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Antimicrobial Activities of Tea-Derived Flavonoids Against Skin *Staphylococci*

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Antimicrobial Activities of Tea-Derived Flavonoids Against Skin *Staphylococci*

A senior thesis submitted to
The Department of Math-Science
College of Arts & Sciences

In partial fulfillment of the requirements
For a Bachelor of Arts degree in Chemistry

by

Ahyun Hong

Faculty Supervisor	**Dr. Mihail Iordanov**	**Date**
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Concordia University
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Abstract

This thesis investigates the antimicrobial activities of tea-derived flavonoids against skin *Staphylococci*. Four hypotheses were tested: (1) Teas that contain high polyphenolic contents possess higher antimicrobial activity against skin bacteria (e.g. *S. aureus* and *S. epidermidis*); (2) Different tea infusions and tea-derived individual catechins show differential inhibition of *S. aureus* and *S. epidermidis*; (3) Different tea infusions and tea-derived individual catechins show differential inhibition of *S. aureus* isolated from individuals with and without chronic atopic dermatitis; (4) EGCg, the most abundant polyphenols found in tea, possess the highest anti-staphylococcal activity. We employed multiple methods such as Folin-Ciocalteau colorimetric method, selective media, Gram staining, the Kirby-Bauer test, and Broth dilution susceptibility test to test our hypotheses. An unpaired t-test was used for statistical analyses. The study strongly supported the first hypothesis: teas with high polyphenolic contents possessed higher antimicrobial activity against skin microbes. However, the study was not able to determine differential inhibition of *S. aureus* and *S. epidermidis*, and of *S. aureus* isolated from volunteers with and without chronic atopic dermatitis. Finally, EGC, and not EGCg, possess the highest anti-staphylococcal activity. We discuss the implications of our findings to the potential utilization of tea-derived catechins in the management of skin microflora in health and disease.

Dedication

To my family who lives 5,272 miles away.

5,272 마일에 떨어져사는 우리 가족들에게 바칩니다.

Acknowledgements

I would never have been able to finish my thesis without the unconditional support, guidance, and patience of my thesis advisor and research mentor, Dr. Mihail Iordanov. There are no words to express my gratitude and respect. Thank you for inspiring me, sharing your deepest passion in science, and helping me to grow as a future researcher.

I would like to express my gratitude to my committee members; Dr. Matthew Wise and Dr. Bertram Coltman for providing me with advice and feedback. I would also like to thank Dr. Jeanette Eggert for supplying LoggerPro software.

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My sincere thanks goes to Summer Undergraduate Research Institute (SURI) and the Math & Science Department of Concordia University-Portland for funding the research.

I would also like to thank my colleague, Joshua Villaro for not just collegial contribution but also friendship.

Last but not the least, I would like to thank my parents, Eunsook Seung and Ranhyeok Hong, and my grandmother, Younghee Kim, for the opportunity to pursue my education and emotional support. I would also like to thank my friends, Raychelle Tiffany Valiente and Kassandra Lopez for keeping me sane.

Antimicrobial Activities of Tea-Derived Flavonoids

Introduction

Tea, one of the most-widely consumed beverages in the world, is an infusion of variously processed leaves of the tea plant, or Chinese camellia, *Camellia sinensis (L.) Kuntze*. Two major varieties of the plant are known: *Camellia sinensis* var. *sinensis* for Chinese teas, and *Camellia sinensis* var. *assamica* for Indian Assam teas ("ITIS Standard Report Page: Camellia sinensis", n.d.).

From fresh tea leaves, different types of tea are produced; Green tea, popular in East Asia, differs from the black tea, consumed in the West, through oxidation (also referred as "fermentation") which occurs during the processing of the leaves (Hamilton-Miller, 1995, p. 2375) [**Figure 1**].

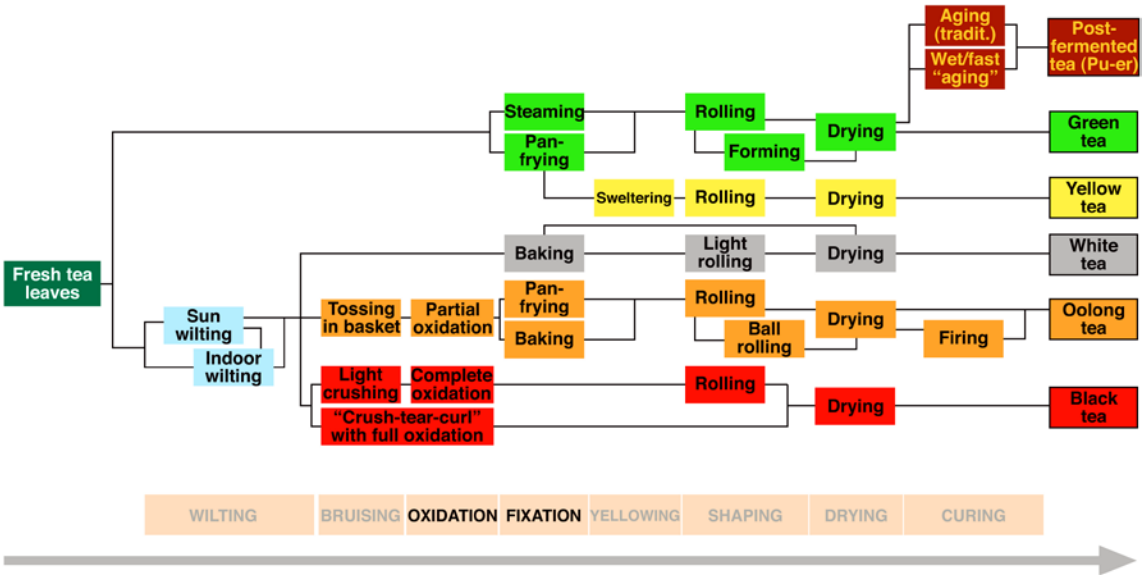

Figure 1. A schematic chart of different tea leaves processing steps: withering, rolling, bruising, oxidation, fixation, and drying.

Oxidation of the active ingredients of tea leaves is a process catalyzed by a cellular enzyme, polyphenol oxidase (Takeo, 1966, p. 529). White tea is the least

processed tea, plucked and quickly treated with high heat to inactivate the polyphenol oxidase, preventing the oxidation step altogether. White tea is much higher in polyphenolic antioxidants and exhibits higher anti-mutagen activity compared to other teas (Hilal & Engelhardt, 2007, p. 415). Green tea is a lightly-oxidized tea, briefly heated by either steaming or pan-frying, then dried without fermentation. Oolong tea and black teas are more processed than green tea, in that oolong tea undergoes partial oxidation and black tea undergoes complete oxidation before the drying step.

Pu-er and Chungtaejeon are post-fermented teas. Traditionally, Pu-er (or Pu-erh) is produced in the Yunnan province, China. Tea leaves are briefly heated by either pan-frying or steaming to prevent full oxidation, pressed into various shapes, then compressed tea cakes undergo a secondary oxidation, or also known as solid-state fermentation (Zhang, 2013). Chungtaejeon is a traditional Korean tea, originated from the Tang Dynasty China (618-907 A.D.). The processing steps for chungtaejeon ("Tea cake") was the official way to produce teas in Tang Dynasty; however when the Song Dynasty (960-1279 A.D.) succeeded the Tang Dynasty, they outlawed the production of tea cake in favor of "powdered tea" (as preserved today in Japan as "Matcha") (Mair & Hoh, 2009). The tea cake tradition was, however, kept alive by a single family in Boseong Country, Korea, and this preserved tradition is known today as chungtaejeon. The processing steps are following: picking, steaming, pounding, packing into round shapes, baking, and stringing cakes together for fermentation and subsequent drying. To brew chungtaejeon, the tea cake is roasted, ground into powder, and a tea infusion is made by boiling adequate amount of powder in a pot [**Figure 2**] (Mair & Hoh, 2009).

Figure 2. An illustration of chungtaejeon tea production steps.
(Retrieved from http://blog.naver.com/kyumjae62)

Chemical Composition of Tea

The chemical composition of tea is complex and not fully understood. The most abundant active ingredients in tea are polyphenols, specifically flavonoids, also known as catechins. Catechins are derivatives of flavan-3-ols; Flavan-3-ols are derived from flavans that use the 2-phenyl-3,4-dihydro-2H-chromen-3-ol [**Figure 3A**]. Catechin possesses two benzene rings, A- and B-rings, and a dihydropyran heterocycle (the C-ring) with a hydroxyl group on carbon 3. The molecule contains four diastereoisomers: two of the isomers are in *trans* configuration (catechin) and the other two are in *cis* configuration (epicatechin). (+)-catechin is the most abundant catechin in nature. The most common epicatechin isomer is (-)-epicatechin (Rinaldo, Batista, Rodrigues et al., 2010). The name of the catechin originated from *Catechu*, which is the tannic juice or boiled extract of *Mimosa catechu* (“Cutch and catechu plant origin”, 2011).

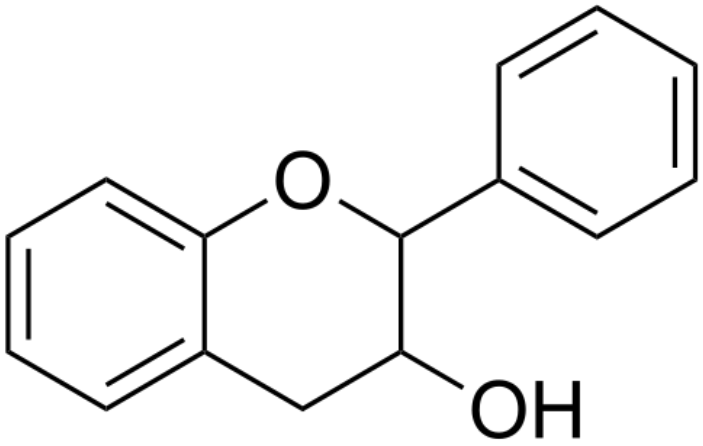

Figure 3A. Chemical structure of Flavan-3-ol (Formula: $C_{15}H_{14}O_2$).

Four major catechins that can be found in teas are (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECg), (-)-epigallocatechin gallate (EGCg). ECg, EGC, and EGCg are derived from EC. In ECg, EC forms an ester bond with gallic acid (GA); EGC has additional hydroxyl group on the aromatic B ring; lastly, EGCg has both additional GA group and hydroxyl group attached on EC [**Figure 3B**].

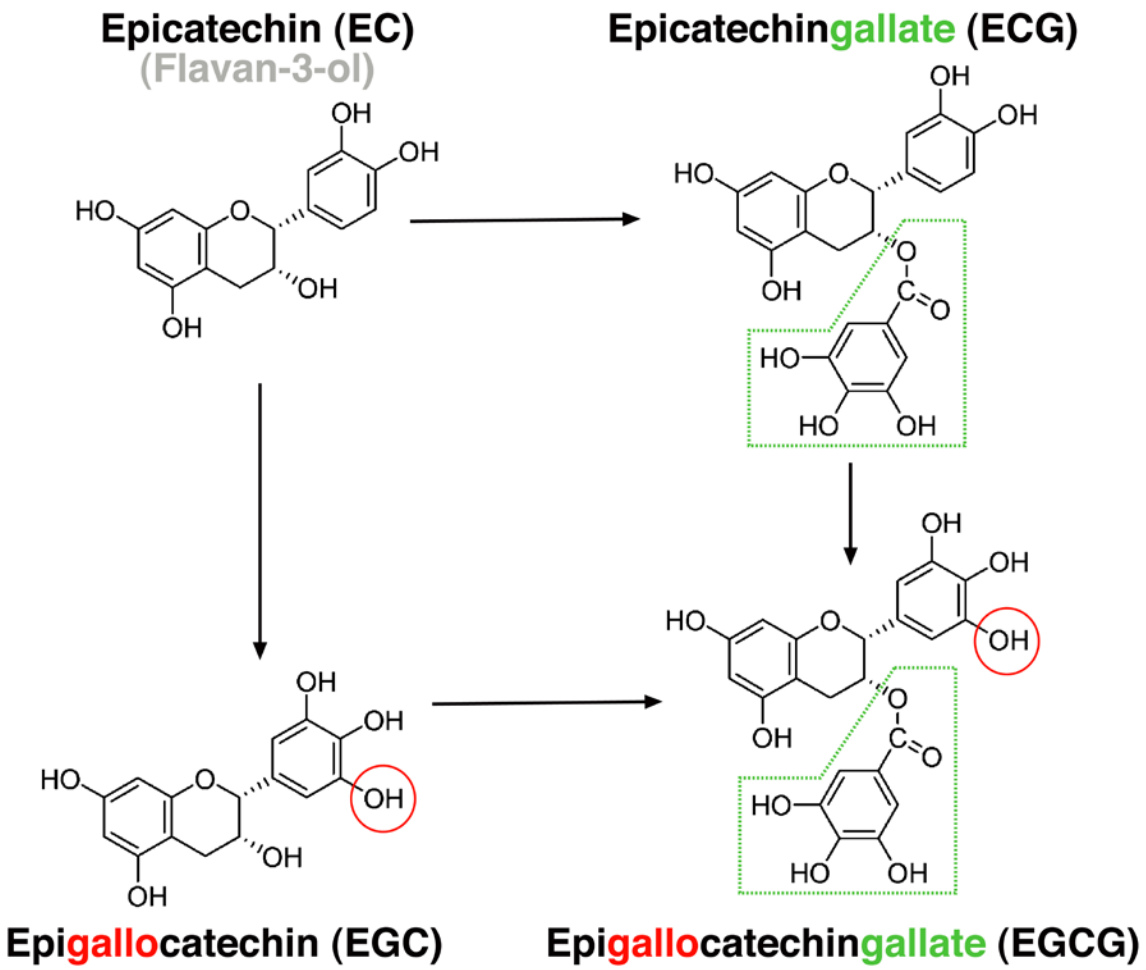

Figure 3B. Four major polyphenols (catechins) in tea.

In addition to polyphenols, or catechins, fresh tea leaves contain caffeine (3.5% of the total dry weight), theobromine (~0.2%), theophylline and other methylxanthines (~0.04%), lignin (6.5%), organic acids (1.5%), chlorophyll (0.5%) and free amino acids (~5.5%), and theanine (4%) (Graham 1992).

Health Benefits of Tea from Historical and Modern Perspectives

In addition to pleasurable aromatic scent and taste, tea possesses potential health-promoting qualities. As early as 3000 B.C., the Chinese used tea as a medicinal drink, as well as a daily beverage because of its benefits (Hilal & Engelhardt, 2007, p. 414). In 760

A.D., Chinese scholar Lu Yu first published his writing on history, cultivation, the processing of tea, and discussed the use of tea as medicine (Yu, 1974).

In modern times, the potential health-promoting properties of tea are continuously and scientifically investigated. The major directions of research on health benefits of tea are following: cancer prevention, dental health (e.g. prevention of cavities), cardio-vascular health, and antimicrobial properties (Taylor, Hamilton-Miller & Stapleton, 2005, p. 74-76).

Cancer Prevention. Several research groups that tea inhibits the cell duplication rates, which suggested that tea may suppress the growth of tumor cell *in vitro* and in animals (Weisburger, 1999, p. 273). Moreover, Chen et al. (2004) reported that EGCg has been shown to induce apoptotic cell death, and cell cycle arrest in tumor cells but not in their normal counterparts.

Cardio-Vascular Health Benefits. Studies have shown that tea also has cardio-vascular health benefits. Adequate cellular antioxidant levels are needed in order to prevent oxygen from generating reactive species such as the superoxide anion radial and other. Oxidation of LDL-Cholesterol is one of factors influencing the incidence coronary heart disease. Several studies have shown that regular tea drinkers have a lower risk of heart disease compared to non-drinkers because certain oxidative processes can be inhibited by antioxidants from tea (Chen et al, 2004, p.272).

Antimicrobial Activity. Tea has been investigated for its potential activity against microbial pathogens. In 1906, McNaught, a major in the British Army Medical Corps, observed that brewed black tea killed *Salmonella typhi* and *Brucella melitensis*. McNaught recommended that his troops drink black tea to prevent outbreaks of infections

due to these agents (Taylor et al., 2009, p. 3). Furthermore, several researchers have discovered that tea catechins can be used against superinfection, such as Methicillin-resistant *Staphylococcus aureus* (MRSA). Studies demonstrated that EGCg is able to synergize with β-lactam antibiotics against MRSA (Hu et al., 2002, p. 558).

This thesis is focused on the antimicrobial activities of various teas and purified tea constituents against the most abundant representatives of the skin microbiome, specifically *Staphylococcus aureus, Staphylococcus epidermidis*.

Hypotheses

A number of previous studies have been published over the years regarding the health benefits of tea. Focusing specifically on antimicrobial activities against skin-resident bacteria, the goal of this thesis is to determine whether tea has antimicrobial effects against primary isolates of skin *Staphylococci* and if so, which tea-derived flavonoids are the most active against skin *Staphylococci*. This thesis addresses the following hypotheses:

1. Teas that contain high polyphenolic contents have higher antimicrobial activity against skin microbiomes (e.g. *S. aureus* and *S. epidermidis*).
2. Different tea infusions and tea-derived individual catechins show differential inhibition of *S. aureus* and *S. epidermidis*.
3. Different tea infusions and tea-derived individual catechins show differential inhibition of *S. aureus* isolate from a healthy volunteer and an isolate from an individual with chronic lifelong atopic dermatitis.
4. EGCg, the most abundant polyphenols found in tea, possess the highest anti-staphylococcal activities.

Materials

Tea Samples

Fourteen different teas from five different geographic sources (e.g. China, Vietnam, Korea, Turkey, and Japan) were used in this research [**Figure 4**]. Tea leaves were processed by traditional methods, thereby creating different aromas, tastes, and amount of its polyphenolic content and composition.

Figure 4. An appearance of the tea samples used in the study. Two black tea samples were from Vietnam and Turkey, two oolong tea samples were from Vietnam and mainland China, five green tea samples originated from China, Korea and Japan, two post-fermented tea (Pu-er) samples were from China, one post-fermented green tea, Chungtaejeon, was from the Republic of Korea, and one sample of white tea was from China.

Catechins

Individual polyphenolic compounds, (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECg), (-)-epigallocatechin gallate (EGCg), and gallic acid (GA) were obtained from Sigma-Aldrich. As a solvent, 10 X Phosphate-Buffered Saline concentration (10X PBS, BioRad) was diluted to 1XPBS. All reagents were dissolved in sterile 1X PBS as 23mM stock solutions and stored at -20°C.

Methods

Preparation of Tea Infusion

To prepare the tea infusion, 0.5g of dry tea leaves were ground to coarse powder using a Power Chopper^{TM} Personal Food Processor. A 0.5 g of grounded tea leaf powder added to 20ml of 1X PBS in a 25ml Erlenmeyer flask, covered with aluminum foil, autoclaved at 110 °C (230 °F), 15psi for 10 minutes, and cooled down for 20 minutes, followed by slow cooling down to room temperature (~30 minutes). Once the tea samples were cooled, two pre-filters (100μm and 40μm) and 0.22μm sterile vacumn filter (Milipore) were used to collect the liquid. Those liquid samples were stored in a 4°C refrigerator until use.

pH Measurement of Tea Samples

To measure pH of tea samples, an electronic pH meter (Model 601A, Orion Research) was used. Before and after measuring different tea samples, standard pH solutions were used to calibrate the pH meter. PBS stock solution was also measured as a control.

Determination of Total Polyphenolic Content

This assay was as described by Wang, Chung, Lee, and Dykes (2013) to quantify the polyphenols specifically in tea. Total polyphenolic content of the tea samples was determined using the Folin-Ciocalteau colorimetric method. Folin-Ciocalteau reagent is a mixture of phosphomolybdate and phosphotungstate used for an *in vitro* colorimetric assay of monophenolic and polyphenolic antioxidants (Singleton, Orthofer & Lamuela-Raventos, 1999, p. 154). A 15µl of tea sample was added to 80µl of 7.5% (wt/vol) sodium carbonate and 75µl of 10% (vol/vol) Folin-Ciocalteau reagent in a well of microtitre plate. The plate was incubated in the dark for 30 minutes before measuring the absorbance of the reaction at 765 nm. A standard curve was plotted using known concentrations of gallic acid, and the total polyphenolic content was expressed in either as mg gallic acid equivalent (GAE) per g of dry leaves or mM gallic acid equivalent (GAE) per g of dry leaves.

Preparation of Bacterial Culture Media

Four different bacterial culture media were used in this study: Tryptic Soy Agar (Carolina Biological Supply Co.), Tryptic Soy Broth (Becton, Dickinson and Company), Mannitol Salt Agar (Neogen), and MacConkey Agar (Becton, Dickinson and Company). To make the media, an adequate amount of each dehydrated media (40g of Tryptic Soy Agar, 30g of Tryptic Soy Broth, 111g of Mannitol Salt Agar, and 50g of MacConkey Agar) was suspended in 1 liter of distilled water, the mixture was autoclaved at 121°C for 15 minutes, and the mixture was dispensed into sterile Petri dishes and/or culture tubes. Then, those agars and broths were incubated at 37°C for 24 hours to check for sterility. The prepared media were stored at 4°C until use.

Isolation of Skin *Staphylococci*

Two different microbes resident in human skin, *Staphylococcus aureus* and *Staphylococcus epidermidis*, were isolated from one healthy volunteer using a standard swap test method. Selective media and Gram staining, as described in "Microbiology: laboratory theory & application" (Leboffe & Pierce, 2012), as shown in **Figure 5**, demonstrated the identity of the isolates. Primary isolates were stored at 4°C until use.

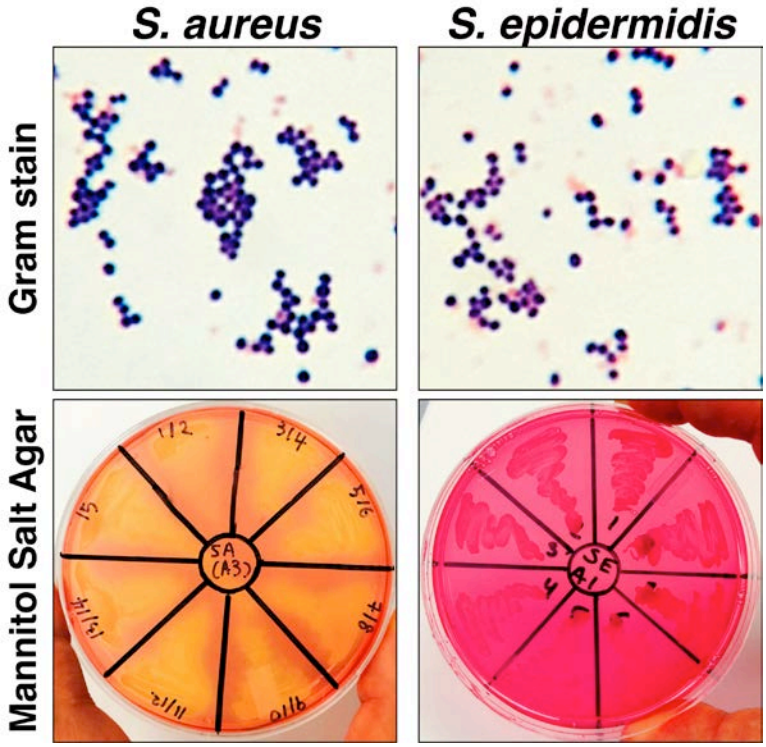

Figure 5. An illustration of *S. aureus* and *S. epidermidis* isolates. Positive Gram staining and morphology suggests that both of the isolates belong to genus *Staphylococcus*. Mannitol Salt Agar was used to differentiate *S. aureus* and *S. epidermidis*. *S. aureus* ferments mannitol manifested by acidification (yellow coloration) and *S. epidermidis* does not.

In addition to the sample from a healthy volunteer, a sample was isolated from one volunteer with a lifelong history of chronic atopic dermatitis.

Antimicrobial Susceptibility Tests

Modified Agar Diffusion Test. To determine the antibacterial activity of tea infusions, the standard agar diffusion test was modified. Bacterial lawns were plated on Tryptic Soy Agar plates. Round wells (The diameter of a well is 7mm) were created in the agar with a plastic straw with applied vacuum. 100 µl of PBS (control) or tea sample solution were added to the wells, and incubated up to 24 hours at 37°C [**Figure 6**].

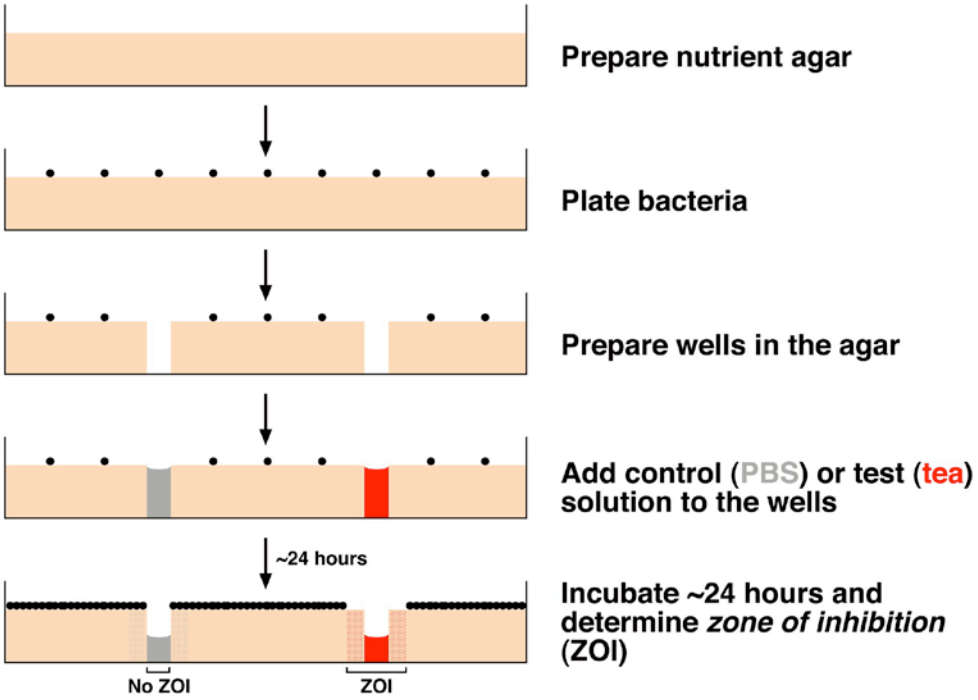

Figure 6. An illustration of modified Agar Diffusion Test for determining the antimicrobial activity of tea infusions. Instead of soaking a paper disk into tea infusions, tea infusions were added into a well and the zone of inhibition was analyzed.

Kirby-Bauer Test. We used standardized Kirby-Bauer, also known as the disk diffusion test, to determine if skin microbes were susceptible or resistant to a selection of catechins. Bacterial lawns (*S. aureus, S. epidermidis, B. cereus* were used in this experiment) were plated using standard microbiology technique on prepared Tryptic Soy Agar. After the plates have been inoculated, paper disks that have been infused with

different concentrations of catechins were placed on the surface of the agar. The plates were, then incubated at 37°C overnight.

Prior to the experiment with catechins, the method was tested employing commonly used antibiotics. Specifically, ampicillin, chloramphenicol, and tetracycline in five different concentrations were used: 0.625mM, 1.25mM, 2.5mM, 5mM, and 10mM. PBS was also included as a solvent control. To proceed with the experiment with catechins, four individual polyphenols, EC, EGC, ECg, and EGCg, and GA were used. The concentration of the catechins used in this experiment was 25mM. The experiment was done in quadruplet for statistical analysis.

Broth Dilution Susceptibility Test

A broth dilution susceptibility test was used to determine the possible effects of catechins against *Staphylococci*. Every culture tube contained 3 mL of TSB and an inoculation loop was used to transfer *S. aureus* or *S. epidermis*. In this experiment, various concentrations of EGCg were tested: 0μM, 15.625μM, 31.25μM, 62.5μM, 125μM, 250μM, 500μM, and 1000μM. The culture tubes were then incubated at 37°C for 24 hours.

Data Analysis

Logger Pro (Vernier Software & Technology) was used to measure the zones of inhibition. The zones of inhibition were averaged from repeated measurements, and standard deviations were calculated using DeltaGraph Software. An unpaired t-test (GraphPad Software) was used for statistical analyses.

Results

pH Measurement of Tea Samples

Prior to the investigation in antimicrobial activities of tea, the pH of each tea sample was assessed (see Methods). The mean of pH measurements was 6.20, the median was 6.32, and the mode was 6.32. PBS was used as a control. These findings indicated that all the tea infusions were slightly acidic with a tendency of the black teas being the most acidic.

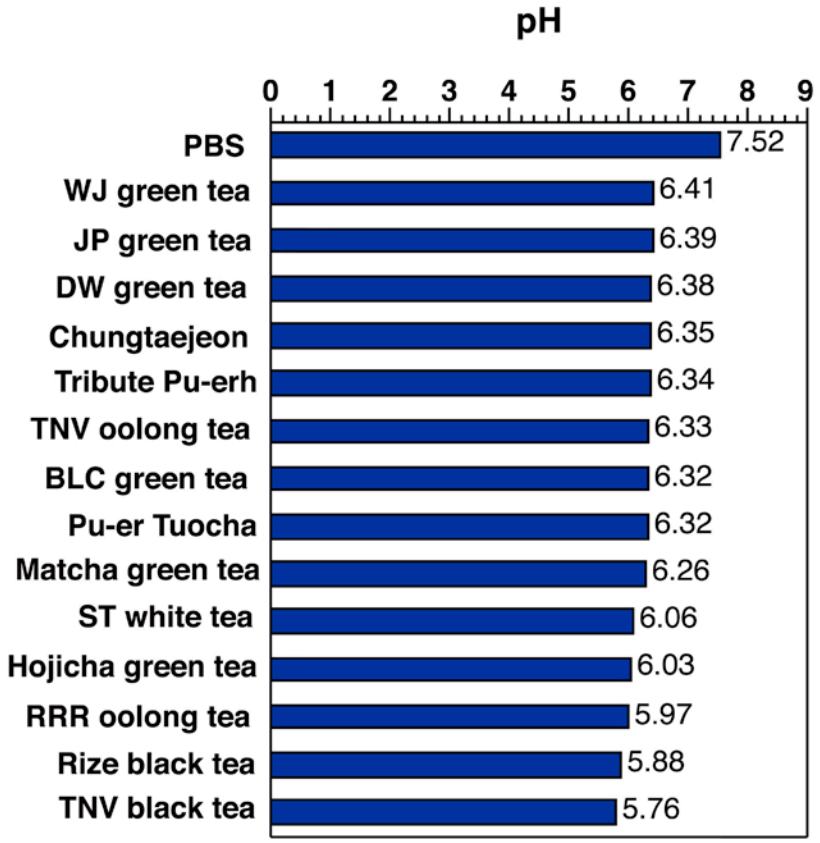

Figure 7. pH measurement of tea samples using electronic pH meter

Antimicrobial Activities of Tea Infusions

To assess the antimicrobial activities of tea infusions, an agar diffusion test was employed, modified for the purpose of this study (see Methods). Fourteen tea infusions

were tested against *S. aureus* and *S. epidermidis*, including *S. aureus* isolate from a volunteer with lifelong chronic atopic dermatitis. *S. epidermidis* was unable to be isolated from a volunteer with atopic dermatitis.

As shown in **Figure 8**, antimicrobial activities of tea infusions were manifested as zones of inhibition (ZOI) around a well. Zones of inhibition (in mm^2) were measured using LoggerPro; the total diameters of the zones were measured, areas were calculated, and the area of the well was subtracted (diameter of 7mm). The inhibition effect of white tea was greatest against both *Staphylococci*. While there was minimal inhibition effect of post-fermented teas and Rize black tea, **Figure 9** reveals that several green teas (e.g. Matcha, Woojeon, and Bi Luo Chun) displayed preferential activity against *S. aureus* and the Tribute Pu-erh tea displayed preferential activity against *S. epidermidis*.

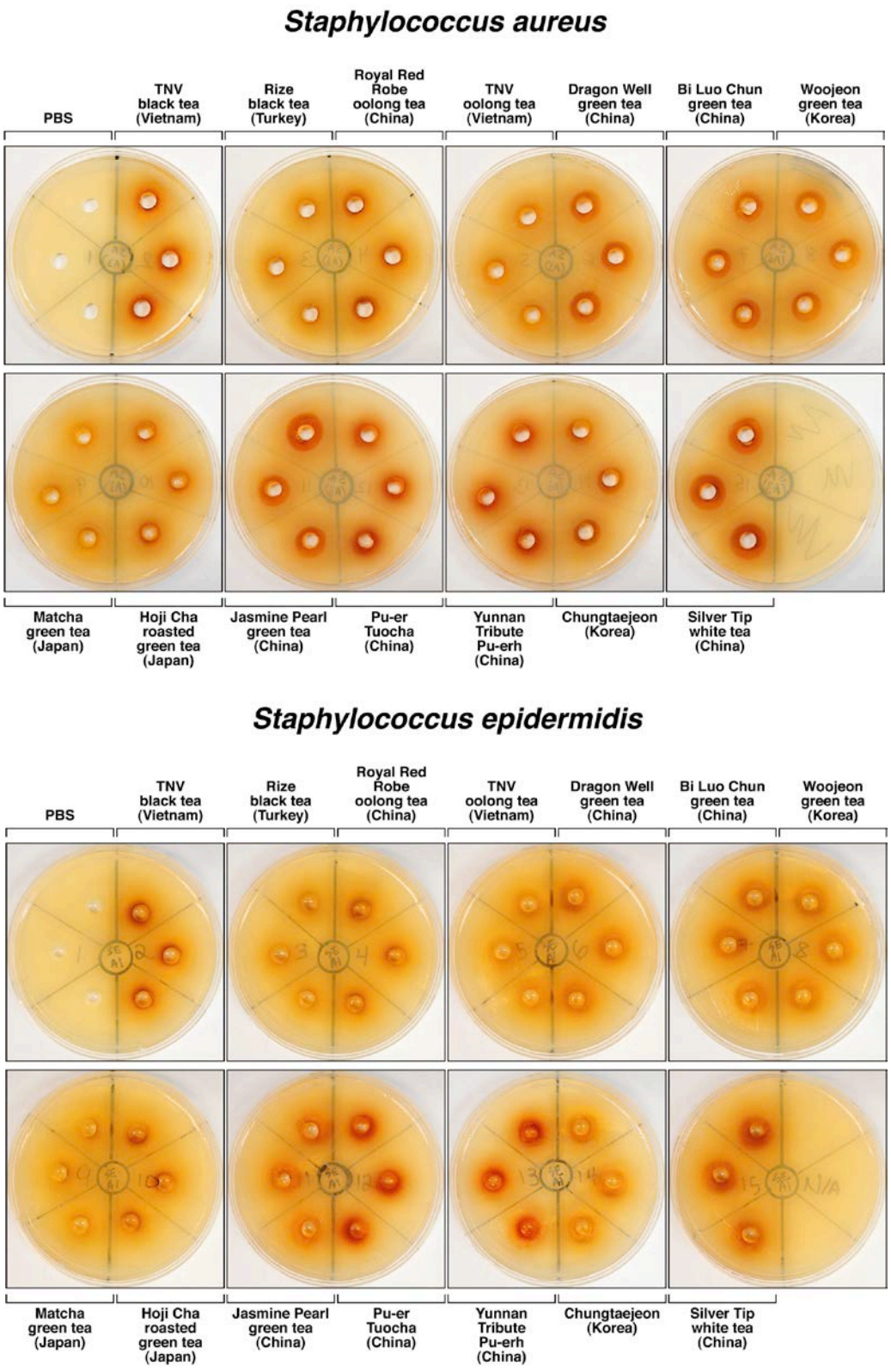

Figure 8. A modified Agar Diffusion test assessing the activities of the fourteen different tea infusions against *S. aureus* (top) and *S. epiderminis* (bottom).

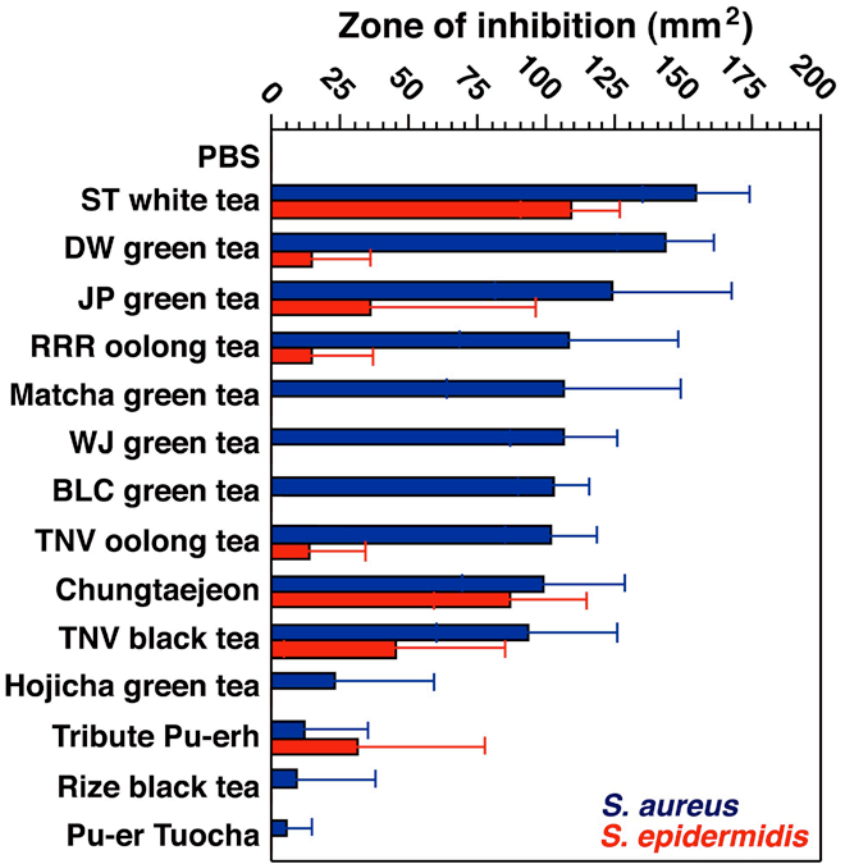

Figure 9. A comparison of the antimicrobial activities of the fourteen tea infusions against *S. aureus* and *S. epidermidis*. The graph represents the averaged zone of inhibition values (in mm^2) from three independently-tested primary isolates of each *staphylococcal* species, each tested in triplicates. Error bars denote standard deviation.

Correlation Between Total Phenolic Content and Antimicrobial Activity of Tea Infusions

Since the exact polyphenol composition of each tea composition is not known, the total polyphenolic content was converted to Gallic Acid Equivalence (see Methods) [**Figure 10**]. As shown in **Figure 11**, the total polyphenolic content of different teas varied from approximately 10% of total dry leaves weight (ST white tea) to less than 5% (Rize black tea). This result indicated that polyphenolic content of various teas does not differ by orders of magnitude.

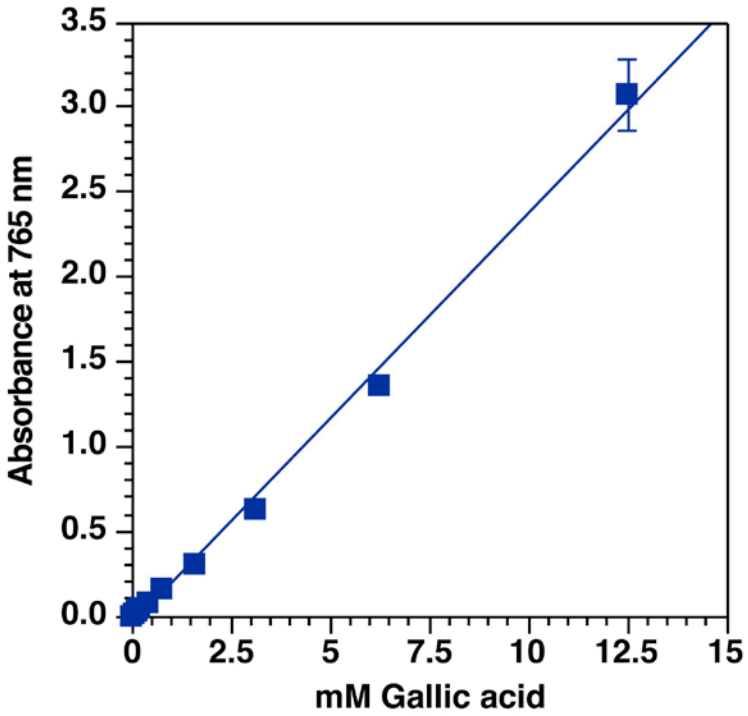

Figure 10. A standard curve using the known concentrations of gallic acid: 0mM, 2.5mM, 5mM, 7.5mM, 10mM, 12.5mM, and 15mM.

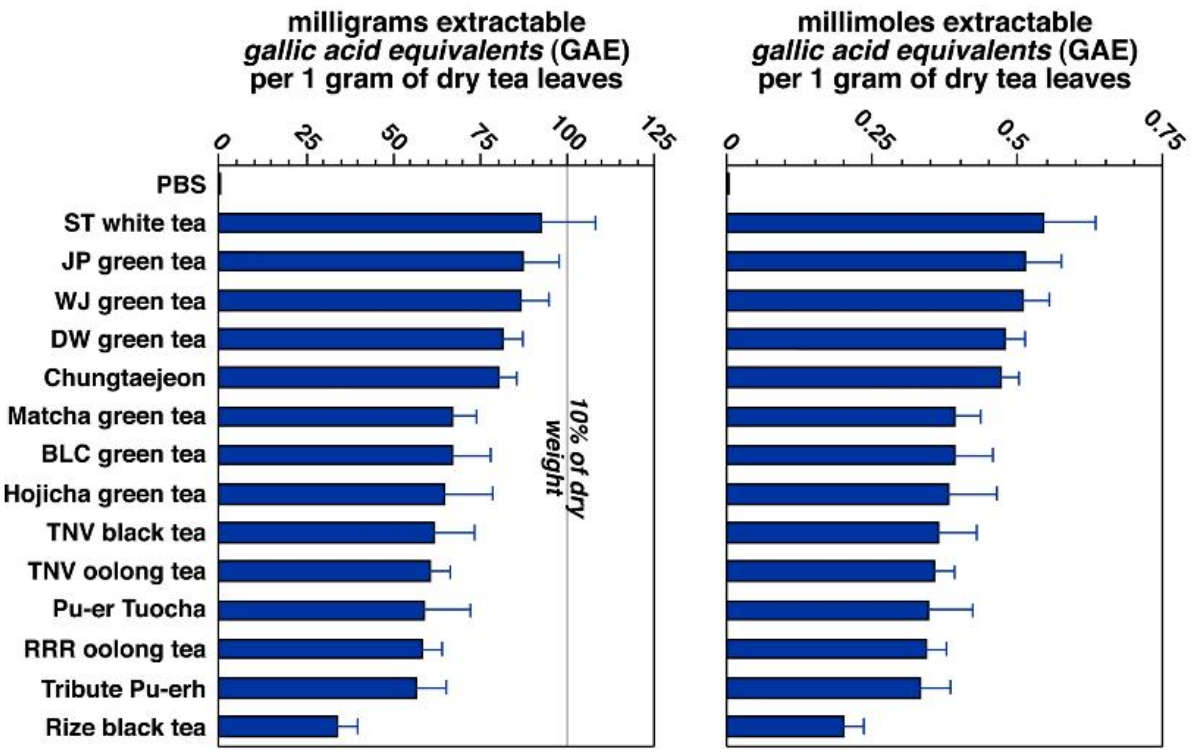

Figure 11. Total phenolic content of the fourteen tea infusions, determined by converting the Gallic Acid Equivalence.

As shown in **Figure 12**, data presented in the previous figures were integrated to assess the possible correlation between the total phenolic content and antimicrobial activities of the tea infusions against *S. aureus* and *S. epidermidis*. To do that, both the phenolic content and the antibacterial activity were converted to relative "*per cent* of maximum" values. The Silver Tip white tea manifested the highest phenolic content and antibacterial activity and was chosen as the "100%" standard for all other tea infusions. The plots shown in **Figure 12**, the corresponding fitted regression lines (one "forced" through the 0 point, the other not), and the R^2-values were obtained using the DeltaGraph5 software. Thus obtained, the plots suggested relatively strong correlation between a specific tea's phenolic content and its antibacterial activity against *S. aureus* ($R^2 = 0.570$). The correlation between the phenolic content and activity against *S.*

epidermidis was, in turn, weaker (R^2 = 0.222). This was largely due to a number of teas (WJ, BLC, MTC, and DW) seemingly displaying weak activity against *S. epidermidis* despite their high phenolic content. It must be emphasized that a careful reproduction of this experiment has not be done; therefore, the validity of the potentially important finding that some teas have preferential activity against *S. aureus* has not been confirmed to date.

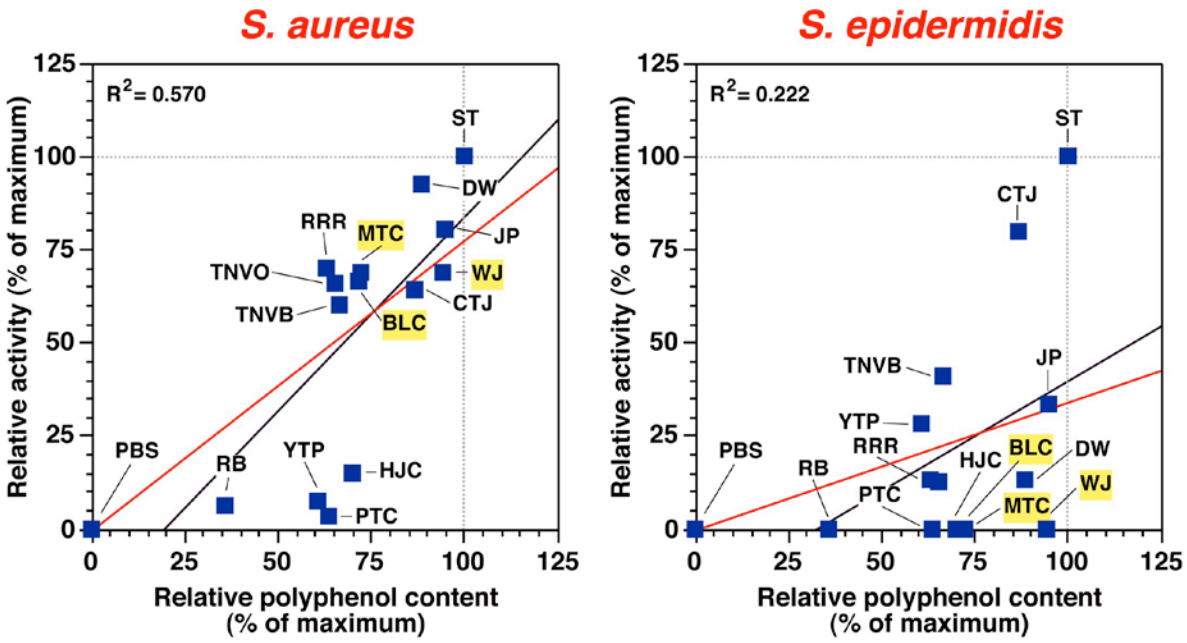

Figure 12. The differential activities of the fourteen tea samples on *S. aureus* verses *S. epidermidis* are evident in the different degrees of correction between the total phenolic content and activity.

Antimicrobial Activities of Individual Catechins

In order to determine the antimicrobial activities of individual catechins, the Kirby-Bauer susceptibility test was utilized. Prior to testing antimicrobial activities of individual catechins, five different concentrations of three commonly used antibiotics were employed: ampicillin, chloramphenicol, and tetracycline (see Methods) [**Figure 13**].

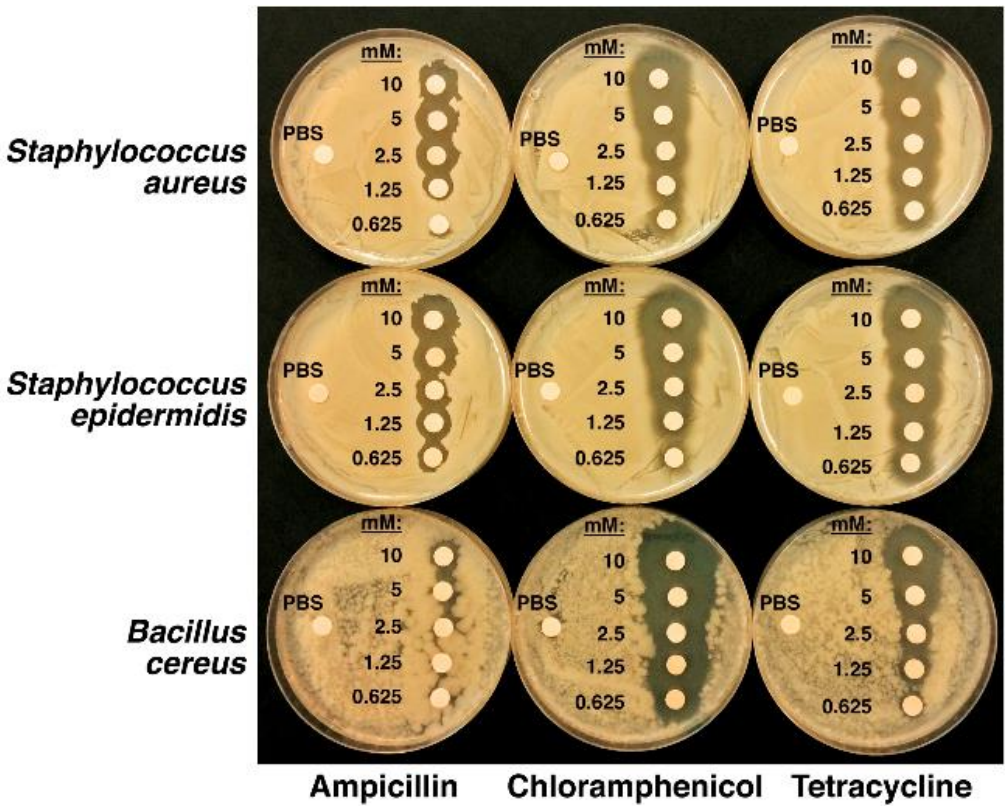

Figure 13. The Kirby-Bauer test using ampicillin, chloramphenicol, and tetracycline against *S. aureus*, *S. epidermidis*, and *B.cereus*. Five different concentrations of antibiotics were used: 0.625mM, 1.25mM, 2.5mM, 5mM, and 10mM, and 1X PBS as a negative control.

After successfully demonstrating the Kirby-Bauer test with known concentrations of antibiotics, individual catechins were tested against *Staphylococci* and *B.cereus* (see Methods). In this particular experiment, as illustrated in **Figure 14**, individual catechins were tested against two *S. aureus* isolates: one from a normal volunteer and the other from a volunteer with atopic dermatitis. This experiment was designed to demonstrate possible differential inhibition.

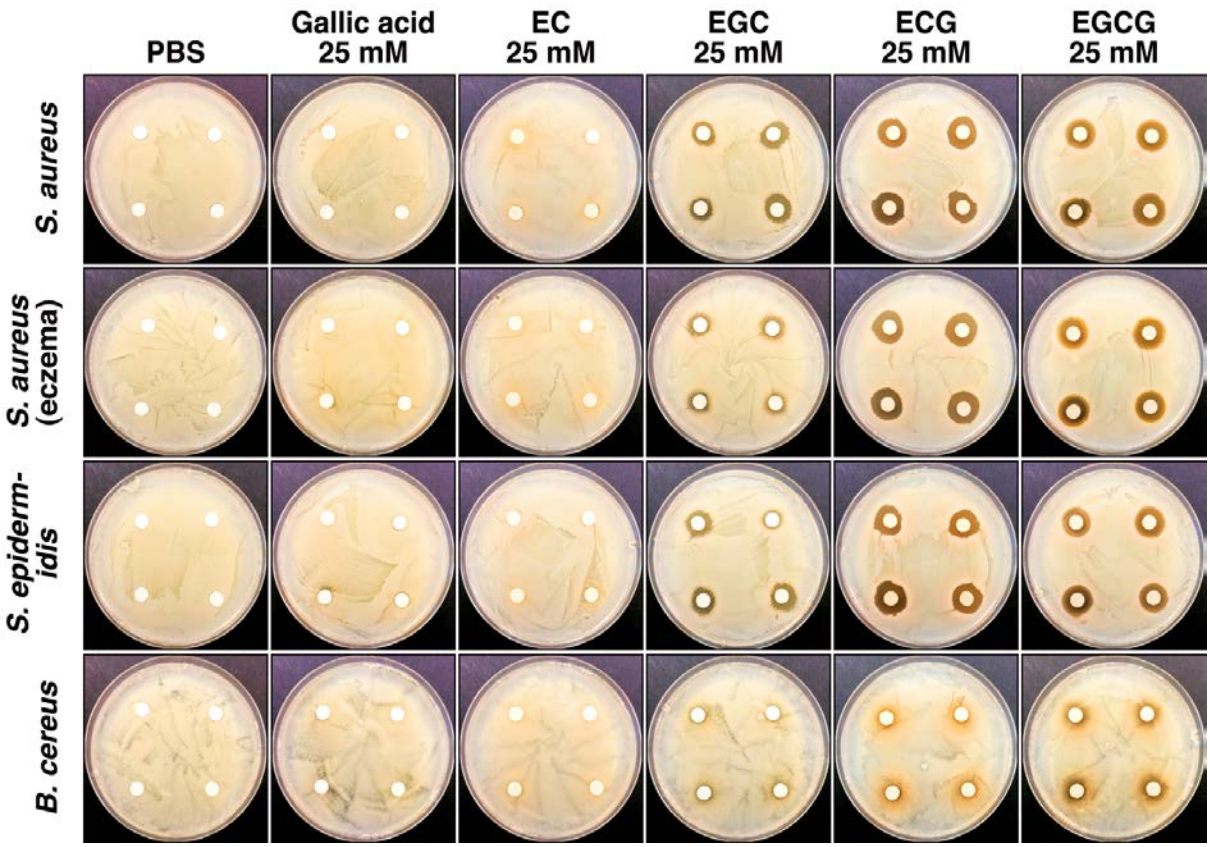

Figure 14. A Kirby-Bauer test assessing the activities of the four catechins and GA against *S. aureus* (isolated from a normal volunteer, and isolated from a volunteer with lifelong chronic atopic dermatitis), *S.epidermidis* (isolated from a normal volunteer; *S.epidermidis* was unable to isolate from a volunteer with lifelong chronic atopic dermatitis) , and *B. cereus* (from a commercial source). PBS was used as a solvent control.

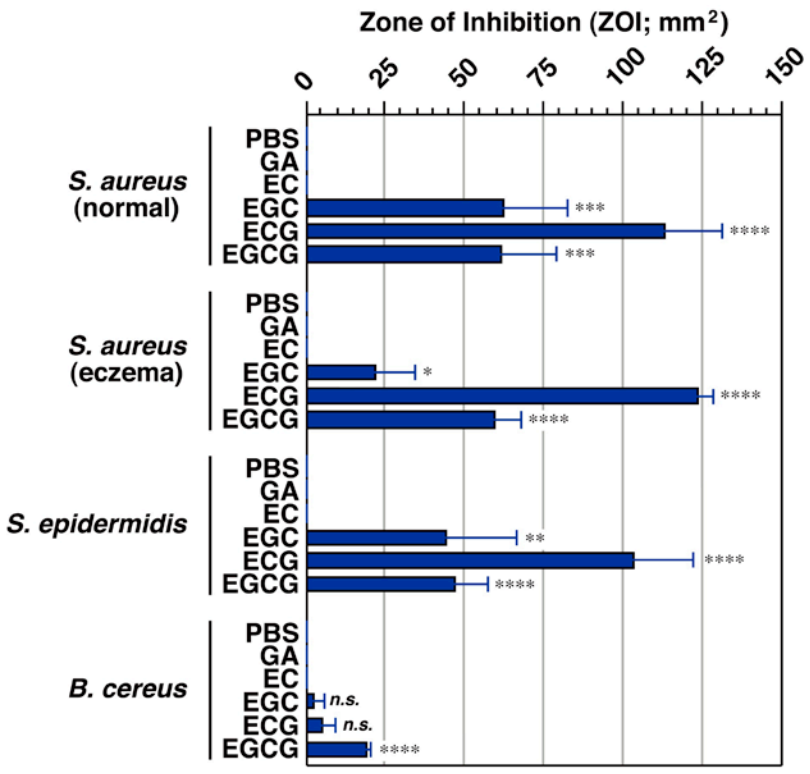

Figure 15. Quantification and statistical analysis of the experiment shown in **Figure 13**. Two-tailed P values are indicated as follows: (****) –P < 0.0001; (***) –P<0.001; (**) –P<0.01; (*n.s.*) –not statistically significant. Error bars –standard deviation.

As illustrated above [**Figure 14**], PBS, GA, and EC did not have any antimicrobial activity against *S. aureus*, *S. epidermidis*, and *B. cereus*. On the contrary, EGC, ECg, and EGCg had antimicrobial activities against *S. aureus* from a healthy volunteer, *S. aureus* from a volunteer with lifelong chronic atopic dermatitis, *S. epidermidis*, and *B. cereus*. Statistical analysis suggests that ECg had the highest antimicrobial activity against skin *Staphylococci*, while EGCg had the highest antimicrobial activity against *B. cereus* [**Figure 15**]. Furthermore, a possible differential inhibition between *S. aureus* from a healthy volunteer and *S. aureus* from a volunteer with atopic dermatitis was not observed.

Effects of Catechins on the Morphology of *Staphylococci*

Previous experiments have demonstrated that EGCg possesses high anti-*Staphylococcal* activities against both *S. aureus* and *S. epidermidis* [**Figure 15**]. To determine possible causes, a broth dilution susceptibility test was used (see Methods).

Figure 16. A broth dilution susceptibility test using serially diluted EGCg: 0μM, 15.625μM, 31.25μM, 62.5μM, 125μM, 250μM, 500μM, and 1000μM. A standard plate streaking method was used to determine whether the effect of catechins is bactericidal or bacteriostatic.

As illustrated in **Figure 16**, in higher concentrations of EGCg, some bacteria were suppressed or dead. To confirm whether EGCg is bactericidal or bacteriostatic, a plate streaking method was used. Utilizing a broth dilution susceptibility test, a tube with

1mM EGCg exhibited dark and clear appearance, suggesting suppression of bacterial growth. However, viable bacteria were still present after 24 hours incubation with 1 mM EGCg, as they were able to grow when streaked on catechin-free agar plates (in comparison to the PBS-incubated bacteria; **Figure 16**).

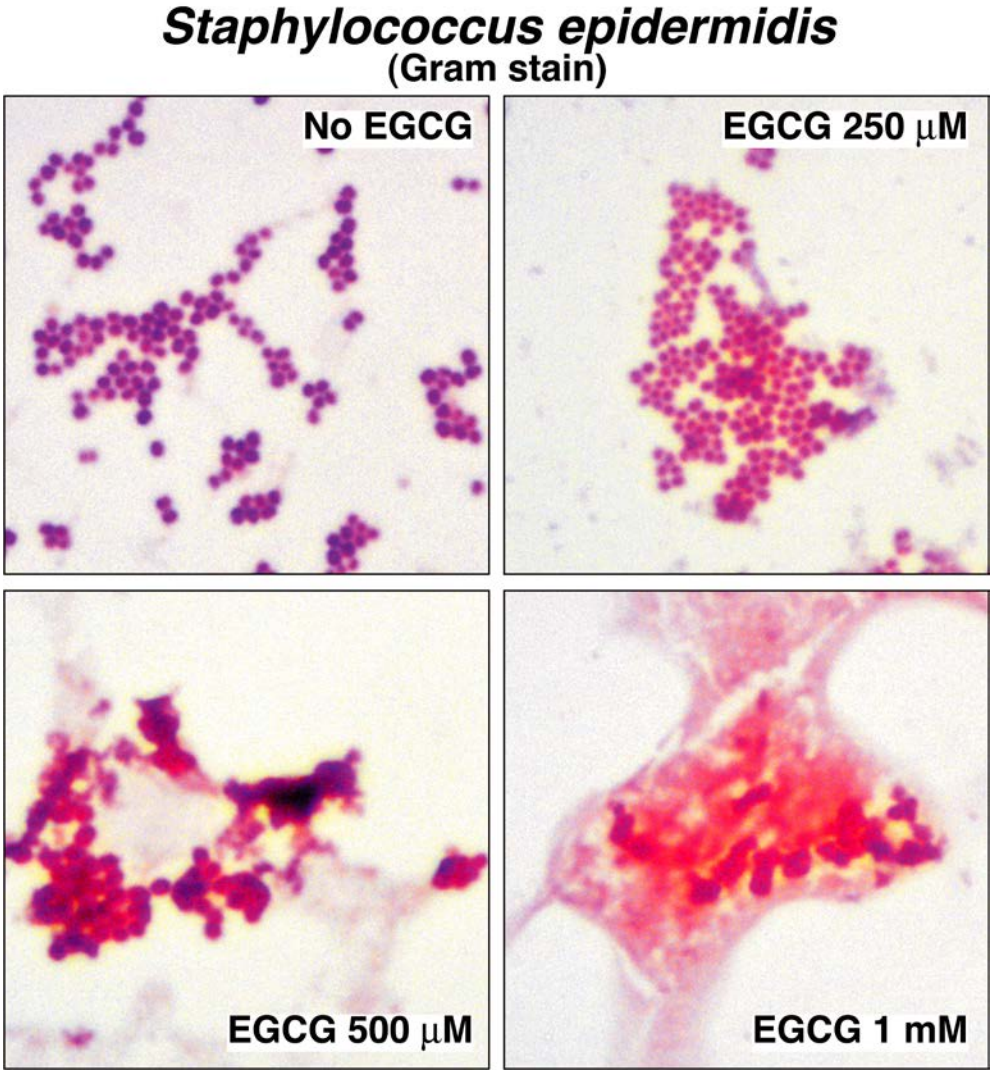

Figure 17. Morphology of *S. epidermidis* after exposure to different concentrations of EGCg (1000X Magnification).

When the bacterial morphology was assesses by Gram staining 24 hours after exposure to various concentrations of EGCg, a relative decrease in the intensity of the staining was observed (**Figure 17,** compare 0 to 0.25 mM EGCg). Furthermore, with the increase of EGCg concentration, clumps of apparently damaged or lysed cells were evident (**Figure 17,** compare 0 to 0.5 and 1 mM EGCg). Taken together, the results shown in **Figures 15** and **16** are consistent with a conclusion that tea catechins exert both bacteriostatic and bacteriocidal effects on skin staphylococci, with the results of the Gram staining suggesting that the integrity of the bacterial cell wall may be a possible target of the catechins.

Effect of Individual EC and GA Compared to EGCg

As shown in **Figure 13**, EC and GA alone did not possess antimicrobial activity against skin *staphylococci* nor *B. cereus*. However, when both EC and GA were presented in one molecule (EGCg), it possessed notable antimicrobial activity. To investigate a possible reason for this phenomenon, an experiment was conducted, using the Kirby-Bauer analysis. In this experiment, 25mM of each EC and GA were used and mixed together. Then, the equimolar mixture and the 25mM of EGCg were compared. PBS, EC, and GA were used as a negative control.

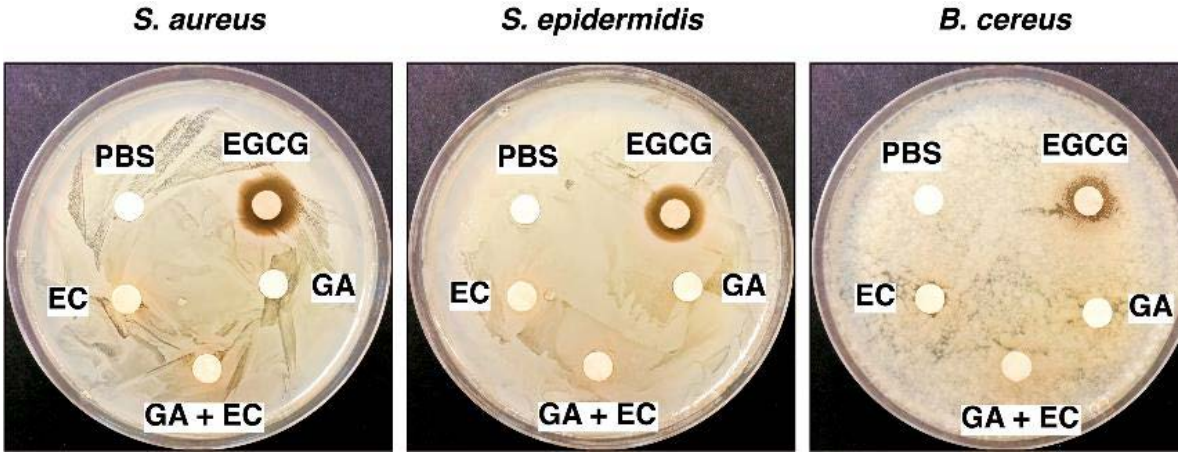

Figure 18. A Kirby-Bauer susceptibility test was performed with the indicated single reagents (25mM) or an equimolar mixture of GA and EC.

As shown on **Figure 18**, there was no observable zone of inhibition of the equimolar mixture. On the other hand, there was a distinct zone of inhibition of EGCg. This result suggested that a covalent bonding between EC and GA is required for the inhibition of bacterial growth.

Discussion

Preferential Activity of Several Teas Against Skin *Staphylococci*

This is one of the first studies that systematically compared fourteen different teas from various geographic locations and tested for antibacterial activities against skin *Staphylococci*. Previously, researchers have compared teas and investigated their antibacterial activities, but it was never done in one lab. In addition, our research was the first attempt to normalize the total polyphenolic content of fourteen different tea samples and correlate the data with antibacterial activities.

After investigating antimicrobial activities of tea infusions against *S. aureus* and *S. epidermidis*, we concluded that *S. aureus* yielded consistent results. However, the *S. epidermidis* result was inconclusive. We speculated that it might be due to the growth rate

inconsistency of *S. aureus* and *S. epidermidis*. As shown in **Figure 8**, wells made on the *S. epidermidis* contained liquid, whereas wells made on the *S. aureus* sample drew out liquid. Although the *S. epidermidis* result and correlation with polyphenolic content is inconclusive, the correlation between antibacterial activity and polyphenolic content of *S. aureus* is undeviating. For future references, leaving *S. epidermidis* plates in an incubator longer than *S. aureus* might provide better results.

Moreover, we observed that Matcha, Woojeon and Bi Luo Chun green teas displayed preferential activity against *S. aureus* [**Figure. 9]**. However, we were not able to confirm this finding in subsequent experiments, possibly due to the growth rate inconsistency between *S. aureus* and *S. epidermidis*.

In addition, two types of post-fermented teas, Chungtaejeon and Pu-erh, displayed different activity against *Staphylococci*. Chungtaejeon demonstrated antimicrobial activity against both *S. aureus* and *S. epidermidis*, while Tribute Pu-erh dispayed preferential activity against *S. epidermidis*. We hypothesized that this difference inactivity between Chungtaejeon and Tribute Pu-erh might be due to the difference in the processing of leaves. Chungtaejeon is produced today, followed by traditional method. The fermentation process takes place slowly after the tea cakes have been formed, under climatic condition. In contrast, modern Pu-erhs are processed by fast fermentation in bulk, prior to the formation of the tea cake.

ECg Possess Higher Anti-*Staphylococcal* Activity Than EGCg

Tea has been used for over 4000 years to promote health benefits. Researchers suggested consuming 5-7 cups of green tea per day could significantly improve one's general health (Weisburger, 1999, p. 272). As shown in **Figures 13** and **14**, catechins

display 10 times less effects than antibiotics. Antibiotics are taken for a short period of time for specific purposes. On the other hand, in some cultures tea is consumed for a long period of time, which suggests that tea is a bioaccumulation to pharmacologically relevant concentration.

EGCg is the most abundant polyphenols found in tea, and a number of studies demonstrated that EGCg is shown to have a direct effect in cancer prevention and antimicrobial activity (Chen et al., 2004; Hu et al., 2002). Prior to our experiment, we hypothesized that EGCg possesses the highest anti-staphylococcal activities. In contrast, shown in **Figure 15**, the result says otherwise. Our statistical analysis suggested that ECg had the highest antimicrobial activity against skin *Staphylococci*, while EGCg had the highest antimicrobial activity against *B. cereus*. This experiment should be repeated and further studies are required to confirm our result.

Lack of Differential Inhibition of *S. aureus* Isolate from a Normal Volunteer and an Isolate from a Volunteer with Atopic Dermatitis

Atopic dermatitis is a chronic and relapsing inflammatory skin disorder. Although it is genetically predisposed, it is also environmentally induced (Kim et al., 2012; Hanifin, Cooper & Roth, 1986). Previous studies reported *S. aureus* to be the predominant strain in atopic dermatitis, and increased *S. aureus* colonization has shown to correlate with increased disease severity for patients with atopic dermatitis (Masenga, Garbe, Wagner & Orfanos, 1990; Williams et al., 2017). As shown in **Figure 14**, we were not able to isolate *S. epidermidis* from a volunteer with atopic dermatitis; Aly and Maibach demonstrated that skin lesions in >90% of patients with atopic dermatitis were

colonized by *S. aureus*, which confirms why we weren't able to isolate *S. epidermidis* (as cited in Masenga et al., 1990, p. 579).

As demonstrated in **Figure 15**, we were not able to observe differential inhibition between *S. aureus* isolate from a normal volunteer and an isolate from a volunteer with atopic dermatitis. Although we were not able to find any differences with *S. aureus*, several researchers have corroborated potential positive effects of catechins against human microbiome. A group of researchers in Korea conducted a clinical study to assess the effectiveness of bath therapy with green tea extracts for the treatment of patients with atopic dermatitis associated with *Malassezia sympodialis*. *M. sympodialis* is an allegen, as it was cultured from 83% of adult patients. They observed that all subject serum eosinophil counts were decreased but the changes were not statistically significant, suggesting that tea extracts can be developed to treat patients with atopic dermatitis through further studies (Kim et al., 2012, p. 126-127). Moreover, Asahi et al (2014) demonstrated that EGCg demolishes established *Porphyromonas gingivalis* biofilms and inhibits biofilm formation. *P. gingivialis* is a Gram-negative anaerobic bacterium, an etiological agent of periodontal disease. The effect of EGCg may represent a novel antibiofilm agent that prevents infections involving bacterial biofilm (p. 1167).

Catechins May Disrupt Cell Wall of Gram-(+) Bacteria

A number of studies demonstrated antibacterial activities of catechins, and our experiments also clearly indicated anti-staphylococcal activities of catechins. Nevertheless, a consistent mechanism of catechins' actions against *Staphylococci* is yet to be demonstrated. As shown in **Figure 17**, we observed lightening around the bacteria, suggesting that EGCg might interfere with the peptidoglycan of Gram-positive cells, thus

causing the thinning of peptidoglycan layers. Lee, Shim, Chung, Lim & Kim's (2009) findings are possibly consistent with our hypothesis. They proposed that catechins irreversibly damage the bacterial cytoplasmic membrane, which can alter the adhesion of bacteria to the cells (as cited in Sharma et al., 2012, p. 672). Additionally, Mori et al. (1987) suggested that antimicrobial activity of tea catechins might be attributable to the inhibition of nucleic acid synthesis (as cited in Asahi et al., 2014, p. 1167). Moreover, several researchers investigated the binding mechanism of catechins using a molecular docking study. Fikrika, Ambarsari, and Sumaryada (2016) used a molecular docking simulation of catechins on Glucosamine-6-Phosphate Synthase, and they demonstrated that glucosamine-6-phosphate synthase inhibition by catechin suppressed the production of a bacterial cell wall and reduced the population of invading bacteria (p. 6). Gradisar, Pristovsek, Plaper, and Jerala (2007) also used molecular modeling to determine antimicrobial activity of catechins. They determined that the catechins inhibit bacterial DNA gyrase by binding to the ATP binding site of the gyrase B subunit. They observed that EGCg and ECg inhibited the formation of supercoiled DNA (p. 264- 267).

Conclusion

In this thesis, we demonstrated antimicrobial activities of tea-derived flavonoids against skin *Staphylococci*. We began our experiments with four hypotheses, and we rejected three out of four hypotheses. We failed to reject our hypotheses 1; teas with high polyphenolic contents possessed higher antimicrobial activity against skin microbiomes [**Figure 12**]. We were not able to confirm differential inhibition of *S. aureus* and *S. epidermidis*, due to inconclusive results with *S. epidermidis* [**Figure 9**]. We also were not able to determine differential inhibition of *S. aureus* isolate from a healthy volunteer and

an isolate from an individual with atopic dermatitis [**Figure 15**]. Lastly, we rejected our hypothesis that EGCg did not possess the highest anti-staphylococcal activity. As illustrated in **Figure 15**, ECg possessed significantly higher anti-staphylococcal activity than EGCg. All the experiments that were done in this study should be replicated and modified to confirm the result.

In addition to our four hypotheses, we observed the effect of catechins morphologically [**Figure 17**]. We hypothesized that the growth of bacteria were suppressed and died due to cell wall disruption. Several literatures supported our hypothesis, but further studies are required to confirm the effect of catechins in molecular level. The determination of the effect of catechins in a molecular level could be used to develop non-toxic and target-specific antimicrobial drugs in the future.

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