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Epigallocatechin Gallate Inhibits Biofilm Production and Attenuates Virulent Factors of *Pseudomonas aeruginosa* and *Psuedomonas fluorescence*

Linda Tran[1], Arjun Naik[1], Brian Koronkiewicz[2], Bela Peethambaran[1,2*]

[1]Department of Biological Sciences, University of the Sciences in Philadelphia, Philadelphia, PA 19107, USA
[2]Department of Chemistry, University of the Sciences in Philadelphia, Philadelphia, PA 19107, USA

Abstract

Catechins especially Epigallocatechin Gallate (EGCG) and its isomers have been shown to constitute the most important and effective antimicrobial part of tea polyphenols. Antimicrobial effects of EGCG on a variety of pathogens have been published previously. The anti-microbial mechanism of green tea polyphenols has also been studied. Effects of green tea extracts on bacterial surviving tactics such as Quorum Sensing (QS) have showed significant inhibitory effects. However, there are no literatures that demonstrate a quantitative assay that would establish the Minimum Inhibitory Concentration (MIC) that can interfere with QS regulated factors. Also, there is no published data that clearly demonstrates which components of green tea or black tea is responsible for the inhibition of QS-regulated virulence factors. In this study we prove that EGCG which constitutes 5% of dry weight of green tea and 10% in green tea is effective in inhibiting the biofilm production of *Psuedomonas aeruginosa* and *Psuedomonas fluorescens* by down-regulating its virulence factors. Our study established a Minimum Inhibitory Concentration (MIC) of 8mM (~4 mg/ml) concentration of EGCG for inhibition of biofilm production in both organisms. QS regulated virulent factors such as pyocyanin, protease and elastase production also decreased with increase in the EGCG concentration.

Keywords: Epigallocatechin gallate, *Psuedomonas aeruginosa, Pseudomonas fluorescens,* pyocyanin, protease and elastase assays

1. Introduction

Epigallocatechin Gallate (EGCG) is a polyphenolic bioflavonoid synthesized in a wide range of plants especially black and green tea. It is a potent antimicrobial compound and is also beneficial to human health [1]. EGCG is an antioxidant that has several health benefits such as inhibition of the cellular oxidation of Low Density Lipoproteins (LDL) [2] and pharmacological effect such as an antihistaminic, anti-inflammatory, and anti-viral agent [3]. EGCG constitutes the most effective antimicrobial agent among the tea polyphenols known [1, 4]. Tea polyphenols are known to play a role in protein precipitation, enzyme inhibition and hence have a broad antimicrobial spectrum to pathogens such as *Escherichia coli*, *Salmonella thyimurium*, *Listeria monocytogenes*, *Staphlococcus aureus* and *Pseudomonas aeruginosa* [1, 4–6]. Shu-min et al. showed in their studies that tea polyphenols inhibit *P. aeruginosa* by damaging the cell membranes [7]. In Shimamura et al. study, tea polyphenols were shown to inhibit influenza virus by inhibiting enzyme activity [8]. Several studies have demonstrated various mechanisms by which EGCG and its isomers Epigallocatechin (EGC) and the flavine 3, 3' gallate can inhibit microbial growth [9–11]. EGCG was also demonstrated to have anti-*L. monocytogenes* activity by inhibiting the hemolytic and cholesterol binding activity of listeriolysin O which disrupts the

*Author for correspondence
Email: b.peethambaran@usciences.edu

phagosomal membrane [9]. Quorum Sensing (QS) is one of the mechanisms by which gram negative bacteria such as *P. aeruginosa* modulates survival, pathogenicity and virulence [11]. Hence development of antimicrobial agents that can inhibit QS can offer a possible alternative to the antibiotic mediated bactericidal and bacteriostatic approaches in addition to reducing the growing resistance to antibiotics. Our investigation was to establish a MIC at which EGCG inhibits biofilm production and is also effective in down-regulating virulent factors of *P. aeruginosa*. In humans, *P. aeruginosa* has been known to cause chronic lung infections in cystic fibrosis and immunocompromised patients [7]. It is an opportunistic pathogen known for its inherent and acquired resistance against a wide variety of antimicrobial agents by forming a matrix of polysaccharide called biofilms that enables the advanced survival capacity on the bacteria by providing a physical barrier against the entry of antimicrobial agents. Hence, there is a constant quest for new anti-microbial compounds that can inhibit its proliferation. Gram negative rods show different susceptibilities to EGCG as it depends on the virulent factors of the bacteria and its interaction with the molecular structure of the antimicrobial compound [12]. In this study, we try to prove that EGCG can attenuate the production of a set of virulence factors and biofilm formation of *P. aeruginosa*. We also present quantitative results that show the EGCG concentration at which there was no visible biofilm production and also a drastic reduction in production of virulent factors such as pyocyanin, protease and elastase.

2. Materials and Methods

All the chemicals were bought from Sigma and *P. aeruginosa* was bought from ATCC (9027). *P. fluorescens* strain 2–79 was borrowed from the USciences microbiology labs.

2.1 Biofilm Formation Assay and Biofilm Quantification

The ability of EGCG to inhibit *P. aeruginosa* and *P. fluorescens* to form biofilm on abiotic surfaces was studied according to a previously described crystal violet assay [9], except that polypropylene instead of polystyrene tubes were used. Briefly, 2 mL of *P. aeruginosa* culture was incubated 37°C overnight prior to the start of the experiment. The following concentrations of EGCG 2 mM, 4 mM, 6 mM, 8 mM, and 16 mM were added to tubes containing 1/100 dilution of an overnight LB broth culture in BDT medium (Bushnell-Haas mineral salts medium supplemented with 0.2% dextrose and 0.5% tryptone), bringing the final volume to 1 mL. The tubes were then incubated at 37°C for 24 hours. The biofilm was qualitatively assayed using crystal violet staining as described in the study [13]. Biofilm formation was quantified by staining the polypropylene tubes with 0.1% (wt/vol) CV in wash buffer (0.15 M ammonium sulfate, 100 mM potassium phosphate [pH 7], 34 mM sodium citrate, and 1 mM $MgSO_4$) at room temperature for 20 minutes. Excess crystal violet was then removed and rinsed with water. The crystal violet that had stained cells was solubilized in 1 mL of 80% (vol/vol) ethanol and 20% (vol/vol) acetone. Biofilm formation was quantified by measuring the OD_{570} for each tube, using a spectrophotometer.

2.2 Pyocyanin Quantification Assay

For the pyocyanin assay, 200 µl overnight *Pseudomonas* culture was added in 20 ml of LB broth. This was incubated overnight at 37°C prior to the start of the experiment. The following concentrations of EGCG 2 mM, 8 mM, and 16 mM were added to the overnight culture tubes bringing the final volume to 5 mL and incubated at 37°C for a minimum of 18 hours. After incubation, the cell free supernatants of *P. aeruginosa* cultivated in the presence of EGCG were extracted with 3 mL of chloroform and then re-extracted into 1 mL of 0.2 M HCl to obtain a pink to deep red solution [14]. The absorbance of the solution was then measured spectrophotometrically at OD_{520}.

2.3 Protease Assay

The procedure for the protease assay was taken from previous studies [15]. Briefly, 24 mL of 1% *P. aeruginosa* or *P. fluorescens* culture was incubated at 37°C for a minimum of 18 hours prior to the start of the experiment. Concentrations of EGCG similar to ones above i.e. 2 mM, 8 mM, and 16 were added to the overnight culture tubes bringing the final volume to 2 mL and incubated at 37°C for a minimum of 18 hours. 1 mL of azocasein in buffer (consisting of 0.05 M TRIS HCl and 0.5 mM $CaCl_2$) were added and incubated at room temperature for 15 minutes

followed by the addition of 0.5 mL trichloroacetic acid. The tubes were then centrifuged at 10,000 rpm for 10 minutes. The absorbance of clear supernatant was measured spectrophotometrically at OD_{400}.

2.4 Elastase Assay

Elastolytic activity was determined by following the method of Ohman et al. [13] using Elastin Congo Red (ECR) as the substrate. 1 mL of 1% *Psuedomonas* culture was incubated 37°C for a minimum of 18 hours prior to the start of the experiment. In brief, 100 µl of treated and untreated *P. aeruginosa* culture supernatant for 2 mM, 8 mM and 16 mM of EGCG concentration was added into 900 µl of ECR buffer (consisting of 100 mM Tris and 1 mM $CaCl_2$) (pH 7.5) containing 20 mg of ECR and incubated with shaking at 37°C for 3 hours. The reaction was then stopped by adding 1 mL of 0.7 M sodium phosphate buffer (pH 6.0). The tubes were placed in an ice water bath for 15 minutes and centrifuged to remove the insoluble ECR. The absorbance of the supernatant was measured at OD_{495}.

3. Results

Catechins extracted from tea have demonstrated anti bacterial activity [6, 7, 12]. Thus, we hypothesize that EGCG will inhibit biofilm production of *P. aeruginosa*. Figure 1 results indicate that *w*ith the increase in EGCG concentration there was a subsequent decrease in biofilm production.

As the concentration of EGCG increases, the amount of biofilm produced decreases. The 2 mM, 4 mM, 6 mM, 8 mM, and 16 mM concentration of EGCG reduced the biofilm formation by 57.7 %, 60.2%, 74.4%, 82.6%, and 94.4%, respectively compared to the untreated *P. aeruginosa*. On the other hand, *P. fluorescens* was more sensitive to EGCG treatment as there was significant decrease in its growth at 2 mM EGCG when compared to growth of *P. aeruginosa* at the same concentration. The Minimum Inhibitory Concentration (MIC) for both the organism was 8 mM which approximately is 3.6 mg/ml of EGCG (Figure 1).

Increase in the concentrations of EGCG also decreased the QS regulated virulent factor pyocyanin. *P. aeruginosa* is only organism that is capable of producing pyocyanin as the *P. fluorescens* strain does not produce pyocyanin [19, 20]. Hence, Figure 2 represents the amounts of pyocyanin produced by *P. aeruginosa*.

As the concentration of EGCG increased, the pyocyanin formation decreased (Figure 2). Untreated *P. aeruginosa* produced 39.2 µg of pyocyanin per 10^8 cells while the replicates for 2 mM, 6 mM, 8 mM and 16 mM of

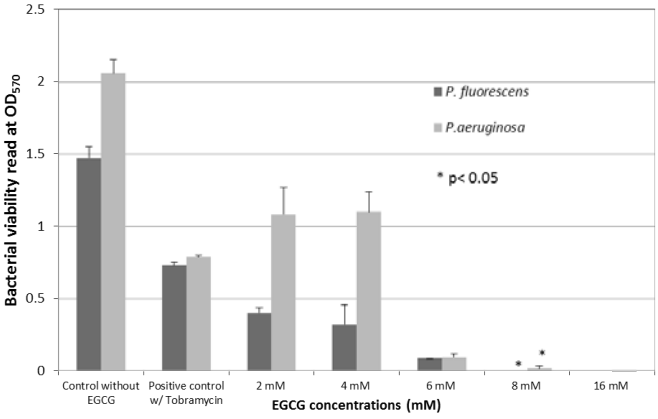

Fig. 1. Graph consisting of the averages of each of the three independent replicates tested for 24 hours after treatment with EGCG. The absorbance at 570 nm represents the amount of biofilm stained. The error bars represent the standard deviation. *p<0.05. The statistical test was performed on the EGCG treated groups versus the non-treated *Psuedomonas* control.

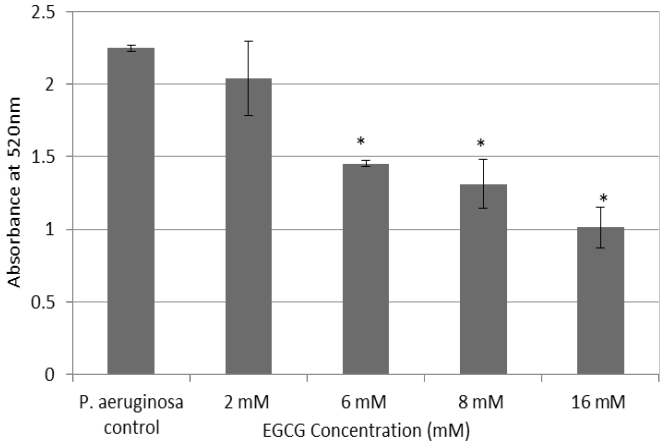

Fig. 2. Graph showing the averages of each of the three independent replicates tested a minimum of 18 hours after treatment with EGCG. The error bars represent the standard deviation. The Y axis represents the amount of pyocyanin produced. *p<0.05 statistical test was performed for each EGCG treated group to the non-treated control.

EGCG produced 32.6, 25.9, 21.3, and 18.4 µg of pyocyanin per 10^8 cells, respectively. Thus, this reduced the pyocyanin formation by 9.2%, 35.3%, 41.5%, and 54.8% for 2 mM, 6 mM, 8 mM and 16 mM of EGCG respectively.

Elastase and protease are important proteolytic enzymes that allow bacteria to invade and colonize host tissues [16], thus the effect of EGCG showed a reduction in the total proteolytic and elastolytic activity as seen in Figure 3 and 4. There was a significant decrease in the protease activity at 16 mM concentration of EGCG both for *P. aeruginosa* and *P. fluorescens*. The elastolytic activity in *P. aeruginosa* was significantly reduced at 8mM EGCG concentration, while in the case of *P. fluorescens* significant reduction was observed only at 16 mM EGCG concentrations.

4. Discussion

The regulation of QS systems in *P. aeruginosa* has made considerable progress but the regulation of QS in other *Pseudomonas* especially in *P. fluorescens* has not been elucidated well [23]. The studies so far show that the complex QS regulation present in *P. aeruginosa* may also occur in other *Pseudomonas*. But on the flip side, these pseduomonads may undergo complex regulation of QS depending on the different growth conditions [23]. Hence there are renewed efforts in studying the molecular mechanisms of QS regulation to design ways to interrupt it by introducing novel antibacterial molecules. Our research is an attempt to show that the catechins present in tea that have been previously demonstrated to reduce growth of two different *Pseudomonas*, affects its growth by down-regulating the QS controlled virulent factors. We have assayed the effects of EGCG on virulent factors such elastolytic and proteolytic activity common in both organism as well as pyocyanin that is exclusively seen in *P. aeruginosa*.

All the results above show that EGCG has an inhibitory effect on the QS regulated factors in *P. aeruginosa* and *P. fluorescens*. The development of biofilm is extremely crucial for the existence of *P. aeruginosa* inside the host. The extracellular polymeric substances secreted by these organisms forms a protective barrier preventing the access of antibiotics as well as the host immune system [17]. In a study conducted by Chou et al. [21] the growth of *P. fluorescens* depended on the amounts of catechin in the tea. Catechin content in tea leaves varies according to the seasons. The content is 22%, 20%, 19.4% and 19.2% respectively in summer, spring fall and winter [22]. Our results show that a concentration of 8mM to 16mM EGCG will cause detrimental effects on the QS regulated virulent factors for both the bacteria.

Pyocyanin is a green antibiotic pigment regulated by genes controlled by Quorum Sensing (QS) secreted by *P. aeruginosa*, which enables it to favourably grow in certain environments [16], thus the inhibitory effect of EGCG on

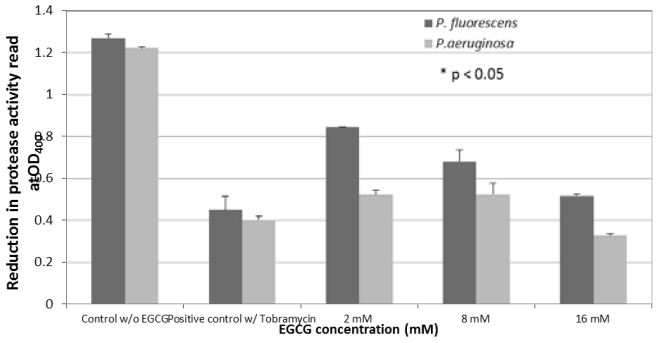

Fig. 3. Graph depicting the averages of each of the three independent replicates incubated for a minimum of 18 hours after treatment with EGCG. The error bars represent the standard deviation. The protease activity is indicated by the absorbance reading at 400 nm. *p<0.05 statistical test was performed for each EGCG treated group to the non-treated control.

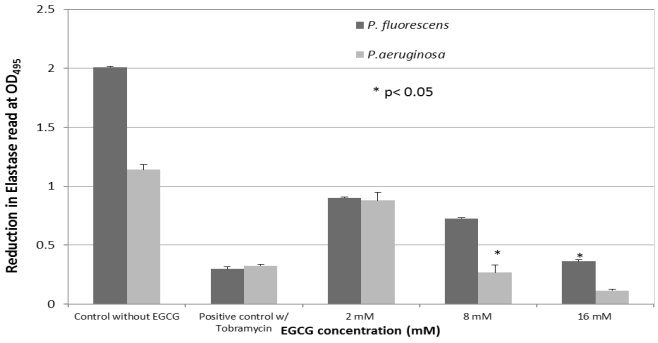

Fig. 4. The above graph consists of each of the three independent replicates incubated for a minimum of 18 hours after treatment with EGCG. The error bars represent the standard deviation. The elastase activity is indicated by the absorbance reading at 495 nm. *p<0.05 statistical test was performed for each EGCG treated group to the non-treated control.

the ability of *P. aeruginosa* to produce and secrete this virulence-associated pigment was studied. There was a linear reduction in the amounts of pyocyanin produced with the increase in the concentration of EGCG. Earlier work by Huber et al. [18] had showed that EGCG in purified form can inhibit QS in chromobacterium system. But another group's preliminary studies [16] showed that EGCG did not have any significant effect in inhibiting QS regulated virulent factors in *Pseudomonas*. Our study supports the work done by Huber et al. [18] and also provided a MIC for the inhibition of QS-related virulent factors.

The conclusion to be drawn from the above work is that EGCG is responsible for altering QS regulated factors and can effectively inhibit the growth of two different strains of *Psuedomonas*. The established MIC of EGCG is 8mM to inhibit biofilm production. We also show here that EGCG concentration as much as 2mM is effective in down-regulating QS-regulated virulent factors.

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References

1. Almajano MP, Carbo R, Jimenez JAL, Gordon MH. Antioxidant and antimicrobial activities of tea infusions. Food Chem. 2008 May; 108(1):55–63.
2. Ou HC, Song TY, Yeh YC, Huang CY, Yang SF, Chiu TH et al. EGCG protects against oxidized LDL-induced endothelial dysfunction by inhibiting LOX-1-mediated signalling. J Appl Physiol. 2010 Jun; 108(6):1745–56.
3. Benelli R, Vene R, Bisacchi D, Garbisa S, Albini A. Anti-invasive effects of green tea polyphenol Epigallocatechin-3-Gallate (EGCG), a natural inhibitor of metallo and serine proteases. Biol Chem. 2002 Jan; 383(1):101–05.
4. Hamilton-Miller JM. Antimicrobial properties of tea (*Camellia sinensis* L.). Antimicrob Agents Chemother. 1995 Nov; 39(11):2375–77.
5. He Q, Lv Y, Yao K. Effects of tea polyphenols on the activities of α-amylase, pepsin, trypsin and lipase. Food Chem. 2007; 101(3):1178–82.
6. Yam TS, Shah S, Hamilton Miller JM. Microbial activity of whole and fractionated crude extracts of tea *(Camellia sinensis),* and of tea components. FEMS Microbiol Lett. 1997 Jul 1; 152(1):169–74.
7. Yi S, Zhu J, Fu L, Li J. Tea polyphenols inhibit *Pseudomonas aeruginosa* through damage to the cell membrane. Int J Food Microbiol. 2010 Nov 15; 144(1):111–17.
8. Shimamura T, Hara Y inventor; Mitsui Norin Co., Ltd assignee. Method of inhibiting and treating infection caused by influenza virus. United States patent 5137922 A. 1992 Aug 11.
9. Frei B, Higdon JV. Antioxidant activity of tea polyphenols in vivo: evidence from animal studies. J Nutr. 2003 Oct; 133(10):3275S–84S.
10. Nakane H, Ono K. Differential inhibitory effects of some catechin derivatives on the activities of human immunodeficiency virus reverse transcriptase and alular deoxyribonucleic and ribonucleic acid polymerases. Biochemistry. 1990 Mar 20; 29(11):2841–45.
11. Raffa RB, Iannuzzo JR, Levine DR, Saeid KK, Schwartz RC, Sucic NT et al. Bacterial communication (quorum sensing) via ligands and receptors: A novel pharmacologic target for the design of antibiotic drugs. J Pharmacol Exp Ther. 2005 Feb; 312(2):417–23.
12. Yoda Y, Hu Z, Zhao W, Shimamura T. Different susceptibilities of staphylococcus and gram-negative rods to epigallocatechin gallate. J Infect Chemother. 2004 Feb; 10(1):55–58.
13. Ohman DE, Cryz SJ, Iglewski BH. Isolation and characterization of a *Pseudomonas aeruginosa* PAO1 mutant that produces altered elastase. J Bacteriol. 1980 Jun; 142(3):836–42.
14. Kessler K, Safrin M, Olson JC, Ohman DE. Secreted LasA of *Pseudomonas aeruginosa* is a staphylolytic protease. J Bio Chem. 1993 Apr 5; 268(10):7503–08.
15. Essar DW, Eberly L, Hadero A, Crawford IP. Identification and characterization of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: interchangeability of the two anthranilate synthases and evolutionary implications. J Bateriol. 1990 Feb; 172(2):884–900.
16. Mihalik K, Chung DW, Crixol SH, McLean RJC, Vattem DA. Quorum sensing modulators of *Pseudomonas aeruginosa* characterized in *Camellia sinensis*. Asian J Tradit Med. 2007 Nov; 3(1):12–23.
17. O'Toole GA, Pratt LA, Watnick PI, Newman DK, Weaver VB, Kolter R. Genetic approaches to study of biofilms. Methods Enzymol. 1999; 310:91–109.
18. Huber B, Eberl L, Feucht W, Polseter J. Influence of polyphenols on bacterial biofilm formation and quorum sensing. Z Naturforsch. 2003 Nov–Dec; 58(11–12): 879–84.

19. Reyes EAP, Bale NJ, Cannon W, Matsen JM. Identifcation of *Pseudomonas aeruginosa* by pyocyanin production on tech agar. J Clin Microbiol. 1981 Mar; 13(3):456–58.
20. Mavrodi DV, Bonsal RF, Delaney, SM, Soule MJ, Phillips G, Thomashow LS. Functional analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from *Pseudomonas aeruginosa* PAO1. J Bacteriol. 2001 Nov; 183(21):6454–65.
21. Chou CC, Lin LL, Chung KT. Antimicrobial activity of tea as affected by the degree of fermentation and manufacturing season. Int J Food Microbiol. 1999 May 1; 48(2):125–30.
22. Kan N. The aroma constituents of various teas. Food Industr. 1980; 12(4):19–22.
23. Venturi V. Regulation of quorum sensing in *Pseudomonas*. FEMS Microbiol Rev. 2006 Mar; 30(2):274–91.