ellagic acid inhibited biofilm formation only in plasma-coated wells, suggesting that the mechanistic basis of EA’s biofilm-inhibiting effects involves surface protein recognition or attachment. doi:10.1371/journal.pone.0028737.g011

**Determination of MIC and MBC**

To determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for each fraction, strains were grown at 37°C in cation-adjusted Mueller-Hinton broth (CAMHB). MIC and MBC were determined following Clinical and Laboratory Standards Institute (CLSI) broth microdilution guidelines [58,59]. Briefly, test strains were inoculated into 0.1 ml CAMHB containing varying concentrations of extract. For MIC, optical density (OD600) was assessed immediately after inoculation and again after 18 hours using a Biotek Synergy II microplate reader. Corrections for extract color were done as previously described [28]. The MIC was defined as the lowest concentration that inhibited growth to a level $\geq 90\%$ (for MIC90) or $\geq 50\%$ (for MIC50) by comparison to untreated control cultures. MBC was assessed by determining the number of colony-forming units...
units (CFU) after 24 hours of exposure and was defined as the lowest concentration at which the initial density of viable cells was reduced to a level by $\geqslant 90\%$ (for MBIC$_{90}$) or $\geqslant 50\%$ (for MBIC$_{50}$) by comparison to the untreated control cultures.

**Assessment of biofilm formation**

The primary biofilm assays, used as a guide during fractionation, were undertaken using a human plasma protein-coated microtitre plate assay as previously described [53]. Wells were pre-coated for 24 h at 4°C with 20% human plasma diluted in carbonate buffer (pH 9.6). After inoculation and addition of the appropriate media (containing drug or excipient alone), the plates were incubated without shaking at 37°C for 24 h, the wells were gently washed twice with 200 µl of phosphate-buffered saline to remove nonadherent cells. Adherent biofilms were fixed with 200 µl of 100% ethanol prior to staining for 2 min with 200 µl of 0.41% (wt/vol) crystal violet in 12% ethanol (Protocol Crystal Violet; Biochemical Sciences, Swedenborg, N.J.). The stain was then aspirated, and the wells were washed several times with phosphate-buffered saline. A quantitative assessment of biofilm formation was obtained by adding 100 µl of 100% ethanol and incubating at room temperature for 10 min. A total of 50 µl of the eluate was then transferred to a sterile polystyrene microtitre plate and the absorbance (OD$_{595\text{nm}}$) was determined using a plate reader. The minimum biofilm-inhibiting concentration (MBIC) was defined as the lowest concentration of extract in which biofilm formation was limited to a level $\geqslant 90\%$ (for MBIC$_{90}$) or $\geqslant 50\%$ (for MBIC$_{50}$) by comparison to the untreated parent control strain. Additional tests with 220D-F2 and EA were conducted to examine the influence of plasma proteins on the relative activity of the drugs. In this case, both coated and uncoated wells were employed in the static microtitre plate assay.

Assays examining the impact of different extracts on antibiotic susceptibility were done using an in vitro model of catheter-associated biofilm formation [34]. Briefly, 1-cm segments of fluorinated ethylene propylene catheters (14-gauge Introcan Safety catheter; B. Braun, Bethlehem, PA) were coated with human plasma proteins (Sigma-Aldrich, St. Louis, MO, USA), placed in the wells of a 12-well microtitre plate containing BM, and inoculated with the test strain at an initial optical density (OD$_{600\text{nm}}$) of 0.05. After 24 hours, the BM was replaced with fresh BM containing 220D-F2 (or excipient alone) with or without antibiotic. Antibiotics tested were daptomycin, clindamycin, and oxacillin, all of which were examined at concentrations corresponding to 1 and 10 times ($1 \times$ and $10 \times$) the CLSI-defined breakpoints for an antibiotic-resistant strain of *S. aureus*. The medium was replaced in its entirety at 24 hour intervals for 7 days. Following each 24 hour interval, catheters were removed and processed to assess viability as previously described [34,35]. More specifically, catheters were dunked into sterile PBS several times using a sterile forceps in order to remove all of the loosely adherent cells from both the interior lumen of the catheter and external surface. In a second set of experiments, extracts were added to BM during colonization of plasma-coated catheters. After 24 hours, catheters were either processed for plate counts as described above or transferred to fresh BM with and without antibiotics.

**Confocal laser scanning microscopy (CLSM) of static biofilms**

Two *S. aureus* strains (UAMS-1 and the USA300 isolate UAMS-1782) and their isogenic *sacA* mutants (UAMS-929 and UAMS-1804) were grown in 96 well microtitre plates (Costar 3603, Corning Life Sciences) as described above. After 20 hours, the well contents were aspirated and the wells gently washed three times with 0.85% (wt/vol) NaCl. The adherent biofilm was then stained with LIVE/DEAD stain (Invitrogen) at room temperature in the dark for 18 minutes. After removal of the stain, the wells were gently washed with 0.85% NaCl before collecting CLSM images using a Zeiss LSM 510 Meta confocal scanning system and inverted microscope. SYTO 9 fluorescence was detected by excitation at 488 nm and emission collected with a 500–530 bandpass filter. All z-sections were collected at 4-µm intervals using a 10× objective lens. A 0.9×0.9 mm section of biofilm was selected from the center of the well for each image. Image acquisition and processing was performed using LSM Image Browser (Carl Zeiss). Identical acquisition settings were employed for all samples.

**Cell culture and cytotoxicity assays**

Normal human kidney proximal tubular (HK-2) cells, normal rat kidney (NRK-52E) cells, and normal mouse hepatocytes (AML12) were purchased from the American Type Culture Collection (ATCC). Normal mouse kidney proximal tubular cells (TKPTS) were developed and gifted by Dr. Elba Bello-Reuss [60]. The four cell lines were cultured with different media, keratinocyte serum free media (K-SFM) supplemented with bovine pituitary extract (BPE) and human recombinant epidermal growth factor (EGF) for HK-2 cells, Dulbecco’s Modified Eagle’s Medium (DMEM) for NRK-52E cells, and ATCC complete growth medium (1:1 mix of DMEM and Ham’s F12 medium supplemented with insulin, transferring, dexamethasone, and fetal bovine serum) for AML12 and TKPTS cells. Cells were maintained in humidified air with 5% CO$_2$ at 37°C. Cells were transferred to 96-well cell culture plates (10,000 cells seeded per well) and incubated for 24 hours prior to aspirating the media, adding extract 220D-F2 in serum-free media and undertaking cytotoxicity tests. To examine the cytotoxic effects of 220D-F2 on normal mammalian liver and kidney cells, a lactate dehydrogenase (LDH) assay was employed. LDH is a stable cytosolic enzyme that is released upon membrane damage in necrotic cells. LDH activity can serve as a useful measure for determining drug toxicity to cell lines.

LDH was measured using a commercial cytotoxicity assay kit (Promega CytoTox 96® Non-Radioactive Cytotoxicity Assay, WI, USA), in which LDH released in culture supernatants is measured with a coupled enzymatic assay, resulting in conversion of a tetrazolium salt into a red formazan product. The cells were treated with concentrations of extract 220D-F2 ranging from 0.1–7,000 µg/mL and incubated in humidified air with 5% CO$_2$ at 37°C for 24 hours. Controls for the extract excipient (20% DMSO in phosphate buffered saline, PBS), positive LDH control, and positive LDH control with media and extract were also included. The sample solution (supernatant) was removed, and the LDH released from the cells into culture medium treated according to kit instructions, then measured at an OD$_{560\text{nm}}$. The maximal release was obtained after treating cells with a lysis solution for 45 minutes, then treating the supernatant according to kit instructions. All tests were performed in quadruplicate. The necrotic percentage (% cytotoxicity) was expressed using the formula: (sample value/maximal release)×100%.

**Statistical analysis**

Pair-wise testing was performed based on *t* tests as formatted in Sigma Stat® Statistical Software Version 2 (SPSS, Inc) with *P* values<0.05 considered significant.
Supporting Information

Table S1: Inhibitory effects of individual phytochemicals reported in the literature for *R. ulmifolius* against biofilm formation and growth of UAMS-1.

(DoCX)

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Author Contributions

Conceived and designed the experiments: CLQ MEC CMC HH MSS KB. Performed the experiments: CLQ GH MEC HH. Analyzed the data: CLQ MSS CMC HH. Contributed reagents/materials/analysis tools: CLQ MEC HH. Wrote the paper: CLQ MESS HH CMC.

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