

The *in vitro* Studies of the Inhibitory Effect of Green Tea (*Camellia sinensis*) on *Pseudomonas aeruginosa* Treated Contact Lenses



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Pseudomonas aeruginosa is the leading cause of ocular infections in those who wear contact lenses. Others have previously done a study using the antioxidant selenium-coated contact lenses to inhibit the bacteria in an animal model. However, selenium is very toxic even in small quantities. In this study, green tea which is known for its antioxidant property was used to treat contact lenses. We did a disc diffusion assay using different concentrations of green tea and compared with black tea to study their inhibitory effect on *P. aeruginosa*. The 100mg/mL of green tea was the most effective concentration that maintained a uniform solution and produced the clear zone. Contact lenses were treated with 100mg/mL of green tea before being exposed to *P. aeruginosa* and another experiment was done by coating the contact lenses with the bacteria and treated with the green tea afterward. We found that green-tea-treated contact lenses had fewer bacteria, with a 41.9% inhibition rate when compared to the control but the results were not significant. However, green tea significantly reduced the bacteria present on contact lenses ($p < 0.05$). In conclusion, green tea shows an inhibitory effect on *Pseudomonas aeruginosa* and has the potential to be used as a cleaning solution on contact lenses.

INTRODUCTION

Contact lenses are known to be susceptible to bacterial attachment and result in infections such as corneal ulcers and microbial keratitis in the eyes (Preechawatmd, Ratananikommd, Lerdvitayasakul, & Kunavisarut, 2007; Stenson, 1986). The risk of infection is often due to poor personal hygiene in the handling of the lenses and the storage cases, which provide the ideal environment for the growth of bacteria (Dantam et al., 2016; Szczotka-Flynn, Pearlman, & Ghannoum, 2010). Many lens cleaning solutions also risk being contaminated with the bacteria from the contact lens (Lin, Kim, Chen, Kowalski, & Nizet, 2016; Posch, Zhu, & Robertson, 2014; Szczotka-Flynn et al., 2010). *Pseudomonas aeruginosa* is the leading cause of contact lens-related ocular infections due to the nature of the bacteria's ability to survive in the eye, on the contact lens, and in the storage case (Stapleton & Carnt, 2012; Weissman, Mondino, Pettit, & Hofbauer, 1984; Willcox, 2007). It is an opportunistic pathogen in humans and can typically be found in a biofilm environment with some surface or substrate (Todar, n.d.). In addition, *P. aeruginosa* is able to attach and cause an infection of the cornea of the eye (Fleiszig, Efron, & Pier, 1992; Klotz, Misra, & Butrus, 1990; Willcox, 2007).

Many studies have been done by using organic and inorganic substances as the coating for the contact lens to prevent bacterial attachment. Concanavalin A, a lectin, was used in an injured rab-

bit's cornea in order to compete with *P. aeruginosa* for the binding of cornea cells (Blaylock, Yue, & Robin, 1990). Despite the ability to reduce the number of bacteria found on the cornea, it is toxic in high amounts (Nopanitaya, Hanker, & Tyan, 1976; Tiegs, Hentschel, & Wendel, 1992). Matthews et al. (2006) performed another study on the coating of contact lenses with selenium to inhibit the growth of *P. aeruginosa in vitro* and *in vivo*. It was found that the coating allowed for extended-wear over a period of two months and prevented *P. aeruginosa* colonization with no adverse effects on the cornea. Selenium is an antioxidant but it is also toxic even in small quantity and can cause neurotoxicity, cancer, and harm to an unborn child (Vinceti et al., Wei, Malagoli, Bergomi, & Vivoli, 2001).

Tea is another very powerful antioxidant, and has been shown to have antibacterial, anti-inflammatory and anticancer properties (Chan, Lim, Chong, Tan, & Wong, 2010; Chan, Soh, Tie, & Law, 2011; Hamilton-Miller, 1995; Piljac-Žegarac, Šamec, & Piljac, 2013; Siddiqui et al., 2016). Flayyih et al. (2013) found black tea was able to inhibit *P. aeruginosa* isolated from the corneal scrapings of various eye infections. Green tea, which comes from the same plant as black tea, *Camellia sinensis*, has also been associated with many medical properties, including anticancer properties, improvement in cardiac health, and lowering stress (Cooper, Morré, & Morré, 2005; Thangapazham et al., 2007). The green tea contains more of the specific antioxidant polyphenols, catechins, than black tea (Hamilton-Miller, 1995), which makes it more potent in antioxidant properties (Ojo, Ladeji, & Nadro, 2007; Serafini, Ghiselli, & Ferro-Luzzi, 1996; Yokozawa et al., 1998). In addition, the catechins play an important role in the inhibition of bacterial growth (Bai et al., 2016; Kumar et al., 2012; Taylor, Hamilton-Miller, & Stapleton, 2005;) by inducing the stress-relat-

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ed genes (Liu et al., 2013).

P. aeruginosa is also known for its biofilm properties and its antibiotic resistance (Costerton, Stewart, & Greenberg, 1999; Mah et al., 2003). Abidi et al. (2014) found different plant extracts exhibited antimicrobial properties against the biofilm of the bacteria and Radji et al. (2013) also incorporated green tea into drug therapy to combat antibiotic resistant bacteria.

The goal of this research was to study the effect of green tea on inhibiting *P. aeruginosa* from attaching and growing on contact lenses in vitro. We hypothesized that the use of green tea, through coating, would effectively inhibit *P. aeruginosa* from attaching and growing on contact lenses and that the use of green tea would effectively reduce the amount of *P. aeruginosa* present on infected contact lenses. The alternate use of organic products as cleaning materials are common nowadays and green tea has been used as a cosmetic for repairing dry skin (Aburjai & Natsheh, 2003). The significance of our study is the indication of the possibility of using green tea as an alternate cleaning solution for contact lenses.

MATERIALS AND METHODS

Relationship Between the Optical Density and Cell Number of *P. aeruginosa*

Seven 1:2 serial dilutions were done using a prepared culture of *P. aeruginosa* (Carolina Biological Supply Company, NC) in 0.1M Phosphate Buffered Saline (PBS) to obtain the relationship between the optical density and cell number of bacteria. Bacteria were grown at 37°C for 24 hours, and the optical densities (OD) of the stock and each dilution (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128) were measured at a 600nm wavelength using a DU 720 General-Purpose UV/Vis Spectrophotometer (Beckman Coulter, NJ). Each dilution was further diluted to obtain the countable numbers between 30-300 colony forming units (CFU), and 0.1ml or 0.5ml were placed on two nutrient agar plates (Carolina Biological Supply Company, NC). All plates were then incubated at 37°C for 24 hours. The number of bacteria that grew on the plates was then used to calculate the original amount of bacteria and was plotted against the optical density. Four trials were done, and a linear regression curve was plotted to obtain the number of bacteria in which OD = 1.

Preparation of Green Tea and Black Tea Solution and Disk Diffusion Assay

Four different concentrations (25mg/mL, 50mg/mL, 100mg/mL, and 200mg/mL) of green tea and black tea solutions were prepared with autoclaved water. We purchased and used organic Green tea Matcha (Kiss Me Organics, WY) and organic Black tea Matcha (Pure Matcha, JP) to prepare 1mL of each concentration. We also measured the pH of each tea solution.

We plated the 10⁷CFU of *P. aeruginosa* on Mueller Hinton Agar plates (Difco Laboratories, MD). Sterile plain disks (Fisher Scientific, MA) were dipped into each of the four different concentrations of prepared black and green tea respectively. The disks were dipped, dried and placed in the center of each section of the plates and grew at 37°C for 24 hours. After examining the plates,

we measured the diameter of the zone of inhibition for each concentration. Four trials were done and the averages of the inhibition clear zones were calculated.

Testing the *P. aeruginosa* on the Green Tea-Treated Contact Lenses

Six new Acuve Moist brand contact lenses (Johnson & Johnson, NJ) of -3.00 prescription strength were dripped dry from the original packaging using forceps sterilized in ethanol and transferred to sterile vials. Three vials each containing 1mL of PBS and three vials each containing 1mL of 100mg/mL of green tea were used. For each vial, one contact lens was placed in the solution for one hour. These treated contact lenses and three more from the original packaging used for a positive control were then removed, placed in separate sterile vials containing 1mL of 10⁶CFU of *P. aeruginosa* and incubated for another hour. The contact lenses from the original packaging were also placed in a 1mL solution of PBS to serve as a negative control. We diluted the solutions from each treatment were diluted at a 1:10 dilution with PBS, and 0.1mL of each was placed on two nutrient agar plates to recover the bacteria. The plates were incubated at 37°C for 24 hours and the bacteria were enumerated. A total of seven trials were done.

The Inhibitory Properties of Green Tea on *P. aeruginosa* Treated Contact Lenses

Another six new -3.00 prescription contact lenses were dripped dry from the original packaging using sterilized forceps and transferred to separate sterilized vials containing 1mL of 10⁶CFU/mL of *P. aeruginosa* and incubated on an orbital shaker rotator (Model KJ-201BD, Laboratory Sky, CN) for one hour. After that, we dripped, dried and transferred the contact lenses to sterile vials with three containing 1mL of autoclaved water, and three containing 1mL of 100mg/mL of green tea. The contact lenses were incubated in these solutions for one hour at room temperature. All these treated contact lenses, along with three more from the original packaging used for a negative control, were removed and placed in sterile vials containing 1mL of PBS to recover the bacteria. The solutions were diluted at a 1:10 dilution with PBS and 0.1mL of each solution was placed on two nutrient agar plates. The plates were incubated at 37°C for 24 hours and the bacteria were enumerated. A total of seven trials were done.

Statistical Analysis

A one-way ANOVA with a post-hoc Tukey test was run on vassarstats.net. The test was used to determine the difference between the treatment groups within each experiment. The significance was set at $p < 0.05$. Figures were created using Microsoft excel with values shown as mean and Standard Error of Mean (SEM) for the discrepancy between different trials.

RESULTS

Optical Density and Cell Number

The relationship between optical density at 600nm and the concentration of *P. aeruginosa* in CFU/mL is shown in Figure 1. The resulting equation of the curve was $y = 9 \times 10^8 x - 3 \times 10^7$. It was then de-

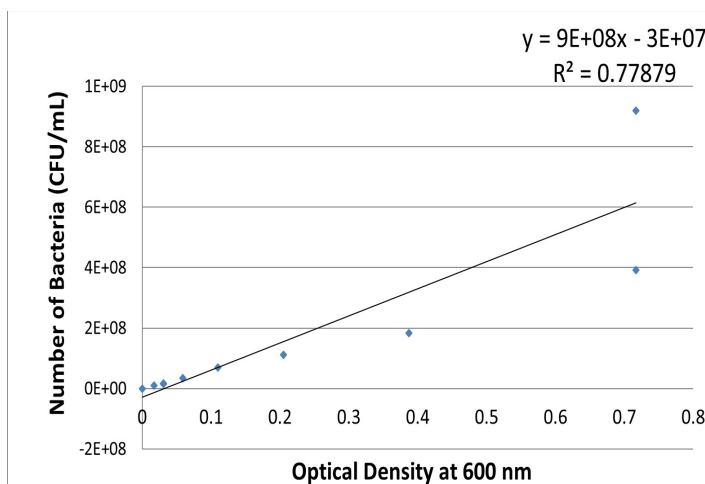


Figure 1. The correlation of the number of *P. aeruginosa* (CFU/ml) versus optical density.

terminated the concentration of the *P. aeruginosa* was 8.7×10^8 CFU/mL when OD_{600} nm is equal to 1.

Disk Diffusion Assay

The pH of both black tea and green tea were 7. Both black tea and green tea were found to have an inhibitory effect on *P. aeruginosa* in the disc diffusion assay, although green tea demonstrated a stronger effect than black tea (Figure 2). Green tea produced larger diameters of the zones of inhibition when compared to those of black tea in the same concentration. The differences between the green and black tea at the concentrations of 50mg/ml and 100mg/ml were significant ($p < 0.05$). However, the differences between the concentrations 25mg/ml and 200mg/ml in both types of tea were marginally significant ($p = 0.06$). The 100mg/ml concentration depicts the most significant difference between the them, with an average of a 2.05cm diameter clear zone from the green tea.

The Inhibitory Properties of Green Tea on Contact Lenses

Contact lenses were treated with different solutions followed by the incubation of 1mL of 10^6 CFU of *P. aeruginosa* for an hour. Contact lenses that were treated with 100mg/ml green tea had recovered 5645.45 ± 2399.70 CFU of *P. aeruginosa* when compared to the control with PBS that yielded 8690 ± 5232.05 CFU (Table 1). The original package of contact lenses that were incubated with an equal amount of *P. aeruginosa* had recovered 9722.2 ± 6287.8 CFU of the bacteria (Table 1). The bacterial inhibition rate in percentage was calculated by the difference between the number of bacteria recovered from the original contact lenses without any treatment and the number of bacteria recovered from the treated contact lenses by PBS or green tea, divided by the bacteria recovered from the original contact lenses without any treatment. The green tea-treated contact lenses had a 41.9% inhibition rate when compared to the control that was not treated ($p = 0.062$). There was no significant difference in inhibiting bacteria when comparing contact lenses treated with green tea to contact lenses treated with PBS ($p > 0.05$). There was also no significant difference in the

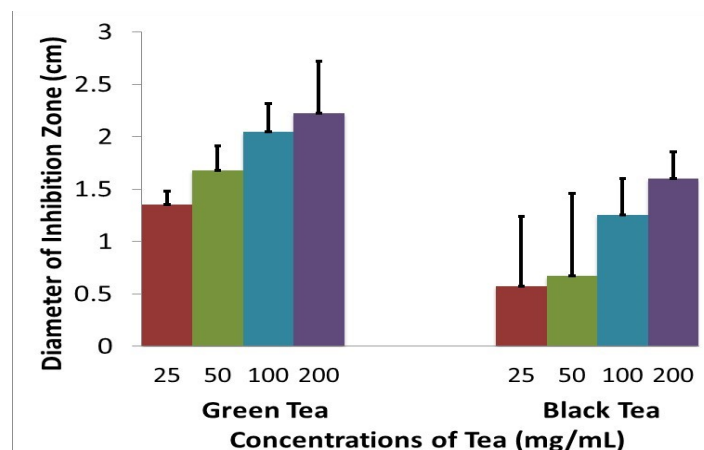


Figure 2. The average diameter (+SEM) (cm) of the clear zones when 10^7 *P. aeruginosa* was treated with green tea and black tea disc at different concentrations.

number of bacteria found in the original package and PBS treatment ($p > 0.05$).

Testing of Green Tea on Bacteria Treated Contact Lenses

The contact lenses that were incubated with 10^6 CFU of *P. aeruginosa* for an hour and treated with green tea afterwards were found to have significantly less bacteria (2887 ± 1441.18 CFU) when compared to contact lenses with equal amount of bacteria and treated with autoclaved water (61500 ± 3535.53 CFU) (Table 2) ($p < 0.05$). The control with the original contact lenses had no bacteria recovered.

DISCUSSION

Contact lenses treatment	Average number of <i>P. aeruginosa</i> (\pm SEM*) recovered (CFU)	Bacterial inhibition rate (%)
Original Packaging (No bacteria)	0.083 \pm 0.287	N/A**
Original Packaging (No treatment)	9722.20 \pm 6287.80	N/A**
Phosphate Buffered Saline	8690.00 \pm 5232.05	10.6%
100mg/ mL of Green Tea	5645.45 \pm 2399.70	41.9%

*SEM= Standard Error of Mean

**N/A= Not applicable

Table 1. The average number (\pm SEM) in CFU and the bacterial inhibition rate in percentage of *P. aeruginosa* recovered from contact lenses when treated with different solutions, followed by the incubation with 10^6 *P. aeruginosa* for an hour.

Contact Lenses Treatment	Average Number of <i>P. aeruginosa</i> (\pm SEM*) Recovered (CFU)
Original Packaging (No bacteria)	0
Autoclaved Water	61500.00 \pm 3535.53
100 mg/mL of Green Tea	2887.78 \pm 1441.18

*SEM= Standard Error of Mean

Table 2. The average number (\pm SEM) in CFU of *P. aeruginosa* recovered from contact lenses when treated with different solutions after the contact lenses were incubated with 10^6 *P. aeruginosa* for an hour.

In this study, we used green tea in an attempt to inhibit the *P. aeruginosa* from growing on the contact lens. The Kirby Bauer disk diffusion assay was done to confirm the bactericidal properties of the green tea. Our results showed that green tea produced a larger diameter of the zone of inhibition when compared to the same concentration of black tea. In previous studies, the minimum inhibitory concentration (MIC) of black tea alcohol extract was found to be 400mg/mL on *P. aeruginosa* isolates with a 20mm clear zone on the agar gel diffusion experiment (Flayyih et al., 2013). This coincides with our findings with the use of green tea on *P. aeruginosa* in the similar experiment, which produced a 20.5mm sized clear zone; however, the concentration of green tea used was only 100mg/mL. When compared to the same concentration of black tea (100mg/ml), only a 12.5mm sized clear zone was produced in this study. Several attempts to obtain the MIC of the green tea used in our studies were failed because of the dark green color of the tea interfering with the optical density reading (data not shown). Overall, green tea is more potent than black tea in inhibiting bacteria as demonstrated in previous studies (Almajano, Carbó, Jiménez, & Gordon, 2008). The stronger antioxidant properties of the catechins in green tea may attribute to its stronger antibacterial power. A previous study confirmed the antibacterial properties of the catechin was correlated to its antioxidant capacity on a phospholipid membrane model (Caturla, Vera-Samper, Villalaín, Mateo, & Micol, 2003).

In the in vitro contact lens studies, when the green tea was used for the coating of the contact lens before the treatment with *P. aeruginosa*, results showed that green tea does not effectively prevent *P. aeruginosa* from attaching and growing on contact lenses. Another experiment was performed by incubating contact lenses with *P. aeruginosa* followed by the treatment of green tea. The bacteria recovered from the contact lenses treated with green tea afterward showed a significant difference when compared to the control treated with autoclaved water. From our studies, it can be concluded that green tea showed a significant inhibitory property on the *P. aeruginosa* treated contact lenses though it was not able to remove all the bacteria from the contact lenses. This may be due to the high inoculum of bacteria (10^6) used in the experiment and the short treatment time of an hour. In a previous in vitro study, maximum numbers of *P. aeruginosa* were found to adhere to the

contact surface within an hour, however, it would take generally 24 hours for the biofilm to be formed (Dutta, Cole, & Willcox, 2012). In addition, different isolates of *P. aeruginosa* may affect the ability of their attachment to the contact lenses (Klotz, Butrus, Misra, & Osato, 1989). The strain used in this experiment was for laboratory teaching purpose and not a clinical isolate, and their attachment on the contact lenses may vary. The Etafilcon A type of contact lenses of Acuve Moist with a high water content of 58% with ionic polymers was chosen to use in our study. The nature of the contact lens material also affects the attachment of the bacteria (Dutta et al., 2012; Miller & Ahearn, 1987). Different strains of *P. aeruginosa* were found to have less adhesion on the lens composed of ionic polymers than non-ionic polymers (Miller & Ahearn, 1987). Therefore, the adhesion measured in this experiment should have fewer bacteria. The pH 7 was found to be the optimal environment for the attachment of *P. aeruginosa* on the contact lens (Miller & Ahearn, 1987) and in our studies, the pH of the green tea was found to be neutral at 7.

Lastly, green tea's active ingredient, epigallocatechin gallate (EGCG), has been shown to have the greatest antioxidant and antibacterial properties (Gordon & Wareham, 2010; Steinmann, Buer, Pietschmann, & Steinmann, 2013; Vidigal et al., 2014). Further studies can be done by testing this active ingredient against the *P. aeruginosa* on the contact lens. In addition, the green tea we used is the Matcha-powdered form, and future experiments can use other forms of green tea to reconfirm the hypothesis. For the future application, the effects of green tea on the contact lenses material and human eye should be tested.

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