Epigallocatechin gallate from *Camellia sinensis* L. (Kuntze) is a potential quorum sensing inhibitor in *Chromobacterium violaceum*

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ABSTRACT

The problem on the widespread occurrence of antibiotic resistant strains of bacteria calls for novel methods of control of bacterial activity. One of the new viable alternatives to antibiotics is the use of substances that inhibit quorum sensing (QS) – a bacterial communication system that has been known to regulate the expression of virulence genes during infection. In this study, epigallocatechin gallate (EGCG) from green tea, *Camellia sinensis* L. (Kuntze) was tested for its ability to inhibit QS in a test organism, *Chromobacterium violaceum*. This microorganism produces a violet-colored substance, violacein, through QS. This study aimed to detect inhibition of QS-regulated violacein production in *C. violaceum* by EGCG and to determine the dynamics of QS inhibition relative to the concentration of EGCG. The effect of increasing concentration of EGCG on both violacein production and cell density of treated and untreated *C. violaceum* was determined in a 96-well-microplate format and read at 570nm and 620nm for violacein production and growth, respectively. The results show that addition of EGCG increased the growth of the organism while there is concentration-dependent decrease in the QS-controlled production of violacein. This study thus establishes that EGCG is a potential QS inhibitor and can be further studied and developed for its use as an anti-pathogenic but non-toxic drug.

Keywords: antibiotic resistance, anti-pathogenic, *Camellia sinensis* L. (Kuntze), *Chromobacterium violaceum*, epigallocatechin gallate, quorum sensing inhibition

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INTRODUCTION

Quorum sensing (QS) is the production and reception of diffusible signal molecules or autoinducers by bacteria to regulate the expression of phenotypes that are dependent on population density (Shauder & Bassler, 2001). After the first mechanism of this communication system in *Vibrio fischeri* was elucidated (Nealson et al., 1970; Eberhard et al., 1981), other studies found the same system at work in many other groups of bacteria – mainly differing in the nature and structure of the diffusible signals and on the phenotypes being regulated (De Kievit & Iglewski, 2000; Schauder & Bassler, 2001). Some of the phenotypes regulated by QS are antibiotic production in *Erwinia carotovora* (Bainton et al., 1992); expression of virulence factor in *Pseudomonas aeruginosa* (Gambello & Iglewski, 1991); swimming and swarming motility in *Yersinia enterocolitica* (Atkinson et al., 2006); and biofilm formation in a number bacterial species including *P. aeruginosa* (Davies et al., 1998; Favre-Bonte et al., 2003), *Vibrio cholerae* (Hammer & Bassler, 2003) and *Serratia marcescens* (Labbate et al., 2004; Rice et al., 2005).

In the quest for QS inhibitors or antagonists, studies have found that many eukaryotes, particularly plants, and even bacteria themselves produce anti-QS substances (Gonzalez & Keshavan, 2006). The first one to be studied was the Australian red alga (*Dilesia pulchra*), which was found to produce furanones that inhibit the QS system of a marine bacterium, *Serratia liquefaciens* (Givskov et al., 1996). Several species of higher plants, including pea seedlings, secrete a series of unidentified signals that are capable of interfering with the QS of reporter strains (Teplitski et al., 2000). The work of Keshavan and colleagues in 2005 found that L-canavanine, secreted by the legume alfalfa (*Medicago sativa*) interferes with the QS of *Sinorhizobium meliloti*, a nitrogen-fixing bacterium that invade its roots.

In vivo studies have documented the anti-pathogenic effects of QS inhibitors. Synthetic furanones were found to enhance bacterial clearance of *P. aeruginosa* from lung infection in mice (Wu et al., 2004). QS inhibitory extract from garlic renders *P. aeruginosa* susceptible to tobramycin in lungs of mice (Bjarnsholt et al., 2005). Disruption of AI-2 quorum sensing by a natural and a synthetic brominated furanone protected gnotobiotic *Artemia* from the pathogenic isolates in *in vivo* challenge tests (Defoirdt et al., 2006). QS inhibitor RIP injected to rats prevented methicillin-resistant *Staphylococcus aureus* infections (Balaban et al., 2007). These studies establish that the use of QS inhibitors is a viable alternative to the use of antibiotics and that there is a need to search for other natural QS inhibitors from plants or other sources.

In this regard, we checked for the possibility that extracts from green tea, *Camellia sinensis* L. (Kuntze) can be a source of such QS inhibitory substances. The main component of green tea extracts, epigallocatechin gallate (EGCG) has been previously shown to inhibit biofilm formation in *Staphylococcus* spp. (Blanco et al., 2005), that it inhibits penicillinase leading to restoration of antibiotic activity of penicillin (Zhao et al., 2002) and that it possesses direct bactericidal activity on certain species of bacteria (Toda et al., 1989, 1991).

The objective of the present study was to evaluate the ability of EGCG to inhibit quorum sensing using a test organism, *Chromobacterium violaceum*. Specifically, it aimed to detect inhibition of QS-dependent violacein production in *C. violaceum* by EGCG and to determine the dynamics of QS inhibition by EGCG in relation to its concentration.

MATERIALS AND METHODS

Bacterial cultures and EGCG

The pure culture of wild type *C. violaceum* JCM1249 was obtained from Japan Collection of Microorganisms (Saitama, Japan) while the non-pigmented mutant strain, *C. violaceum* CV026 (NCTC13278) was acquired from the National Collection of Type Cultures (London, U.K.). These were maintained and cultured in Luria-Bertani (LB) broth and LB agar. The EGCG used was Teavigo® green tea extract powder (DSM Nutritional Products, Philippines) with a claimed 94 to 99% EGCG content derived from *Camellia sinensis* L. (Kuntze).

Tube-based qualitative assessment of QS inhibition by EGCG

A stock solution of 10,000 µg mL^{-1} EGCG was prepared. From this stock solution, dilutions using nutrient (NB) as diluent were prepared. Three lanes composed of seven sterile test tubes at varying concentrations were set. A single lane contained tubes with 5 mL of 1000, 500, 250, 125, 62.5, 31.25 and 0 µg mL^{-1} of EGCG in nutrient broth. Two lanes were inoculated with 100 µL of *C. violaceum* culture with an OD_{660} of 1.6. The remaining lane was used as a negative control. The whole set-up was then incubated at 37°C for 48 hours. Inhibition of quorum sensing or of growth was seen as a reduction in violet pigmentation of the treated broth cultures.

Quantitative QS inhibition and toxicity assays in C. violaceum

The effect of EGCG on the QS-controlled production of violacein was determined using the wild type pigment-producing strain of *C. violaceum* while the potential toxic effects was monitored using non-pigmented *C. violaceum* strain to avoid inaccuracy due to light scattering of violacein at 620 nm. This protocol was a modified version of that described by Martinelli *et al.* (2004). A two-fold serial dilution of the sample was prepared in 8-well lanes of a 96-well microtiter plate (1000, 500, 250, 125, 62.5, 31.25, 15.625 and 0 ug/mL) at 150 uL each using LB broth as diluent. Fifty microliters per well of an overnight culture of the wild type *C. violaceum* in LB broth was added to three lanes. The same amount of an overnight culture of CV026 in LB was added to the next three lanes. Another three lanes contained serially diluted EGCG at the indicated concentrations dissolved in LB but without any culture added to measure the absorbance of EGCG in LB alone for normalization purposes. The plate was then incubated at 37°C for 24 hours; after which, it was read at 570 nm (for violacein) and 620 nm (for cell density) using a microplate spectrophotometer (Tecan, Spectra III, Austria).

Data analyses

The data was analyzed using ANOVA followed by DMRT when the data set satisfy the assumptions of parametric tests; while Friedman test followed by Conover's post hoc test was used for the data sets that are non-parametric. Statistical analyses were done using SPSS 15 and StatsDirect 2.6.5.

RESULTS AND DISCUSSION

The test organism, *C. violaceum*, produces a violet-colored substance, violacein, which has been identified to have antibiotic properties. McClean et al. (1997) have shown that the production of this antibiotic is regulated by QS following the LuxI-LuxR circuit. This became the basis for the development of a simple procedure of screening for QS inhibitors using the decrease in violet pigmentation of *C. violaceum* as indicator of QS inhibition (McLean et al., 2004). But since any reduction in violacein production could also be a result of reduction in bacterial cell density, another criterion has to be met in order to consider the violacein-inhibiting substance as a QS inhibitor. Methods should be done to check the growth or cell density of the test organism. If the violacein-inhibiting substance causes a corresponding decrease in cell density, then it is not to be considered as a QS inhibitor. An effective and pharmacologically important QS inhibition should be able to considerably reduce the expression or production of a QS-regulated phenotype without significantly affecting the population density of the treated bacteria.

The results for the violacein inhibition assay based on violacein's absorbance at 570 nm revealed that its production is negatively affected by increasing concentration of EGCG in the medium (Figures 1 & 2). ANOVA and Duncan's post hoc test for this data set have shown that violacein production has started to significantly decrease at EGCG concentration of 250 µg mL^{-1} and that pigment production is significantly decreasing from this point when EGCG concentration is further increased. It is quite interesting that at concentration lower than 125 µg mL^{-1}, violacein production is enhanced, which correlates with an increase in cell density of the non-pigmented *C. violaceum* at this range of concentration. This could be due to the presence of other components (e.g. impurities in the Teavigo® preparation of EGCG) that may have been utilized by *C. violaceum* as a carbon or nitrogen source. This may also have been elicited

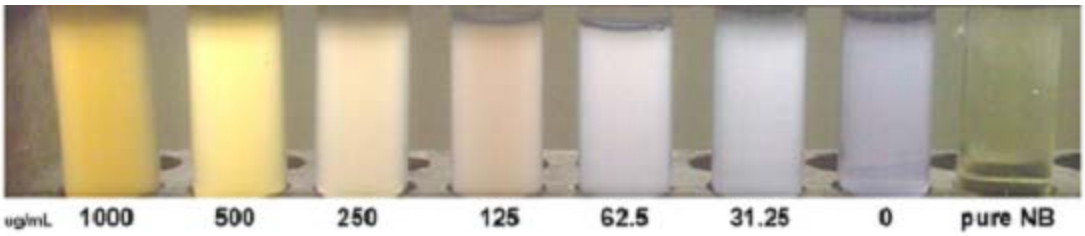

Figure 1. Photograph showing the increasing production of violacein by *C. violaceum* at decreasing concentrations of EGCG. (Colored image appears online.)

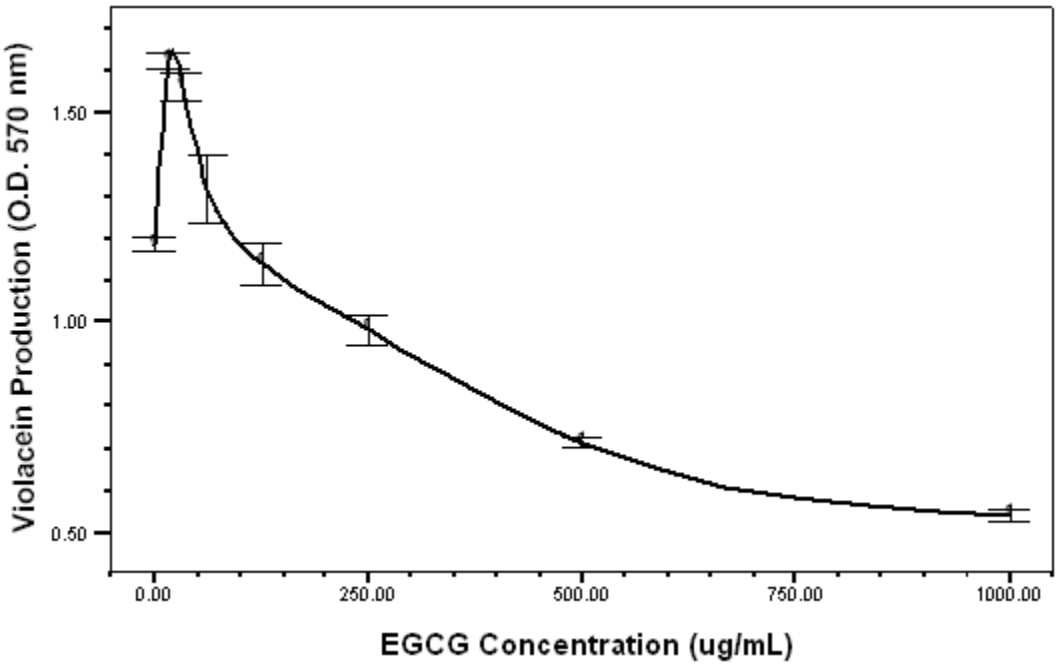

Figure 2. Graph showing the relationship between epigallocatechin gallate (EGCG) concentration and the amount of violacein produced by treated *C. violaceum* JCM1249.

by EGCG itself, which the test organism may have construed to be a chemical offensive from a possible competitor microbial population.

The data on effects of EGCG on growth (see Figure 3) have shown that there was a general increase in *C. violaceum* CV026 cell density in the presence of increasing concentration of EGCG. Friedman test and Conover's post hoc test have confirmed that from the lowest (15.625 μg mL^{-1}) to the highest EGCG concentration (1000 μg mL^{-1}), the growth of the test organism has gone significantly higher than that of the untreated. The slower growth of the test organism at concentrations between 15.625 and 62.5 μg mL^{-1} then a sudden increase in growth from 125 to 250 μg mL^{-1} can be thought to be a result of interactions between various components in the Teavigo® preparation, which may contain components other than EGCG. Otherwise, this could be due to changes in binding preference of EGCG to different targets at different levels or concentrations. It may, for instance, bind or react to certain metabolites at a particular range of concentration that may adversely affect the growth of the organism but may preferentially bind other targets at higher concentrations. However, what is worth noting is that growth is generally enhanced by EGCG at a

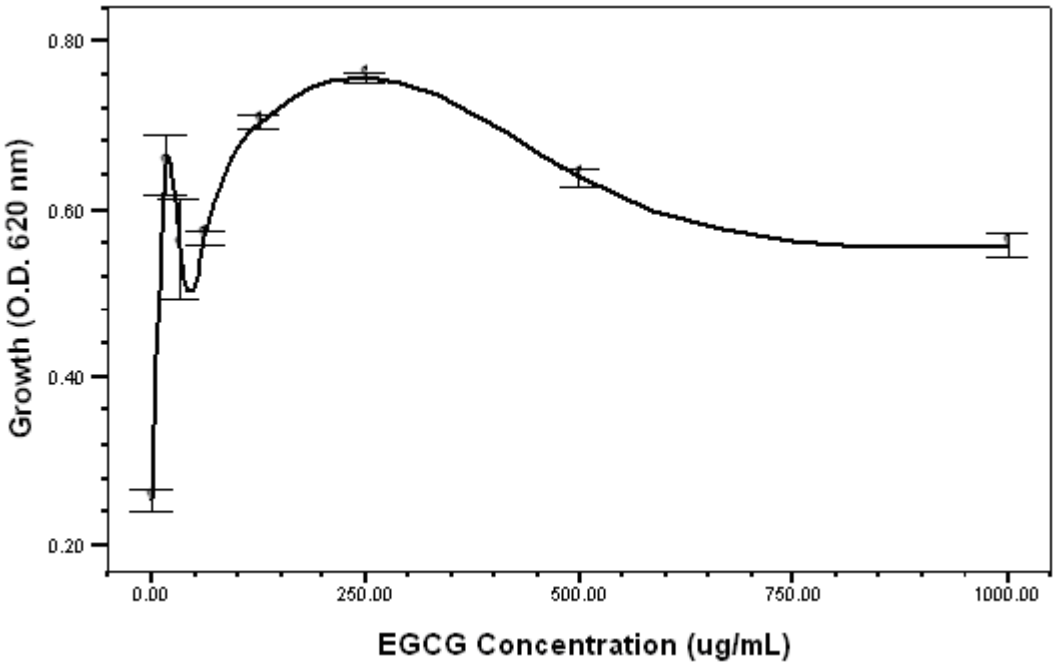

Figure 3. Graph showing the growth or cell density of *C. violaceum* CVO26 cells at increasing concentration of epigallocatechin gallate (EGCG).

concentration range when violacein production is significantly reduced starting at around 250 μg mL^{-1}. While a previous work by Huber *et al.* (2003) already found that EGCG antagonizes QS in two recombinant reporter strains – *Escherichia coli* MT102 (pSB403) and *Pseudomonas putida* (pKR-C12), the present work proceeded to test the anti-QS activity of this compound on another test organism, *C. violaceum*, that normally expresses an easily observable and quantifiable QS-controlled phenotype. Furthermore, the Teavigo® powder is a food grade EGCG preparation, which has less purity than the analytical grade EGCG used by the previous report; and yet, it is still QS inhibitory at a higher concentration but within the working formulations of many Teavigo®-supplemented food products.

Therefore, the findings of the present work strongly indicate that EGCG derived from *C. sinensis* L. inhibits QS in *C. violaceum*, which strengthens a previous report that it can be a potential QS inhibitor in other Gram-negative bacteria and can be further studied and developed for its use as an anti-pathogenic but non-toxic drug. Other plants, especially those that are used in traditional medicine, can be screened for QS inhibitory compounds. Screening, isolation and purification of these types of compounds from Philippine medicinal plants are currently in progress, of which this study is a preliminary work for assay optimization.

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