

The Antimicrobial Activity of (–)-Epigallocatechin-3-Gallate and Green Tea Extracts against *Pseudomonas aeruginosa* and *Escherichia coli* Isolated from Skin Wounds

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Background: Skin infections with Gram-negative bacteria are sometimes challenging to treat, because these bacteria show multidrug resistance against commonly used antibiotics and patients with Gram-negative bacterial infection overall have deteriorated in conditions in many cases. Studies have shown that epigallocatechin gallate (EGCG) and green tea extracts (GTE) inhibit the growth of several Gram-positive bacteria species. **Objective:** The purpose of this study was to investigate the minimum inhibitory concentrations (MICs) of EGCG and GTE in *Pseudomonas aeruginosa* and *Escherichia coli*, and assess the use of these chemicals as an alternative or adjunct topical antimicrobial agent against *P. aeruginosa* and *E. coli* with multidrug resistance. **Methods:** The MICs of EGCG, GTE, and other tested antibiotics were measured and compared to determine the antibacterial efficacy and the differences in pattern of resistance. **Results:** The *P. aeruginosa* and *E. coli* strains used in this study showed multidrug resistance. EGCG inhibited the growth of *P. aeruginosa* at a MIC level of 200 ~ 400 μ g/ml. The MIC of GTE was a 1 : 16 dilution for *P. aeruginosa*. EGCG showed antimicrobial activity against *E. coli* at a MIC of 400 μ g/ml. In the case of GTE, the MIC was a dilution between 1 : 8 and 1 : 4 for *E. coli*. **Conclusion:** EGCG and GTE showed potential as alternative or adjunct topical antimicrobial agents for

infections that are resistant to traditional antibiotic therapy. (Ann Dermatol 26(5) 564~569, 2014)

-Keywords-

Escherichia coli, Epigallocatechin gallate, Gram-negative bacterial infection, Microbial sensitivity tests, *Pseudomonas aeruginosa*

INTRODUCTION

Skin infections with *Pseudomonas aeruginosa* and *Escherichia coli* occur more frequently in patients who are immunologically compromised, elderly, or who are in intensive care units (ICU). These patients may have other comorbidities, including deteriorated renal and hepatic function, administration of multiple other drugs, and complications from chronic diseases. Thus, the administration of systemic antibiotics to these patients has a higher risk of side effects. In addition, there is a narrow range of antibiotics that can be used to treat infection, because many strains of *P. aeruginosa* and *E. coli* show resistance to certain antibiotics.

Useful topical treatment for Gram-negative bacterial infection is limited. The use of topical antibiotics and antiseptics are also limited due to bacterial resistance and the inherent nature of treatment agents. For instance, some antibiotics/antiseptics contain superoxide-generating hydrogen peroxide. Further, potassium permanganate and chlorhexidine are cytotoxic and can inhibit the regeneration of cells for wound repair^{1,2}.

Epigallocatechin gallate (EGCG) is a major catechin found in green tea extracts (GTE), and has remarkable antibacterial activity as well as anti-oxidant, anti-inflammatory, and anti-cancer effects. Further, EGCG can induce epithelial

Received June 22, 2013, Revised August 19, 2013, Accepted for publication September 20, 2013

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proliferation and differentiation³⁻⁵. EGCG is known to be active against Gram-positive bacteria^{4,6}. It is also known that the minimum inhibitory concentration (MIC) of EGCG in Gram-negative bacteria appears to be 8- to 16-fold higher than that in Gram-positive bacteria⁶. The relatively lower antimicrobial activity of catechins against Gram-negative bacteria is due to the protection of the Gram-negative bacteria by the outer membrane and lipopolysaccharides.

This study was conducted to assess the possible use of EGCG and GTE as antibacterial agents in Gram-negative *P. aeruginosa* and *E. coli*, regardless of antibiotic resistance status.

MATERIALS AND METHODS

Bacterial strains

The studied bacterial strains were isolated from the skin wounds of patients admitted to the ICU at our University Hospital. Twenty-two strains in total were studied, and consisted of 10 *P. aeruginosa* strains, 10 *E. coli* strains, and 2 reference strains (*P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922) for quality control. Cultured strains were kept at a turbidity matching a 0.5 McFarland standard and placed in each well with a final concentration of 2.5×10^5 colony-forming unit (CFU) /ml.

Antibiotic susceptibility tests of the bacterial strains

Specimens from the skin wounds of the ICU patients were inoculated and cultured on blood agar medium and MacConkey agar medium for 24 to 48 hours at 35°C. The identification of bacterial isolates was performed manually and processed simultaneously using the ATB ID system (BioMérieux, Marcy l'Etoile, France). Antibiotic susceptibility was assessed using the disk diffusion method following the guidelines published by the Clinical and Laboratory Standards Institute (CLSI)⁷ for ampicillin, amikacin, aztreonam, ceftazidime, cefepime, cephalothin, cefotaxime, ciprofloxacin, gentamicin, imipenem, piperacillin, piperacillin/tazobactam, and sulperazone. The results of the antibiotic susceptibility tests of isolated strains were compared with the results of EGCG and GTE susceptibility tests.

EGCG and GTE preparation

EGCG (Sigma, St. Louis, MO, USA) in powder form with 95% purity was dissolved in normal saline and serially diluted with cation-adjusted Mueller-Hinton broth (MHB) starting from a maximum concentration of 800 μ g/ml to 0.4 μ g/ml with 12 levels of dilution. Two grams of dried green tea leaves (product name: Halla; Amorepacific

Corporation, Seoul, Korea) were brewed with 100 ml of boiling water for 10 min. After removing the tea leaves, the infusion was left for another 10 min and then cooled down to below 40°C. The green tea infusion was filtered twice using Whatman filter paper (Whatman International Ltd., Maidstone, UK). Final filtration by a filter with a pore size of 0.22 μ m was performed to remove possible microbial contaminants. Crude GTE was diluted from undiluted extract in 12 dilution stages (1 : 1 ~ 1 : 2,048) by successively adding two-fold MHB.

Measuring minimal inhibitory concentration

Gentamicin (Sigma) and ciprofloxacin (Fluka, Saint Gallen, Switzerland) were used as reference antibiotics to assess the antimicrobial activity of EGCG and GTE against *P. aeruginosa*⁸. Ampicillin (Sigma) and ciprofloxacin were tested against *E. coli*. Ciprofloxacin was dissolved in 0.1 N HCl, and other antibiotics and EGCG were dissolved in normal saline. The antibiotics were serially diluted with cation-adjusted Muller-Hinton broth from a concentration of 256 μ g/ml to 0.125 μ g/ml, and 200 μ l of the serially diluted antibiotics, EGCG or GTE was placed in a 96-well plate (Corning, Acton, MA, USA). *E. coli* and *P. aeruginosa* were inoculated in every well at a concentration of 2.5×10^5 CFU/ml. The MIC was visually determined after 16~20 hours of incubation at 37°C according to the broth microdilution methods suggested by the CLSI⁸.

RESULTS

Antibiotic susceptibility of the studied strains of *P. aeruginosa* and *E. coli*

1) *P. aeruginosa*

Five of the ten collected strains and the reference strain were susceptible to all of the tested antibiotics, while the other five strains showed multidrug resistance. These multidrug-resistant strains were commonly resistant to ciprofloxacin, gentamicin, piperacillin, and piperacillin/tazobactam. All ten of the studied strains were susceptible to imipenem and ceftazidime (Table 1).

2) *E. coli*

Only one of the collected *E. coli* strains and the reference strain were susceptible to all of the tested antibiotics. Nine of the ten strains were resistant to at least one antibiotic (up to nine antibiotics), although they did not show any similarities in their patterns of resistance. All of the *E. coli* strains were susceptible to ceftazidime, amikacin, imipenem, piperacillin/tazobactam, and cefoperazone/sulbactam (Table 1).

Table 1. Antibiotic susceptibility test results of 20 clinical isolates used in this study

Strain	AM	CP	CTX	CE	CI	CX	GM	AN	AZ	CZ	IM	PI	PT	SP
1 <i>Pseudomonas aeruginosa</i>					R	R	R	R	I	S	S	R	R	R
2 <i>P. aeruginosa</i>					S	S	S	S	S	S	S	S	S	S
3 <i>P. aeruginosa</i>					S	S	S	S	I	S	S	S	S	S
4 <i>P. aeruginosa</i>					I	R	R	R	I	I	S	R	R	I
5 <i>P. aeruginosa</i>					S	S	S	S	S	S	S	S	S	S
6 <i>P. aeruginosa</i>					S	S	S	S	I	S	S	S	S	S
7 <i>P. aeruginosa</i>					R	R	R	R	I	S	S	R	R	R
8 <i>P. aeruginosa</i>					S	S	S	S	S	S	S	S	S	S
9 <i>P. aeruginosa</i>					R	R	R	S	R	S	S	R	R	I
10 <i>P. aeruginosa</i>					S	S	S	S	S	S	S	S	S	S
ATCC27853					S	S	S	S	S	S	S	S	S	S
<i>P. aeruginosa</i>														
1 <i>Escherichia coli</i>	R	R	S	S	S	R	R	S	S	S	S	R	S	S
2 <i>E. coli</i>	R	S	S	S	S	R	S	S	S	S	S	S	S	S
3 <i>E. coli</i>	S	S	S	S	S	R	S	S	S	S	S	S	S	S
4 <i>E. coli</i>	R	R	R	S	R	S	S	S	R	R	S	R	S	S
5 <i>E. coli</i>	R	R	R	S	R	R	R	S	R	R	S	R	S	S
6 <i>E. coli</i>	S	S	S	S	S	S	S	S	S	S	S	S	S	S
7 <i>E. coli</i>	R	R	R	S	R	R	R	S	R	R	S	R	S	S
8 <i>E. coli</i>	R	R	R	S	R	R	R	S	R	R	S	R	S	S
9 <i>E. coli</i>	R	S	S	S	S	R	S	S	S	S	S	R	S	S
10 <i>E. coli</i>	R	S	S	S	S	S	R	S	S	S	S	R	S	S
ATCC25922	S	S	S	S	S	S	S	S	S	S	S	S	S	S
<i>E. coli</i>														

AM: ampicillin, CP: cephalothin, CTX: cefotaxime, CE: ceftiofloxacin, CI: cefepime, CX: ciprofloxacin, GM: gentamicin, AN: amikacin, AZ: aztreonam, CZ: ceftazidime, IM: imipenem, PI: piperacillin, PT: piperacillin/tazobactam, SP: sulperazone, R: resistant, I: intermediate, S: sensitive.

Table 2. MICs of tested antibiotic agents against *Pseudomonas aeruginosa*

Strain number	Ciprofloxacin* (μ g/ml)	Gentamicin [†] (μ g/ml)	EGCG (μ g/ml)	Green tea extracts (diluted)		
1	2	I	>256	R	200	1 : 16
2	<0.125	S	2	S	200	1 : 16
3	0.5	S	1	S	400	1 : 16
4	32	R	>256	R	200	1 : 16
5	<0.125	S	1	S	200	1 : 16
6	<0.125	S	1	S	400	1 : 16
7	16	R	>256	R	400	1 : 16
8	<0.125	S	1	S	400	1 : 8
9	>256	R	>256	R	400	1 : 16
10	<0.125	S	1	S	400	1 : 8
ATCC27853 [†]	0.25	S	1	S	200	1 : 16

MIC: minimum inhibitory concentration, EGCG: epigallocatechin gallate, I: intermediate, R: resistant, S: sensitive. *MIC breakpoints of gentamicin: S, $\leq 4 \mu$ g/ml; I, 8μ g/ml; R, $\geq 16 \mu$ g/ml. [†]MIC breakpoints of ciprofloxacin: S, $\leq 1 \mu$ g/ml; I, 2μ g/ml; R, $\geq 4 \mu$ g/ml. [‡]Acceptable limits for quality control to monitor the accuracy of the MICs of ATCC27853: gentamicin, $0.5 \sim 2 \mu$ g/ml; ciprofloxacin, $0.12 \sim 1 \mu$ g/ml.

MICs of ciprofloxacin, gentamicin, EGCG, and GTE against *P. aeruginosa*

1) Three *P. aeruginosa* strains were resistant to ciprofloxacin. However, the growth of one strain was not suppre-

ssed, even at a concentration of 256μ g/ml. The reference strain and six of the studied *P. aeruginosa* strains were susceptible to gentamicin, with MICs around 1μ g/ml. The other four *P. aeruginosa* strains showed a high degree of resistance to gentamicin, even up to 256μ g/ml (Table 2).

Table 3. MICs of tested antibiotic agents against *Escherichia coli*

Strain number	Ciprofloxacin* ($\mu\text{g/ml}$)		Ampicillin [†] ($\mu\text{g/ml}$)		EGCG ($\mu\text{g/ml}$)	Green tea extracts (diluted)
1	16	R	> 256	R	400	1 : 8
2	64	R	256	R	400	1 : 8
3	32	R	4	S	400	1 : 4
4	0.25	S	> 256	R	400	1 : 4
5	128	R	> 256	R	400	1 : 4
6	<0.125	S	4	S	400	1 : 8
7	32	R	> 256	R	400	1 : 4
8	256	R	> 256	R	400	1 : 8
9	16	R	> 256	R	400	1 : 4
10	0.25	S	> 256	R	400	1 : 4
ATCC25922 [‡]	<0.125	S	2	S	400	1 : 16

MIC: minimum inhibitory concentration, R: resistant, S: sensitive. *MIC breakpoints of ciprofloxacin: S, $\leq 8 \mu\text{g/ml}$; I, $16 \mu\text{g/ml}$; R, $\geq 32 \mu\text{g/ml}$. [†]MIC breakpoints of ampicillin: S, $\leq 1 \mu\text{g/ml}$; I, $2 \mu\text{g/ml}$; R, $\geq 4 \mu\text{g/ml}$. [‡]Acceptable limits for quality control to monitor the accuracy of the MICs of ATCC25922: ampicillin, $2\sim 8 \mu\text{g/ml}$; ciprofloxacin, $0.004\sim 0.016 \mu\text{g/ml}$.

2) The MIC of EGCG against six of the *P. aeruginosa* strains was $400 \mu\text{g/ml}$, while the MIC of the other four strains and reference strain was $200 \mu\text{g/ml}$. Two of the ten *P. aeruginosa* strains were susceptible an 8-fold dilution of crude GTE, whereas the other eight strains and the reference strain were susceptible at a 16-fold dilution (Table 2).

3) The MIC of EGCG and the dilution levels of GTE were not related to the degree of resistance of *P. aeruginosa* to ciprofloxacin and gentamicin.

MICs of ciprofloxacin, ampicillin, EGCG, and GTE against *E. coli*

1) Three of the ten strains of *E. coli* and the reference strain were susceptible to ciprofloxacin. The *E. coli* studied had a higher MIC and a higher incidence of resistance to antibiotics than *P. aeruginosa*. Eight of the *E. coli* strains were resistant to ampicillin, with uninhibited growth at a concentration of $256 \mu\text{g/ml}$ (Table 3).

2) The MIC of EGCG against all *E. coli* strains, including reference strain, was $400 \mu\text{g/ml}$. In the case of GTE, the growth of the reference strain was inhibited at a 1 : 16 dilution. The MIC of GTE for four *E. coli* strains was an 8-fold dilution, whereas the MIC of the other six *E. coli* strains was a 4-fold dilution (Table 3).

3) The degree of resistance of the *E. coli* strains to ciprofloxacin and ampicillin was not related to the MIC of EGCG and GTE.

DISCUSSION

Skin and soft tissue infections with *P. aeruginosa* and *E. coli* have been an issue since both incidence and drug resistance are increasing. In healthy people, *P. aeruginosa* rarely causes infection; however, it poses a serious health risk in hospitals, where it is responsible for about 10% of in-hospital infections in immunocompromised patients, including those with cancer, diabetes, and hematological disorders, as well as patients undergoing transplant, receiving implants, and being treated with corticosteroids and antibiotics⁹. In Taiwan, it was reported that *E. coli* was responsible for almost one-fifth of skin and soft tissue infections¹⁰. In Europe, *E. coli* accounted for 10.8% of infections, and the corresponding rate was 7.2% for North America¹¹. With the observed increase in the rate of bacterial resistance to an extended spectrum of antibiotics, clinical cures and the selection of active antibiotics for empirical treatment may be more difficult to achieve¹². A major component of green tea is a flavonol known as catechin¹³. (–)-Epicatechin (EC), (–)-epigallocatechin (EGC), (–)-epicatechin gallate (ECG), and EGCG are the four main catechins. In particular, EGCG is found only in green tea, comprising 40% ~ 50% of green tea catechins and is thought to be primarily responsible for the antibacterial and bactericidal properties of green tea^{4,6,14,15}. Gallo-catechin and gallate are necessary moieties for antibacterial activity, with gallate-conjugated (–)-ECG and (–)-EGCG demonstrating more powerful antibacterial activity than non-gallate-conjugated (–)-EGC and (–)-EC¹⁴. It has been reported that the bactericidal effect of EGCG is stronger in Gram-positive bacteria than in Gram-negative bacteria, owing to the different amounts of EGCG absor-

bed by the bacterial cells^{6,15,16}. While the MIC of EGCG against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus hominis*, and *Staphylococcus haemolyticus* has been reported to be 50~100 $\mu\text{g/ml}$, the MIC of EGCG against *Klebsiella pneumoniae*, *Salmonella typhi*, and *Proteus mirabilis* is much higher (800 $\mu\text{g/ml}$)⁶. GTE has also shown various degrees of antibacterial activity, and a wide range of susceptibility against different strains of the same species^{6,17,18}. One proposed mechanism for the bactericidal action of catechin is that the negatively charged EGCG combines with the positively-charged bacterial lipid polysaccharide membrane generating hydrogen peroxide (H_2O_2), which damages the bacterial membrane^{3,15}. Gram-negative bacteria are generally more resistant to catechins than Gram-positive bacteria, due to the presence of strong negative charge of lipopolysaccharides on the exterior outer member of Gram-negative bacteria¹⁶. EGCG is known to have unique dual actions, and it protects human keratinocytes and fibroblasts against H_2O_2 by reversing the H_2O_2 -induced decrease of superoxide dismutase (SOD) and glutathione peroxidase^{19,20}. The amount of EGCG or catechins in green tea differs depending on the product and extraction method. However, when green tea is infused in hot water for 3 min in a proportion of 1 g of leaves to 100 ml of water, the tea usually contains 250~280 mg of solids, of which 30%~42% are catechins³. When green tea or EGCG capsules are orally administered, only 0.2%~2.0% of the ingested EGCG is intestinally absorbed and appears in the blood^{17,18}. Considering its low plasma concentrations and the reported MIC of EGCG against Gram-negative and Gram-positive bacteria, topical application of EGCG on infected lesions is more desirable than systemic administration, since the concentrations required to treat bacterial infections of the skin cannot be reached through drinking green tea.

Based on the previously reported proportion of catechins in green tea infusions, the estimated concentration of EGCG in the crude infusion of GTE prepared in this study was roughly 400~800 $\mu\text{g/ml}$ ^{21,22}. At a GTE dilution level of 1 : 16, the EGCG concentration was about 25~50 $\mu\text{g/ml}$. Despite the low concentration of EGCG in the tested GTE, GTE showed antibacterial activity equivalent to 400 $\mu\text{g/ml}$ EGCG. This can be explained through the synergistic antibacterial action of other polyphenols in GTE, such as ECG. Considering that even a 16-fold dilution of crude GTE showed effective antibacterial activity, GTE may be practical and economically feasible for use as an alternative for topical antibiotics or as dressing agents in clinical practices. Furthermore, the clinical application of catechin is plausible, since catechin is very stable under

physical manipulations, such as freezing and heating, and can be refrigerated in an aqueous solution for over a month with good stability^{23,24}.

The bacterial strains in this study were isolated from the ulcers and sores of long-stay patients admitted to the ICU. Thus, the incidence of multidrug resistant strains was higher than community acquired strains. However, bacterial strains showing multidrug resistance did not have the same pattern of resistance against EGCG or GTE, and showed susceptibility to EGCG or GTE independent of the antibiotic resistance status. Though the antibacterial effects of EGCG and GTE varied with the individual strains of bacteria, consistent levels of effectiveness were seen regardless of the susceptibility of bacteria to the reference antibiotics. Since EGCG exerts antibacterial effects through diverse mechanisms *in vivo*, the effective MICs of EGCG against *P. aeruginosa* and *E. coli* on skin lesions will be lower than the MICs seen in this study.

Better antimicrobial effects can be expected from GTE than EGCG, since GTE contains various types of catechins with antibacterial activity other than the four major catechins: EGCG, ECG, EGC, and EC. Furthermore, GTE is easily available and would be more cost-effective. Although more studies on their mechanisms of action are needed, EGCG and GTE have great potential for use as topical antimicrobial agents with systemic antibiotics to manage skin infections. Several experiments that tested the ability of EGCG to synergistically inhibit methicillin-resistant *S. aureus* with concomitant use of oxytetracycline, carbapenem, and ampicillin/sulbactam *in vitro* demonstrated that EGCG synergistically inhibited bacterial growth²⁵⁻²⁷. An *in vivo* study of chronic *E. coli* bacterial prostatitis rat model showed synergistic effects between an oral gavage of 300 mg/kg body weight of catechin concentrate and ciprofloxacin²⁸.

The clinical application of EGCG and GTE is worth considering as a therapeutic in pursuit of overcoming the increasing antibiotic resistance of bacteria and further studies are needed²⁹.

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