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Biofilm Formation**

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Running title: Tannins modulate behaviour of *C. violaceum*

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Abstract

Chromobacterium violaceum is an opportunistic pathogen which causes infections that are difficult to treat. The goal of this research was to evaluate the effect of selected tannins (tannic acid (TA) and gallic acid (GA)) on bacterial growth, motility, antibiotic (carbenicillin, tetracycline) susceptibility, and biofilm formation. Both tannins, particularly TA, impaired bacterial growth levels and swimming motilities at sub-MIC concentrations. In combination with tannins, antibiotics showed increased minimum inhibitory concentration (MIC) values, suggesting that tannins interfered with antibacterial activity. Sub-MIC concentrations of tetracycline or TA alone enhanced biofilm formation of *C. violaceum*; however, in combination, these compounds inhibited biofilm formation. In contrast, carbenicillin at sub-MIC was effective in inhibiting *C. violaceum* biofilm formation; however, in combination with lower concentrations of TA or GA, biofilms were enhanced. These results provide insights into the effects of tannins on *C. violaceum* growth and their varying interaction with antibiotics used to target *C. violaceum* infections.

Key words: antibiotic, biofilm, *Chromobacterium violaceum*, motility, tannins

Introduction

Chromobacterium violaceum is a Gram-negative rod shaped bacterium found in soil and aquatic environments. This bacterium is not commonly studied; however, increasing number of *C. violaceum* infections have been reported in recent years that warrant attention (Chen et al. 2003; Alves de Brito et al. 2004; Yang and Li 2011). The bacterium is associated with abscesses of the lung, liver, spleen, skin and can lead to fatal bacteremia (Sirinavin et al. 2005; Lim et al. 2009) and secondary infections such as diarrhea (Ballal et al. 2000; Chang et al. 2007; Yang and Li 2011). These infections rapidly disseminate and due to failure of antibiotic treatment the mortality rate is over 60% (Yang and Li 2011).

Multiple virulence factors affect bacterial pathogenesis including motility, biofilm formation, and production of toxins (Bernard et al. 2012). *C. violaceum* cells can form biofilms adhering to each other or to surfaces (Becker et al. 2009). Type III secretion, type IV fimbriae, lipopolysaccharide synthesis, toxin and violacein production also influence pathogenicity of *C. violaceum* (Alves de Brito et al. 2004). Beta-lactam antibiotics, cefoxitin, ticarcillin, and tetracycline are used to treat *C. violaceum* infections (Farrar and Odell 1976; Aldridge et al. 1988; Lee et al. 1999; Fantinatti-Garboggini 2004; Yang and Li 2011; Kumar 2012). However, recently, the bacterium showed resistance to β -lactam antibiotics (penicillin and cephalosporin) due to acquired beta-lactamase and multidrug resistance genes (Martinez et al. 2000; Fantinatti-Garboggini et al. 2004). Due to the presence of multiple virulence factors (motility, biofilm formation), multidrug resistance and scanty reports or guidelines on treatment methods, there is a growing interest in the study of alternative agents that may be effective against *C. violaceum*.

Plant products containing tannins are promising candidates as alternatives to antibiotics due to their antimicrobial properties (Haslam 1996; Cowan 1999; Serrano et al. 2009). Tannins are water-soluble polyphenols found in herbaceous and woody plants (Schofield et al. 2001). Their antimicrobial activity may be due to (i) astringent property that may induce complexation with enzymes or substrates, (ii) tannin's action on bacterial cell membrane, or (iii) complexation of metal ions by tannins (Chung et al. 1998). Recent studies show that tannins (e.g., tannic acid (TA), epigallocatechin gallate) can modify bacterial virulence, motility and biofilm formation (Rasko and Sperandio 2010; O'May et al. 2012). With increasing occurrence of antibiotic resistance and need for alternative chemical agents, there is a necessity to understand the synergistic and antagonistic effects of tannins when used in combination with common antibiotics. This information will provide insights into the potential clinical applications and limitations of these phytochemicals (Chung et al. 1998). Hence, this study examines the effects of the tannins TA and gallic acid (GA) on growth, motility and biofilm formation by the less explored pathogen, *C. violaceum*.

Materials and methods

Bacterial strain and growth conditions

Chromobacterium violaceum strain CV026 (NCTC 13278) is a double-Tn5 mutant of the wild type strain *C. violaceum* (ATCC 31532) obtained by mini-Tn5 transposon mutagenesis (McClean et al. 1997). The transposon insertion sites have been mapped to a putative repressor locus and to a *luxI* homologue (*cviI*) respectively. The wild type *C. violaceum* (ATCC 31532) produces a violet color pigment, violacein, whereas the mutant *C. violaceum* CV026 is unable to produce this pigment. We used the mutant strain (CV026) in this study

because violacein interfered with the biofilm quantification assay. The bacterium was grown in lysogeny broth (LB) (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) and on LB agar (supplemented with 1 % [w/v] agar). Pure stock cultures were maintained at -80 °C in 30 % (v/v) frozen glycerol solutions in LB broth. Frozen cultures were streaked onto the LB agar and incubated (37 °C, 24 h), and a colony of bacteria was inoculated into LB broth (15 mL) and incubated (37 °C at 200 rpm) for preparation of the bacterial inocula for the experiments.

Chemical agents

Tannic acid (TA) and gallic acid (GA) were purchased from Sigma-Aldrich (Canada). Stock solutions (5 mg/mL) were prepared in deionized water (DI), the pH was adjusted to 7.0 (with NaOH), filter sterilized (0.1 µm filters) and stored at 4 °C, protected from light. Antibiotics, carbenicillin (CAR) and tetracycline (TET), were obtained from Sigma-Aldrich (Canada). The antibiotics were dissolved in DI water, filter sterilized and stored at 4 °C.

MIC determination using standard agar well diffusion assay

To determine the MIC of TA and GA on *C. violaceum* growth, the standard agar well diffusion method was performed (Bennett et al. 1966). Briefly, exponentially grown cells of *C. violaceum* were adjusted to 1×10^6 cells/mL and an aliquot (100 µL) was spread onto an LB agar surface. Wells of diameter 6 mm were made in the agar plates and 50 µL of tannins (15-2000 µg/mL) were added to the respective agar wells. Petri dishes were incubated at 37 °C for 24 h and inhibition zones were recorded. The experiments were performed in triplicate. The MIC was defined as the lowest concentration of antibacterial agent that showed a zone of inhibition ≥ 1 mm around the well.

MIC determination using microtitre growth assay

Growth assays were performed in LB broth in non-tissue culture treated 96 well polystyrene microplates (BD Falcon, USA). The culture was diluted (1:100) with LB broth in the presence or absence of tannins and an aliquot (200 μ L) of this mixture was added to microtiter plate wells. Medium without inoculum was used as controls. The plates were incubated at 37 °C in a microtiter plate reader (Tecan Infinite M200 Pro, Switzerland) and OD₆₀₀ was measured at 30 min intervals for 24 h with shaking. The minimum inhibitory concentration (MIC) was determined as the minimum concentration of TA or GA that showed no visible bacterial growth in the wells (by OD₆₀₀).

MIC determination using checkerboard assay of tannins and antibiotics against *C. violaceum*

In the checkerboard assay, serial dilutions of antibiotics, CAR or TET, with tannins (TA or GA) were mixed together in the microtiter plates so that each row or column contained a fixed amount of one agent and increasing amount of the second. The concentrations of CAR ranged from 0.04-50.0 mg/mL. TET concentrations ranged from 0.4-500 μ g/mL. TA and GA concentrations ranged from 0-2000 μ g/mL. The experiments were carried out in triplicate at 37 °C, for 24 h under shaking conditions of growth. After 24 h, growth (OD₆₀₀) was determined using the microtitre plate reader (Tecan Infinite M200 Pro). MIC was defined as the minimum concentration tested that resulted in no visible bacterial growth in the wells (clear well).

129 **Effect of tannins and antibiotics on biofilm formation**

130 Biofilm formation was studied in flat bottom polystyrene 96 well microtiter plates (BD
131 Falcon). The antibiotics (CAR and TET) and tannins (TA and GA) were tested for their
132 ability to inhibit biofilm formation (for 24 h at 37 °C) in the microtiter plates. Antibiotics and
133 tannins were used either alone or in combination at equivalent concentrations to those used in
134 the checkerboard assay. *C. violaceum* CV026 was inoculated in 10 mL LB broth in 50 mL
135 Falcon tubes (Ultident) and incubated at 37 °C overnight at 150 rpm shaking. The culture was
136 diluted (1:100) with LB broth in the presence or absence of tannins and used for biofilm
137 assays. Biofilms of *C. violaceum* were formed in non-tissue culture treated 96 well
138 polystyrene microplates (BD Falcon, USA) for 24 h (37 °C, static conditions) in the presence
139 or absence of sub-MIC concentrations of tannins (0-125 µg/mL in case of TA and 0-1000
140 µg/mL for GA) and antibiotics (0-6 mg/mL in case of CAR and 0-7 µg/mL for TET). After
141 incubation, the optical density (OD₆₀₀) of the culture was measured before removing the
142 planktonic cells. Biofilms were analyzed by staining with crystal violet (CV) as described
143 elsewhere (O'Toole et al. 1999). Briefly, wells were gently washed twice with DI water to
144 remove non-adherent, planktonic cells. After air drying (10 min), 200 µL of a 0.1 % CV
145 solution was added to each well (15 min). Plates were rinsed with DI water, air dried and then
146 200 µL of 95 % ethanol was added to solubilize the CV stain bound to the surface-attached
147 biofilms. The absorbance at OD₅₉₅ (Tecan Infinite M200 Pro, Switzerland) was measured to
148 estimate the biofilms that were formed. Biofilm levels (OD₅₉₅) were normalized to the level of
149 bacterial growth (OD₆₀₀, determined separately) to decrease the potential bias in the
150 measurements introduced due to differences in growth rate (Dunne 1990; Hosseinidoust et al.

2013; Dusane et al. 2014). The biofilm levels reported represent the biofilm absorbance (OD₅₉₅) normalized by the level of growth (OD₆₀₀).

Bacterial motility assays

Effect of tannins on bacterial motility was analyzed using swimming assays. Motility assays were performed in polystyrene Petri dishes (diameter of 82 mm) containing swim agar (LB supplemented with 0.3 % (w/v) agar) and TA or GA at different concentrations. The plates were dried for 1 h and inoculated with 5 µL aliquots of mid-exponentially grown culture of *C. violaceum* CV026. Inoculation was done within the agar (Tremblay et al. 2008) and the diameters of the swimming motility zones were measured after incubation (for 20 h) at 37 °C. The experiments were carried out in triplicate.

Statistical analysis

Where indicated, a Student's *t* - test was performed to compare the significance of the results, with a *P*-value < 0.05 considered as statistically significant.

Results

TA and GA growth effects on *C. violaceum* CV026

In the agar-diffusion method, TA and GA exhibited MIC values of 250 and 2000 µg/mL, respectively (Table 1). When growth levels (24 h) were assessed in the microtiter plates, TA exhibited a MIC of 500 µg/mL, but concentrations as low as 7 µg/mL resulted in a decreased growth rate. For GA, only the maximum concentration tested (2000 µg/mL) exhibited an inhibitory effect (Fig. 1A and 1B).

Combined effect of tannins and antibiotics on growth of *C. violaceum* (checkerboard assay)

The MICs of the antibiotics (CAR) and (TET) were assessed alone and in combination with TA and GA (Fig. 2A-D). In the checkerboard assay, TA and GA alone exhibited an MIC value of 250 and 2000 $\mu\text{g/mL}$, respectively. CAR alone exhibited an MIC of 12 mg/mL , while TET alone inhibited *C. violaceum* growth at a much lower MIC (15 $\mu\text{g/mL}$). The difference in MIC values for TA in the agar-diffusion assay (Table 1) and the checkerboard assay may be attributable to differences in the growth conditions (static versus shaking). When tannins and antibiotics were combined, the MIC values of both antibiotics were increased (Fig. 2A-D). In the presence of low concentrations of TA or GA, the MIC of CAR increased from 12 to 50 mg/mL (Fig. 2A and 2B). For TET, the MIC increased from 15 to 30 $\mu\text{g/mL}$ in TA and to 125 $\mu\text{g/mL}$ in case of GA. Low concentrations of antibiotics also enhanced the MIC for TA, but not for GA at the concentrations tested. With CAR, the TA MIC increased from 250 to 1000 $\mu\text{g/mL}$, while TET (≤ 3.5 $\mu\text{g/mL}$) increased TA's MIC to 500 $\mu\text{g/mL}$.

Effect of tannins and antibiotics on biofilm formation by *C. violaceum*

Tannins and antibiotics were tested alone and in combination to determine their effect on biofilm formation in 96 well microtiter plates. Biofilm values (OD_{595}) were normalized by growth levels (OD_{600}) to compensate for differences in the growth rate as described in the literature (Deighton and Borland 1993; Pompilio et al. 2011; Hosseinioust et al. 2013). Differences in biofilm formation were observed in both normalized and non-normalized values, especially when tannins were used in combination with the antibiotic TET (Figs. 3C, 3D and S1C, S1D). Only sub-MIC concentrations of the antibiotics are included in Figs. 3 and S1. The MIC of CAR was previously

shown to be 12 mg/mL (Fig. 2A and 2B). With $CAR \geq 0.16$ mg/mL, reduction in the levels of biofilm was observed as compared with controls without the antibiotic (Fig. 3A and 3B). At all concentrations of $CAR \leq 0.7$ mg/mL, addition of TA resulted in an enhancement of biofilm formation (Fig. 3A). This occurred consistently with low TA (15 and 30 μ g/mL) concentrations, but there were exceptions seen at the higher TA levels of 60 and 125 μ g/mL (Fig. 3A). GA also reduced the effectiveness of CAR to inhibit biofilm formation at certain concentrations (Fig. 3B). However, the enhancement of biofilm by GA was less than with TA (Fig. 3A). Biofilm formation was enhanced at CAR concentrations of 0.16 and 0.35 mg/mL and at 3 and 6 mg/mL in combination with GA, especially at lower concentrations (30-125 μ g/mL). The difference was approximately two fold with OD_{595}/OD_{600} ranging from 0.2 to 0.4 (Fig. 3B).

The MIC of TET was shown to be 15 μ g/mL (Fig. 2C and 2D). With TET alone, increase in the levels of biofilm was observed (Fig. 3C and 3D). When TA was added in the absence of TET, biofilm formation was enhanced at concentrations ≤ 30 μ g/mL, but decreased at TA concentrations ≥ 60 μ g/mL (Fig. 3C). Interestingly, when TA was combined with any given concentration of TET, there was a significant impairment of biofilm formation ($P < 0.05$) compared to when no TA was present (Fig. 3C). This behavior was not generally observed with the antibiotic CAR (Fig. 3A). GA was effective in inhibiting the biofilm formation of *C. violaceum* when no antibiotic was present, and in the presence of CAR (at higher concentrations of GA), whereas this inhibition did not occur in the presence of TET (Fig. 3D). In summary, the combination of TA and TET was shown to be effective in reducing the biofilms of *C. violaceum*. GA was not an effective anti-biofilm agent in combination with the antibiotic TET.

Effect of tannin materials on motility

Motility is important for the dissemination of pathogenic bacteria and the spread of infection (McDougald et al. 2011; Rasko and Sperandio, 2010). Swimming motility was characterized by migration of cells from the point of bacterial inoculation (Fig. 4A and 4B) in the swim medium in the presence or absence of sub-MIC concentrations of tannins. *C. violaceum* showed adequate swimming motility in the absence of tannins with swim zone diameters of 40 ± 6 mm. In the presence of TA, swimming motility was significantly impaired at all concentrations tested, with virtually no swimming at TA concentrations ≥ 30 $\mu\text{g/mL}$ (Fig. 4A). GA also significantly impaired swimming motility at all concentrations tested, with virtually no motility seen at GA concentrations ≥ 125 $\mu\text{g/mL}$ (Fig. 4B).

Discussion

The study was undertaken to understand the synergistic and antagonistic effects of tannins (TA and GA) in combination with antibiotics (CAR and TET) used in the treatment of *C. violaceum* infections. Specifically, we investigated the effects of TA and GA (Fig. 5) against *C. violaceum* with the goal of assessing their feasibility to be used as alternatives to antibiotics or to enhance the effectiveness of existing antibiotics to mitigate bacterial growth and biofilm formation. TA and GA impaired bacterial growth at MIC concentrations of 500 and 2000 $\mu\text{g/mL}$, respectively (Fig. 1), demonstrating that TA has more antimicrobial activity in comparison to that of GA (Table 1; Fig. 1). Similarly, Akiyama et al. (2001) reported that TA exhibited greater antimicrobial activity in comparison to that of GA against different strains of *Staphylococcus aureus*; with MIC values for TA of 250 $\mu\text{g/mL}$ compared to MIC values of 8000 $\mu\text{g/mL}$ for GA. These reports demonstrate that TA has more antimicrobial

activity in comparison to that of GA (Table 1; Fig. 1). One of the mechanisms of the effective antibacterial activity of TA could be attributed to the presence of ester linkage between GA and glucose (to form TA) (Chung et al. 1998) (Fig. 5). The inhibitory activity of GA at higher concentrations could be due to the chelation of metal ions (cofactor) necessary for the enzymatic activity and growth of bacterial cells (Liu et al. 2003; Yahiaoui et al. 2008). Chung and Murdock (1991) reported the inhibitory effect of TA against *Listeria monocytogenes*, *Escherichia coli*, *Salmonella enteritidis*, *S. aureus*, *Aeromonas hydrophila* and *Streptococcus faecalis*. They also suggested that when TA is hydrolyzed, the ester bond is broken to form gallic or ellagic acid resulting in the loss of antimicrobial property of TA. Also, the enhanced antibacterial activity of TA, but not GA, could be due to the enhanced iron binding ability of TA (Chung et al. 1998).

We tested the effectiveness of tannins (TA and GA) to influence the MIC of the antibiotics, CAR and TET. These antibiotics were selected based on the reports of sensitivity of *C. violaceum* towards the antibiotic CAR (Neu and Swarz 1969; Farrar and Odell 1976; Yang and Li 2011) and TET (Lee et al. 1999; Kumar 2012). Of the 72 isolates of *C. violaceum* tested against different antimicrobial agents, 42% were susceptible to CAR and 92% to TET (Yang and Li 2011). *C. violaceum* has often been reported to develop resistance to multiple antibiotics, including CAR and TET (Aldridge et al. 1988), so it can be useful to identify adjunct therapies that enhance the activity of antimicrobials; therefore, the use of tannins alone and in combination with selected antibiotics was tested. CAR is a carboxy-penicillin antibiotic and TET is a broad spectrum polyketide antibiotic. In this study, the MIC of CAR was 12 mg/mL and that of TET was 15 µg/mL (Fig. 2A-D). The resistance towards CAR antibiotic is attributed to the ability of *C. violaceum* to produce beta-lactamase that

breaks down the beta-lactam ring present in the penicillin group of antibiotics (Farrar and Odell 1976; Fantinatti-Garboggini 2004). Reports also suggest the increased resistance of *C. violaceum* over years of treatment towards the antibiotics CAR, cefoxitin and ticarcillin (Aldridge et al. 1988). In the present investigation, when tannins were used in combination with antibiotics, an increase in the MIC values of both antibiotics was observed (Fig. 2A-D). We hypothesize that the reduction in antibacterial activity could be due to a) binding of the antibiotic to tannins (Neyestani et al. 2007), b) competitive binding of the tannins to beta-lactamase (in case of CAR) or similar enzymes responsible for antibiotic hydrolysis (Chung and Murdock, 1991; Chung et al. 1998) or, c) tannins causing a modification of the bacterial properties such as biofilm formation and/or secondary metabolite synthesis, rendering them more protected to the effects of antibiotics (O'May et al. 2012).

This study shows how different tannins affect the MIC of antibiotics against *C. violaceum*. It is known that combination of antibiotics with natural agents can be an interesting approach to control infections. As reported by Takahashi et al. (1995), an increase in antibacterial activity against *S. aureus* was observed when antibiotics (oxacillin, methicillin, aminobenzyl-penicillin, TET, and chloramphenicol) were used in presence of tannins (catechin). This suggests that tannin containing compounds can enhance the antibacterial activity of selected antibiotics. However, as per the results herein, this is not always true. The MIC of CAR was increased from 12 to 50 mg/mL in presence of TA and GA, and for TET there was an increase from 15 to 30 µg/mL with TA and 15 to 125 µg/mL with GA (Fig. 2A-D). This has also been documented elsewhere, for example, green tea extracts (containing tannins) reduced the anti-bacterial activity of amoxicillin and cephalexin antibiotics against *Streptococcus pyogenes* (Neyestani et al. 2007). Another study showed

combination of TA and EGCG (epigallocatechin gallate) to enhance biofilm formation and reduce antibacterial activity of tobramycin (O'May et al. 2012).

Bacterial motility is important for dissemination of the pathogen in the host, acquisition of nutrients and in the establishment of bacterial virulence (McDougald et al. 2011). Plant materials containing tannins have been reported to restrict surface-associated motility and, in turn, enhance biofilm formation in *P. aeruginosa* PAO1 (O'May et al. 2012). The ability of the anti-motility compounds to enhance biofilm formation may be concerning as the biofilm mode of growth is associated with enhanced resistance to antibiotics (Costerton et al. 1999). Because *C. violaceum* is resistant to several antibiotics (Fantinatti-Garboggini 2004), we were interested in examining whether tannins are able to affect bacterial motility and lead to the enhancement of biofilms. O'May et al. (2012) showed that the swarming motility in *P. aeruginosa* PAO1 was effectively blocked by the application of TA, epigallocatechin gallate, and cranberry powder; whereas, the formation of biofilms was enhanced in the presence of these materials. In contrast, GA was less effective in blocking the swimming motility and did not significantly affect the biofilms. To date, there is no evidence in the literature that points to the inverse relationship between motility and the biofilm mode of growth of *C. violaceum*. However, it would be expected that a reduction in bacterial motility would correspond with an enhancement in the bacterial sessile lifestyle. In the present study, TA significantly reduced swimming motility (Fig. 4A) and enhanced biofilm formation of *C. violaceum* at lower concentrations (sub-MIC), but impaired biofilm formation at $TA \geq 60 \mu\text{g/mL}$ (Fig. 3A and 3C). Thus, the study herein provides support for an inverse relationship when *C. violaceum* is exposed to sub-MIC concentrations of TA. However, there was no increase in the biofilm levels at lower concentration of GA (in absence of antibiotic)

and the inverse relationship (motility versus biofilm) was not observed in the presence of GA, similar to the observations made by O'May et al. 2012. This study provides further support that when identifying any anti-motility compound, care needs to be taken to understand whether there will be any possible enhancement in biofilm formation or increased MIC of already utilized antibiotics.

To evaluate an alternative strategy to combat *C. violaceum* infections, we tested the susceptibility of this strain towards tannins (TA and GA) and antibiotics (CAR and TET). Biofilm formation in 96 well plates was studied in the presence and absence of tannins and antibiotics. TA at sub-MIC concentrations (15 and 30 µg/mL) showed enhancement in the formation of biofilms, but impaired biofilm at higher concentrations but lower than MIC (Fig. 3A and 3C). On the other hand, addition of GA alone resulted in the reduction of biofilms at sub-MIC concentrations 250-1000 µg/mL (3B and 3D). The enhancement of biofilms in presence of sub-MIC concentrations of tannins has been shown earlier by O'May et al. (2012). The authors provided some evidence that interaction of TA with *P. aeruginosa* flagella could be responsible for the enhancement of biofilms (O'May et al. 2012). The study reported less biofilm formation of *P. aeruginosa* with flagellar mutants and tannins. They hypothesized that tannins could cross-link the bacterial flagellum and result in inhibition of swarming motility and enhanced biofilm formation. Similar interactions between the *C. violaceum* flagellum and TA could have led to the increased biofilms in microtiter plates observed herein. Tannin rich extracts of the plant *Terminalia catappa* have been reported to inhibit biofilms of *P. aeruginosa* (Taganna et al. 2011). In a recent study, Trentin et al. (2013) reported the ability of three plant extracts to prevent growth and inhibit the formation of *P. aeruginosa* biofilms. They suggested the bacteriostatic effect of these tannin rich extracts to

be responsible for the anti-biofilm effects. However, tannins may also interfere with the activity of antibiotics. Herein, the antibiotic CAR alone was found to be effective in inhibiting biofilms of *C. violaceum* at concentrations that did not prevent bacterial growth (Fig. 3A and 3B). However, in the presence of selected sub-MIC concentrations of TA or GA + antibiotics, we observed enhanced biofilm formation (Fig. 3A and 3B). This shows that interaction of tannins (especially TA) with CAR can result in antagonistic interactions, decreasing the efficacy of the antibiotic (Fig. 2) and promoting biofilm formation (Fig. 3A and 3B). This reduced efficacy of antibiotics could be due to binding of the tannins to antibiotics, thereby reducing their bioavailability. However, when TA (15-125 µg/mL) was used in combination with sub-MIC concentrations of TET, it effectively prevented formation of *C. violaceum* biofilms (Fig. 3C). This result is interesting because bacterial growth was not inhibited (Fig. 2C), and bacteria were not forming biofilms either. Similarly, GA in combination with CAR at selected concentrations (0.08 and 1.5 mg/mL CAR) showed reduction in biofilms (Fig. 3B). The reduction in biofilms was however to a lower extent as compared with TA + TET. This suggests that TA can work synergistically with TET to prevent biofilm development. The synergistic relationship was not observed with TA and CAR. TET antibiotic inhibits bacterial protein synthesis by binding to the 30S ribosomal subunit (Chopra and Roberts 2001) and CAR is a beta-lactam antibiotic that inhibits bacterial cell wall synthesis (Butler et al. 1970). TA has a greater relative binding efficiency to iron than GA, wherein TA acts like siderophores and chelates iron from the medium making it unavailable to the microorganisms for growth and virulence. This could be one of the mechanisms of increased efficacy of TA (Chung et al. 1998) compared to GA. It is also worth noting that biofilm levels appear to be enhanced when the data is not normalized by the level of bacterial growth (OD₆₀₀) (Fig. S1).

These results illustrate the importance of considering the normalized extent of biofilm formation which takes into account the growth inhibitory effects of TA, GA or antibiotics (Fig. 3). The study also suggests that the combined application of TA with TET or GA with CAR (at selected concentrations) for preventing the formation of bacterial biofilms might be a useful treatment approach. However, caution must be applied to avoid the development of enhanced bacterial resistance.

This study showed that tannins could have either positive or negative effects towards the bacterium, *C. violaceum*. Overall, tannins were effective in modulating the behaviour of the relatively less explored pathogen, *C. violaceum*. TA was more effective in controlling bacterial growth and motility as compared to GA. At sub-MIC concentrations (15-30 µg/mL), TA enhanced biofilm formation; however, in the presence of the antibiotic TET (0.4-7 µg/mL), it inhibited biofilms of *C. violaceum*. Further study with tannins in combination with different antibiotics and using different biofilm models is needed to obtain an improved understanding of the underlying mechanisms of action for such alternative strategies that could be made available to control *C. violaceum* infections.

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Table 1. Growth inhibitory activity of tannins determined by the agar well diffusion method

Tannins (µg/mL)	0	15	30	60	125	250	500	1000	2000
Tannic acid (TA)	+	+	+	+	+	–	–	–	–
						(MIC)			
Gallic acid (GA)	+	+	+	+	+	+	+	+	–
									(MIC)

Note: “+” corresponds to no inhibition zone, “–” corresponds to inhibition zone with diameter of ≥ 1 mm.

Figure captions

Fig. 1. Growth of *C. violaceum* CV026 in 96 well polystyrene microtiter plates. Growth of the bacterium was estimated at OD₆₀₀ in presence and absence of (A) tannic acid (TA) and (B) gallic acid (GA). Selected concentrations of TA and GA are shown in the graph.

Fig. 2. Checkerboard assay to determine the effect of (A) tannic acid (TA) and carbenicillin (CAR); (B) gallic acid (GA) and carbenicillin (CAR); (C) tannic acid (TA) and tetracycline (TET) and (D) gallic acid (GA) and tetracycline (TET) on growth of *C. violaceum*. The black colored wells indicate growth of *C. violaceum* and the white wells represent no growth.

Fig. 3. Effect of tannic acid (TA; A, C) and gallic acid (GA; B, D) in presence of carbenicillin (CAR) and tetracycline (TET) on biofilm formation by *C. violaceum* CV026. Biofilm absorbance was taken at OD₅₉₅ and normalized with growth (OD₆₀₀). The bars represent biofilm formation by *C. violaceum* at sub-MIC concentrations of TA (0-125 µg/mL), GA (0-1000 µg/mL) and antibiotics, CAR (0-6 mg/mL) and TET (0-7 µg/mL). † indicates statistically different values ($P < 0.05$) of antibiotics (CAR or TET) without TA or GA when compared with control (values without CAR or TET) and * indicates statistically different values ($P < 0.05$) of TA or GA when compared with control (values without TA or GA) at different concentrations of antibiotics (CAR or TET).

Fig. 4. Effect of (A) tannic acid, TA and (B) gallic acid, GA on swimming motility of *C. violaceum* CV026. * indicates statistically different values ($P < 0.05$) when compared to control without tannins.

523

524 **Fig. 5.** Molecular structures of tannin compounds used in this study.

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Draft

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