THE EFFECT OF EPIGALLOCATECHIN GALLATE (EGCG) ON *MYCOBACTERIUM TUBERCULOSIS* AND *MYCOBACTRIUM SMEGMATIS*

by

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B.S. in Microbiology, Wagner College, 2009

Submitted to the Graduate Faculty of

the Department of Infectious Diseases and Microbiology

the Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Master of Public Health

University of Pittsburgh

2011

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

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Objectives/Research Questions: (-)-Epigallocatechin-3-gallate (EGCG) is a major catechin (antioxidant) component of green tea. Recent studies have shown that it possesses many health benefits, including antimicrobial activity against some bacteria and viruses. Due to its known antimicrobial activity, this study focused on exploring the effects of EGCG on the growth of *Mycobacterium tuberculosis*, the causative agent of tuberculosis, as it remains a major public health concern. Through the use of a model mycobacterial species, *Mycobacterium smegmatis*, the experiments investigated the effects of varying concentrations of EGCG on mycobacterial growth.

Methods: The effects of EGCG on the growth of *M. tuberculosis* and *M. smegmatis* were explored by setting up serial dilutions with varying concentrations of EGCG and carrying out cell counts over certain time periods. High Pressure Liquid Chromatography (HPLC) was used to fractionate the EGCG in order to better understand its behavior in the culture media.

Results: The study has found that medium containing EGCG has anti-mycobacterial activity, but it is not EGCG itself that carries it out but a degradant molecule. This observation was achieved following experiments showing that a 3-day pre-incubation of EGCG in the medium increase the anti-mycobacterial activity. However, the HPLC analysis showed that EGCG is fully degraded by day 1. Therefore, by day 3, there are only degradant molecules in the medium. Findings were

consistent with an experiment which found that a pre-incubated green tea extract has more anti-mycobacterial activity against the bacterial cells than a fresh one.

Conclusions: A 3-day pre-incubation of green tea extract at 37°C enhances anti-mycobacterial activity, which has implications in considering green tea as a prophylactic agent.

Implications for public health: If EGCG has antimicrobial activity green tea could be used as a prophylactic agent against *Mycobacterium tuberculosis*.

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1.0   INTRODUCTION

Tea is the most widely consumed beverage worldwide, second only to water.[1] It is produced from the dried leaves of a plant known as *Camellia sinensis*. Although this plant originated in Southeast Asia and was identified more than 4000 years ago, it is now grown in over 30 countries throughout the world.[2] It has a per capita consumption of over 120 ml/day.[3] Although there are thousands of different flavors of tea, the three major types – green, black and oolong- are all produced from this one plant. The only difference between the three sorts is the way in which the plant leaves are processed. Green tea is produced from freshly harvested leaves which are rapidly steamed to inactivate the enzymes present, thereby preventing fermentation. Whereas black and oolong teas are produced by allowing the fresh leaves to wither first. They are then rolled and crushed, which initiates fermentation. Since green tea is made from unfermented leaves, it retains the highest amount of polyphenols which are attributed to its vast health benefits. [4]

Green tea has been used in traditional Chinese and Indian medicine since ancient times as a stimulant, diuretic, in healing wounds and improving heart health. [2] In recent years, there has been a lot of research done to understand the composition of green tea and how it is beneficial. [5] A large amount of the research has been focusing on a particular component of green tea called epigallocatechin gallate (EGCG), a type of catechin. EGCG is believed to be responsible for many of the health benefits of the tea. [6]

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1.1   EPIGALLOCATECHIN GALLATE

Green tea leaves contain various compounds such as polyphenols, polysaccharides, amino acids and vitamins. [7] Catechins are a major component found in the green tea extract, as they get oxidized during the processing in black and oolong tea. They are a type of polyphenol and have strong antioxidative properties, which are believed to be responsible for the health benefits of green tea. There are four main types of catechins: epigallocatechin gallate (EGCG), epicatechin gallate (ECG), epicatechin (EC) and epigallocatechin (EGC) (Fig. 1). [8] EGCG is the most abundant one and makes up approximately 59% of all the catechins in the green tea.
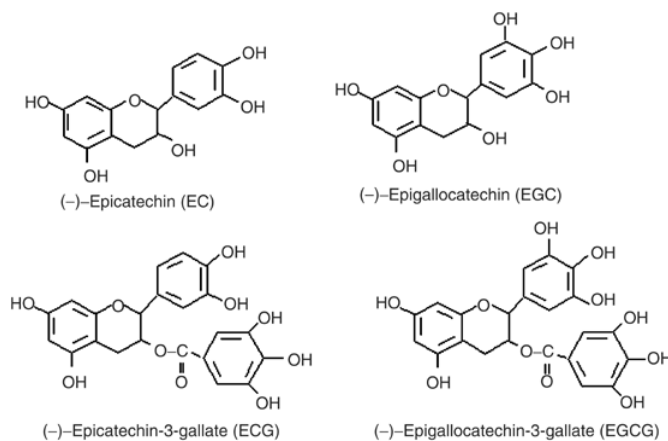
Figure 1. Structure of the four types of green tea catechins
R L H Bigelow and J A Cardelli *The green tea catechins, (-)-Epigallocatechin-3-gallate (EGCG) and (-)-Epicatechin-3-gallate (ECG), inhibit HGF/Met signaling in immortalized and tumorigenic breast epithelial cells.* Oncogene (2006) 25, 1922–1930.) with the permission from Nature Publishing group

The content of catechins in green tea depends on a few variables: how the leaves are processed before drying, the geographical location of the tea plant and its growing conditions (soil, climate, fertilizers), the type of green tea that is being made (instant, decaffeinated, etc.), and the preparation of the infusion. A cup of green tea, which comprises of 2.5 g of green tea leaves brewed in 200 mL of water, may contain approximately 90 mg of EGCG. [9]

EGCG (CAS: 989-51-5) is one of the most researched components of green tea. However, there are many inconsistencies and questions that still remain, concerning its activity and health benefits. The pharmacokinetics studies of EGCG have been limited in scope. The Laboratory for Cancer Research, at the Rutgers University in New Jersey, has performed numerous studies to identify the behavior of green tea catechins in human subjects. During their experiments, the patients were given a single oral dose of green tea solids, derived from freeze-drying the water extract of green tea leaves (6g of dry leaves yielded 1 g of powder), dissolved in 200ml of water in the morning. Blood and urine samples were collected at varying time points and analyzed using HPLC. The experiments showed that most of the ingested EGCG does not get into the blood and is actually excreted through the bile to the colon. EGCG appears to have a longer half life than the other catechins and is present mostly in the free form as opposed to a conjugate form. The study also found that there is an appearance of metabolites in the plasma within the first 12 hours after tea consumption. It was notes that the data varied throughout the experiments and among the different human subjects. [10]

In terms of health benefits, some emerging studies have suggested that EGCG may play a role in the prevention of cancer. One such study, performed at the Saitama Cancer Center Research Institute in Japan, found that EGCG does so by preventing the release of tumor necrosis factor-alpha, as it is believed to stimulate tumor promotion, via inhibiting its mRNA expression.[11] This study was performed using BALB/3T3 embryonic mouse cells. A different study found that when non-Hodgkin's lymphoma cells were transplanted into mice, green tea was responsible for inhibiting tumor growth in 50% of the mice used. The green tea utilized in this experiment was prepared differently than previously described, with a cup consisting of 12.5 g of tea leaves brewed in 500ml of water, with an EGCG plasma concentration measured at

708ug/ml. The EGCG plasma concentration was reported to be equivalent to that of humans after drinking two to three cups of tea daily. The experiments were performed using the Namalwa cell line, since it is one of the more aggressive lymphoid lines.[12] Other studies have shown that due to the presence of EGCG, green tea protects against a range of cancers such as lung, prostate and breast. [13]

Research has also shown that tea consumption has an inverse correlation with the incidence of cardiovascular diseases although the mechanisms of that are not yet clearly understood. [14] Furthermore, research has been done to explore the antimicrobial activity of green tea and EGCG. Studies have shown that green tea inhibits and kills a wide range of bacteria such as *Salmonella, Shigella* and *Staphylococcus* species. [15] Another study, performed at the Showa University School of Medicine in Tokyo, found that EGCG caused damage to the membrane of *Staph. aureus* by targeting the liposomes and has antimicrobial activity against Methicillin-resistant *Staph. aureus* (MRSA).[16] Other than containing activity against bacteria, green tea extracts have also been shown to have such effects against viruses such as the herpes simplex virus-1 and HIV. [17,18] Many research studies have looked at the effect of EGCG on HIV, including one performed at the Heinrich-Pette-Institute for Experimental Virology and Immunology in Germany. That study was able to demonstrate that EGCG inhibits the semen-derived enhancer of virus infection which is an important component of sexual transmission of the infection, making it a promising supplement in reducing sexual transmission of the HIV-1 virus.[18]

However, not much research has been performed to study the effects of EGCG on *Mycobacterium tuberculosis*, the causative agent of tuberculosis which is of high concern for global public health. One study has shown that the presence of this catechin resulted in the

inhibition of mycobacterium survival within macrophages. Specifically, EGCG does so by down regulating the expression of a coat protein called TACO, which prevents the maturation of phagosomes containing *M. tuberculosis* within the macrophages. [19]Another study discovered that green tea catechins are able to reduce the high oxidative stress following early stages of tuberculosis infection in mice. [20]

As mentioned before, each of these studies used varying approaches in their research and different concentrations of EGCG. However, the benefits of the green tea and its major catechin constituent are apparent.

1.2   TUBERCULOSIS

Each year, 9 million individuals around the world acquire tuberculosis (TB) and each year two million die.[21] It is estimated that, in this day and age, a third of the world's population have TB, which affects individuals of all ages and ethnicities.[22] According to the World Health Organization (WHO) the region with the highest incidence and prevalence of TB is South East Asia where it affects mostly young adults. However, the disease has also been a major problem in countries such as Russia and still affects individuals in the Unites States. A total of 11,545 tuberculosis cases were reported in the US in 2009, which represents approximately a 11.3% decrease since 2008. [23,24]

Tuberculosis is a highly contagious disease and has always been a very serious public health concern. However, it has gained even more attention partly due to its high rates of co-infection with human immunodeficiency virus (HIV), being the leading cause of death in HIV-positive individuals.[25] In addition, within the past decade, there has been an increasing

emergence of drug-resistant forms of the disease which have further complicated the fight against TB. In 2006, the estimated global incidence rate of the disease fell to 139 cases per 100,000 population due to the implementation of programs such as the Directly Observed Therapy (DOT), where in 2004 it was at 143. The rates have been falling slowly but the total number of deaths and cases is still on a rise due to an ever increasing population.[26]

1.2.1   What is tuberculosis?

Tuberculosis is a bacterial disease caused by the organism known as *Mycobacterium tuberculosis*. It is a highly contagious illness and, most often, affects the lungs of the infected individual. However, it can also attack others parts of the body such as the brain. When the organism enters the system, it either causes a dormant state of the disease, known as latent TB, or an active state where the bacteria multiply and cause damage to the host.[27]

There are many differences between an individual with latent TB and one with active disease. A person with the latent form of tuberculosis is asymptomatic and shows no apparent signs of sickness, which explains why some individuals have the disease and do not know about it.[27] They only way to tell that an infection has occurred is through a tuberculin skin test or blood test. An individual with active TB, on the other hand, usually feels sick. The symptoms may include a bad cough lasting for 3 weeks or longer, chest pain, coughing up blood or sputum, fatigue, weight loss, lack of appetite, fever and chills, and night sweats. An individual with latent TB would have a normal chest x-ray and negative sputum smears, whereas one with active TB may have an abnormal chest x-ray and a positive sputum culture.[27]

An individual with active TB of the lungs or throat can spread the disease through coughing, sneezing, talking or spiting. The very infectious pathogens spread through the air,

where they can stay for several hours and then infect another individual. The disease is not spread through other forms of contact such as handshakes, sharing food or drinks, through toothbrushes, or kissing.[27]

1.2.2 Diagnosis and treatment

The two tests used to diagnose tuberculosis are the tuberculin purified protein derivative (PPD) skin test and a blood test.[28] The PPD skin test is performed by injecting a small amount of tuberculin fluid under the skin on the arm and measuring the swelling 2-3 days after the injection. A positive result is indicative of the fact that the patient has been. The TB blood test is a recent technique which detects TB proteins in the individual. If either of the two tests is positive, further testing is required to identify if the patient actually has active TB and that it was not a false positive. Such tests may include chest x-rays, blood or urine tests and others.[27]

Individuals with latent TB may never develop the active disease. Individuals at high risk of the transition occurring include those with HIV infection, those recently infected (within the last 2 years), infants and young children, drug users, elderly, immunocompromised individuals and those who were not properly treated for the infection. Drug therapy is necessary to keep TB in the latent stage and involves a regiment of about 6 to 9 months.[28]

Tuberculosis is a treatable and curable disease. The most common medications used are isoniazid (INH), rifampin (RIF), ethambutol, and pyrazinamide. For the first few weeks of infection, the person is contagious and must be kept away from others. Once the drugs take effect then the patient can be cleared to interact with other individuals. The medication may have some side effects but it is important to take it regularly and as prescribed by the physician.[27]

The treatment regimen for tuberculosis lasts approximately 6 months as that is how long it takes to fully kill the bacteria. If the medication is not properly adhered to, there is a risk of the bacteria becoming resistant to the drugs which is a very big public health issue.[25] There are two types of drug resistant forms of TB: multi-drug resistant (MDR-TB) and extremely drug-resistant tuberculosis (XDR-TB). MDR-TB has been documented to be present in over 90 countries worldwide and involves the resistance to the first line drugs (INH and RIF). The treatment for it includes second-line drug therapy, including cycloserine and kanamycin, and to be taken over 2 years, which is more expensive and the effective drugs against the resistant strain have more side-effects. XDR-TB refers to resistance to the second line of drugs and has been found in all parts of the world.[21] The severity of some of the side-effects associated with these drugs has also lead to termination and decreased rates of adherence to the treatment. [29]

The World Health Organization (WHO) has created Directly Observed Therapy (DOT) as a way to reduce the rate of drug resistance. This approach involves healthcare workers reminding their patients to take the medication and monitor their progress. [30]

1.2.3   Prevention

Due to the difficulties and high costs of treatment, prevention becomes crucial in dealing with tuberculosis. Early diagnosis of the disease is critical as it is important to prevent the progression of the disease from a latent form to an active one. If an individual is exhibiting symptoms of infection, it is important to isolate himself away from healthy individuals. Education is a very important part of prevention as populations need to be educated on the risks of drug resistance, HIV co-infection and what the disease is.[27]

1.3   PUBLIC HEALTH SIGNIFICANCE

In eighteenth century Europe, tuberculosis was responsible for about 20% of all deaths even though the cause of death was not yet known at the time. In the mid-1990s, the first effective TB treatment regiments were developed and used in the developed world countries. That is when drug resistant forms of TB started to emerge. In addition, there was the emergence of the HIV pandemic in the late 1980s and the number of TB cases increased from 6.6 million in the 1990s to over 9 million in 2007.[31] Today, tuberculosis is included as one of the UN Millennium Goals, where the objective is to reverse the incidence of the disease by 2015.[26]

Tuberculosis has been a serious public health issue for hundreds of years and has become even more complex in the recent decades. Due to the costs, lack of availability and the length of the treatment, TB is a difficult disease to fight and have a disproportionate effect on the poor as compared to the general population. This is a result of overcrowding and substandard living or working conditions, poor nutrition, and co-infection with various diseases such as HIV. The high costs of treatment prevent the poor populations from gaining access to the necessary treatments. In more remote geographical areas, people live in areas with no roads and difficult terrains, and have to travel a long time to reach any healthcare facility, making the trip more expensive and more burdensome to make.[32] That is why affordable and easy to use prophylactic methods are so important.

If it indeed has anti-mycobacterial activity, green tea would be a very useful prophylactic agent. It is already widely used and therefore many people, even in developing regions, could have access to it. It would also be more cost effective. This study explored whether EGCG mechanistically affects mycobacterial growth.

2.0   THESIS AIMS

Although it has been around for many decades now and there have been many medical and scientific advances made, tuberculosis still remains a large public health issue. Since the emergence of the HIV virus and the difficulties in treating TB and HIV co-infection, as well as the emergence of antibiotic resistant strains, it has become increasingly more complex to battle the disease. Also, the treatment for tuberculosis is very long and involves taking a combination of different medications, making it difficult to adhere to. Therefore, it is important to focus on finding new ways of dealing with the disease, in terms of new treatment methods or prevention.

The goal of the research study was to explore if green tea, specifically one of its components known as EGCG, had any effect against the causative agent of tuberculosis, in order to determine if there was a possibility of it ever being developed into a prophylactic agent. Due to the wide array of literature describing the benefits of EGCG and the few studies that have been performed in studying its effect on tuberculosis, the study hypothesized that the compound will show microbicidal activity against *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*. The research study was divided into two specific aims:

- Aim 1: Exploring the effect of EGCG on the growth and survival of *Mycobacterium smegmatis*, a non-pathogenic species closely related to *M. tuberculosis.*

- Aim 2: Exploring the effect of EGCG on the growth and survival of *Mycobacterium tuberculosis*.

The effects of green tea on the bacteria were also explored in the study. The objective of these aims was to identify if EGCG and green tea had microbicidal activity against both strains through exposing the cultures to the catechin and performing cell counts. The majority of the experiments focused on *M. smegmatis* as it is non-pathogenic and a much faster growing bacterium than *M. tuberculosis*, making it a good model organism.

3.0   MATERIALS AND METHODS

3.1   ORGANISMS USED AND THEIR GROWTH CONDITIONS

3.1.1 *Mycobacterium smegmatis*

Mycobacterium smegmatis is a non-pathogenic acid-fast bacterium. It is a fast growing organism and is therefore an appropriate model species to study the basic biological characteristics of mycobacteria. In this study we used a high-frequency transformable strain, $mc^2 155$, obtained from Dr. Graham Hatfull's laboratory (University of Pittsburgh). The stock was grown in a 15ml plastic tube containing 7H9ADC medium with 0.05% Tween-80. Without the presence of the detergent, the bacteria have tendency to grow in large aggregates which are difficult to disperse and count. The cells were initially grown in a 37^0C incubator for a duration of 2-3 days until the culture was saturated. While used in experiments, the stock was held at room temperature in the laboratory.

Most of the experiments involving *M. smegmatis* were performed in a buffered medium, with a pH of 7.2. Since this medium was previously used to grow *M. smegmatis* biofilms, it will henceforth be called biofilm media in thesis. The medium was composed of 13.6g of KH_2PO_4 and 2g of ammonium sulfate dissolved in 900ml of Millipore water, with the pH adjusted to 7.2 using KOH pellets, and the addition of 0.5mg of $FeSO_4$ and 5g of casamino acid. Following

liquid cycle autoclaving for 20 minutes, the medium was reconstituted as follows (per 100ml): 94ml of medium, 5ml of 40% glucose, 1ml 0.1M $CaCl_2$ (anhydrous form) and 100ul of 1M $MgSO_4$ (anhydrous form). Some experiments were performed with the base of the biofilm medium, which lacked casamino acid and glucose.

Colony cell counts were performed using 7H10ADC plates, which were made using Difco Middlebrook 7H10 Agar . Each plate contained 25ml of the medium

3.1.2 *Mycobacterium tuberculosis*

Mycobacterium tuberculosis is a pathogenic bacterium which is the causative agent of tuberculosis. *M. tuberculosis* is a very slow growing organism and can take around 3-4 weeks to grow. Since it is a air-borne pathogen, working with it requires Biosafety level (BSL) 3 laboratory capabilities. However, the strain used in the study was mc²7000, which is a genetically attenuated strain and is approved for use in a Biosafety Level 2 laboratory.[33] The stock was grown in a 50ml polystyrene bottle containing 7H9OADC media with the addition of 0.05% tween-80 and pantothenic acid (100mg/ml), which is required for the growth of the mc²7000 strain. The cells were grown in a 37°C incubator throughout the experiments.

All of the experiments were carried out using Sauton's medium, which consisted of 0.5g of KH_2PO_4, 0.5g of $MgSO_4$, 4g of L-asparagine, 2g of citric acid, 0.05g of iron ammonium citrate, and 60ml of 40% glycerol dissolved in 900ml of Millipore water. The pH was adjusted to 7.0 using NaOH and the media was autoclaved for 25 minutes in a liquid cycle. Before use, it was reconstituted with the addition of 2ul of $ZnSO_4$, 20ul of pantothenic acid and 50ul of tween-80 per 20ml of Sauton's medium. Cell counts were performed using 7H11OADC plates, which were made using Difco MB7H11 agar (Ref# 283819).

3.2   EGCG USED

The 50 mg stock of EGCG used in the study was obtained from Sigma-Aldrich (Lot number 079K1007). A 10uM stock solution was made by dissolving 45.8 mg of EGCG in 10ml of Millipore water and filtering it into a 15ml plastic tube using a 10mL syringe and a Arodisc syringe filter with a 0.4um HT Tuffryn Membrane. The filtrate was aliquoted into 10 x 1ml plastic tubes and stored at -20°C freezer until use.

3.3   SPECIFC AIM#1: EXPLORING THE EFFECT OF EGCG ON THE GROWTH OF *M. SMEGMATIS*

3.3.1   Effect of EGCG on growth of *M. smegmatis*

The experiment was performed by setting up three different samples of biofilm media in 15ml plastic test tubes, without the addition of 0.05% tween-80. Each tube was inoculated with 10ul of *M. smegmatis* (1:1000 dilution). The first tube, the control, did not receive any EGCG. Two hundred microliters of EGCG were added to the second tube, making it a 200uM concentration of the catechin. The third tube was labeled 1mM EGCG and 1ml of the catechin was added to it. The tubes were shaken and poured into sterile petri plates. The petri plates were incubated at 37^0C for 3 days and then observations of biofilm formation were made.

3.3.2 Effect of 200uM and 1mM EGCG on the growth of *M. smegmatis*

The experiment was performed by setting up a no EGCG control sample, a 200uM EGCG sample and a 1mM one in 15ml plastic test tubes. Each test tube consisted of 10ml of biofilm media, 25ul 0.05% Tween-80 and 10ul of bacterial cells (1:1000 dilution). The control tube did not contain any EGCG, while the 200uM sample contained 200ul of the catechin and the 1mM sample had 1ml of the catechin. Serial dilutions of the culture and plating for colony cell counts were performed at 0, 3, 6, 9, 12 and 24 hours. In between the time periods the samples were kept in a 37^0C shaker. Serial dilutions were performed in the following manner:
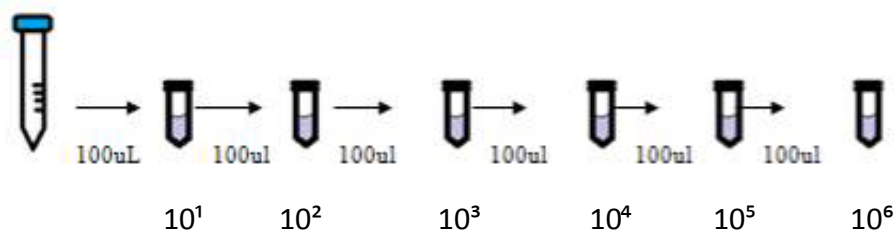
Figure 2. Serial dilutions

Six dilution tubes were set up with 900ul of reconstituted biofilm media with 0.05% Tween-80. A 100ul aliquot of the "No EGCG" sample was pipetted into the 10^1 dilution tube which was then vortexed. The pipette tip was discarded and a fresh one was used to transfer 100ul from 10^1 tube into the 10^2 tube, from 10^2 to 10^3 and so on. The pipette tip was not replaced for the remainder of the transfers and after each transfer the tubes were vortexed. Once the last dilution was made, a 7H10ADC agar plate was labeled as follows:
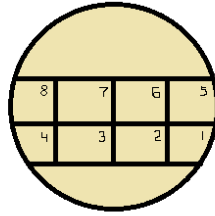
Figure 3. Dilution plate

10ul were transferred from the 10^6 dilution tube to the square labeled 8 on the plate, making it a 10^8 dilution. Using a fresh tip, 10ul were transferred from the 10^5 dilution tube to the square labeled 7. This continued on with the rest of the tubes. 10ul were taken from the original sample an transferred into the square labeled 2.

The procedure was repeated for all samples three times each, at each time point. Once the plates were dried they were stored at 37^0C for 3 days. After 3 days, the plates were taken out and the number of colonies was counted for all of the samples.

3.3.3 The effect of EGCG pre-incubation in the media on its activity

The experiment was performed by setting up two sets of samples, each set consisting of a no EGCG control and a 500uM EGCG sample. The first two tubes were set up as usual: 10ml reconstituted biofilm media with 0.05% Tween-80 and 500ul of EGCG in one of the tubes. These two test tubes were incubated for 48 hours at 37^0C without the addition of any bacterial cells. At 48 hours another identical set was set up with a no EGCG control and 500uM EGCG. At this point, 10ul of bacterial cells were added to all four test tubes. Serial dilutions were set up as described in the previous experiment with 3 replicates for each sample. The dilutions were

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performed at 0, 8, and 24 hours after the addition of bacterial cells. Plates were stored at 37^0C for 3 days and the colonies were then counted.

3.3.4   Examining 0, 1, 2, 3, and 4-day EGCG pre-incubation

The experiment was set up to observe the effects of 0, 1, 2, 3, and 4 days of EGCG pre-incubation.. The experiment was performed with 500uM EGCG. A stock sample was set up with 15ml of biofilm media with the addition of tween and 750ul of EGCG, resulting in a final concentration of 500uM EGCG. Each day, 2ml of the sample were taken out and put into a separate test tube and 2ul of bacterial cells were added to the tube. Serial dilutions were performed as soon as the bacterial cells were added (0 hours) and 24 hours later. Plates were stored at 37^0C for three days and the colonies were counted.

3.3.5   High Pressure Liquid Chromatography (HPLC)

High Performance Liquid Chromatography (HPLC) was performed through collaboration with Dr. Lisa Rohan's laboratory at Magee Women's Research Institute. The HPLC was performed by Phillip Graebing, using a reverse phase Luna C18 column from Phenomenex (Torrance, CA) which is 4.6mm diameter x 250mm length, 5μ particle size, and 100Å pore size, with a gradient elution. The mobile phase used was 17% acetonitrile in 0.05% trifluoroacetic acid. The flow rate of the machine is 1ml/min with a detection of UV 280 nm.

Base biofilm medium was used for HPLC in order to reduce interference of the medium in the chromatogram.

3.3.5.1 Activity of EGCG in base biofilm media

The experiment was set up by first creating a stock solution of 500uM EGCG base media. The stock solution consisted of 15ml of reconstituted base biofilm media with the addition of 750ul of EGCG, resulting in a 500uM EGCG concentration. Each day, for 5 consecutive days, 2ml of the stock solution were transferred into a separate test tube and 2ul of *M. smegmatis* cells were added (1:1000 dilution). Serial dilutions were performed at 0 hours and at 24 hours. Samples were created for 0, 1, 2, 3, and 4 days of pre-incubation. Plates were kept at 37^0C as usual and colonies were counted after 3 days of incubation.

3.3.5.2 HPLC of 1mM EGCG at 0, 1, 2, 3 and 4-day pre-incubation

The experiment was performed by setting up 1mM EGCG samples for 5 consecutive days and running them through the HPLC machine. An EGCG standard and a blank base biofilm media control were also run through the machine. The resulting chromatographs were studied to understand the activity profile of EGCG.

3.3.6   Effect of green tea extract in water on the growth of *M. smegmatis*

The experiment was set up using Twining's Gunpowder Green Tea leaves, purchased at the local supermarket. A cup of green tea was brewed up in sterile Millipore water. Forty milliliters of sterile Millipore water were heated until boiling. They were allowed to cool for a few minutes and then 0.5g of green tea leaves were added. The tea was allowed to brew for 5 minutes as noted in the instructions on the tea container. After 5 minutes, the tea was poured through a strainer and then filtered through a 0.4um HT Tuffryn membrane using a 30ml syringe. The sample was allowed to cool. Two sets of samples were then set up: one set with no pre-

incubation of the green tea and another with a 3-day pre-incubation before any bacteria would be added. Each set consisted of a water control, a 1:4 diluted sample of the green tea in water, and a regular sample of green tea (as it was prepared). The 1:4 diluted sample consisted of 1ml of the green tea extract mixed in 3 ml of sterile Millipore water, giving it a net total of 4ml of sample. Four microliters of bacterial cells were added into the first set of samples (no pre-incubation) consisting of 4ml of medium. Serial dilutions were performed for each sample at 0 and 24 hours 3 times. The procedure was repeated for the second set of samples after a 3-day pre-incubation of the green tea extract at 37^0C. Plates were stored for 3 days and colonies were counted for each.

3.3.6.1 Repeat of the green tea experiment

The previous experiment was repeated one more time. The experiment was set up as mentioned before. A 250uM EGCG sample was also set up in water. Each sample was observed with no pre-incubation and after a 3-day pre-incubation. Cell counts were carried out at 0 hours and at 24 hours after the addition of *M. smegmatis* cells (1:1000 dilution).

3.4 SPECFIC AIM#2 EXPLORING THE EFFECT OF EGCGC ON THE GROWTH OF *M. TUBERCULOSIS*

3.4.1 The effect of 1uM EGCG on the growth of *M. tuberculosis*

The experiment was set up by using a 1mM EGCG concentration in reconstituted Sauton's media. Ten milliliters of the media were used, with the addition of 0.05% Tween-80 and 10ul of *M. tuberculosis* cells. A no EGCG control was set up in the same way. The samples were kept at

37^0C for 48 hours. Serial dilutions were then performed using 7H11OADC agar plates, which were then incubated for 3.5 weeks, once again at 37^0C. Colony counts were then carried out.

4.0   RESULTS

4.1   SPECIFIC AIM#1: *M. SMEGMATIS*

4.1.1   Effect of EGCG on biofilm formation in *M. smegmatis*

The initial experiment was performed as a preliminary means to observe if EGCG had any effect

on the growth of *M. smegmatis.* The study was carried out by inoculating *M. smegmatis* on three

different plates each with 10mL of detergent-free biofilm media and containing a no EGCG

control, 200uM EGCG or 1mM EGCG. Under these conditions in normal biofilm media, *M.*

smegmatis forms a robust pellicle-like biofilm on the air-media interface. [27]After four days of

incubation at 30°C the plates were observed for differences in biofilm formation. The data are

presented in Figure 4.

A B C
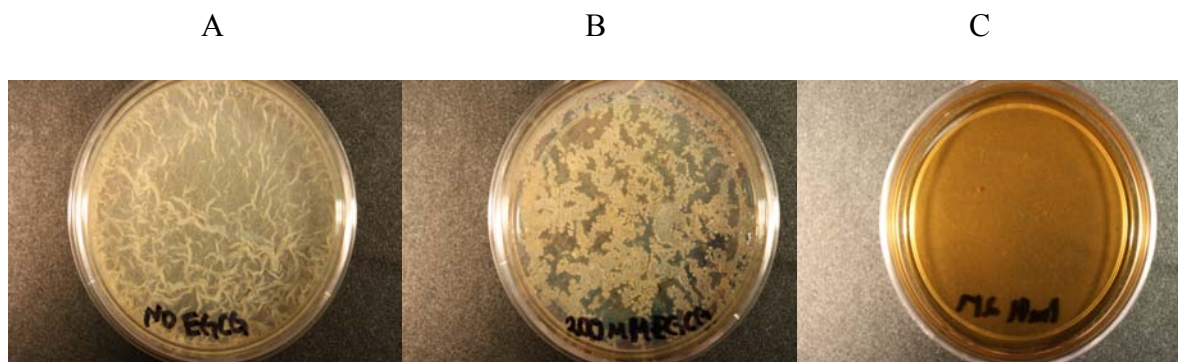
Figure 4. Effect of 200uM and 1mM EGCG on biofilm formation of *M. smegmatis*.
(A) represents the No EGCG control, where 10ul of bacterial cells were inoculated into 10ml of Biofilm media and naturally formed a biofilm. (B) depicts a slight disruption in the formation of biofilm in the presence of 200uM EGCG. (C) shows the complete absence of biofilm formation in the presence of 1mM EGCG.

In the absence of EGCG, *M. smegmatis* is able to grow normally and form robust biofilms at the air-media interface (Fig. 4A). While 200uM EGCG had moderate but visible effects on the growth of bacilli (Fig. 4B), increasing the concentration to 1mM completely inhibited the growth of the cell (Fig. 4C). The results showed that EGCG does in fact disrupt the normal behavior of the bacterium, but it was unclear if it holds any antimicrobial activity. The experiment was repeated 2 times.

4.1.2   Time-dependent effect of 200uM and 1mM EGCG on the growth of *M. smegmatis*

Following the preliminary observation of inhibitory effects of EGCG the next goal was to investigate how varying concentrations of EGCG affect the growth of *M. smegmatis,* specifically looking for antimicrobial activity. *M. smegmatis* bacilli were inoculated in no EGCG control, 200uM EGCG sample and a 1mM EGCG sample, and their viabilities were monitored at 0, 3, 6, 9, 12, and 24 hours by plating serial dilutions. Colony forming units (cfu) were counted

following 3 days of incubation of the plates at 37°C. Average cell counts of three replicates were

plotted in a line graph. Figure 5 represents the observations of the experiment.
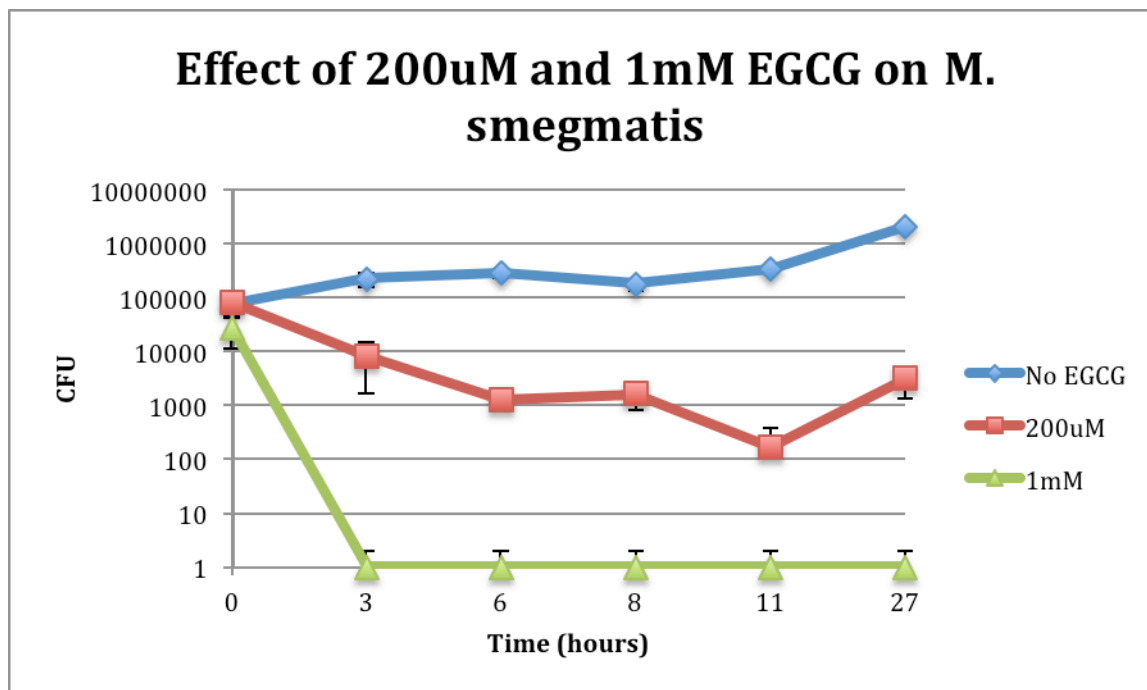
Figure 5. Effect of 200uM and 1mM EGCG on *M. smegmatis*.
       The experiment was performed by setting up a no EGCG control, 200uM EGCG sample and a 1mM EGCG sample, each inoculated with a 1:1000 concentration of bacterial cells. The data represent the averages of 3 replicates. The blue line depicts the growth of the bacterial cells in the no EGCG control. The red line represents 200uM EGCG sample and the green line the 1mM EGCG sample.

As seen in the figure, in the no EGCG control, which is represented by the blue line, there is an

increase in the cell counts from 0 hours through 24 hours. In the 200uM EGCG sample, which is

represented by the red line, there is approximately a two-fold decrease in cfu from 0 hours to 12

hours and increasing again, suggesting possible instability of EGCG in the media. In the 1mM

EGCG sample, which is depicted by the green line in the figure, there is a sharp decrease in the

number of colonies from 0 hours and complete killing of cells by 3 hours. The cell counts do not

increase over the period of observation.

4.1.3 The effect of EGCG pre-incubation n the media on its activity

It was observed in the previous experiment that growth of the culture starts to increase after 12 hours of exposure to 200uM of EGCG. It was hypothesized that EGCG either gets consumed by the bacteria or gets degraded in the media and therefore loses its activity. The experiment was then performed to look at the activity of 500uM EGCG after a 48 hour pre-incubation in the biofilm medium. The 500uM of EGCG was appropriate because 200uM had moderate anti-microbial activity and 1mM would kill most of the cells. A no EGCG control and a 500uM EGCG sample were set up with no pre-incubation and 48 hour pre-incubation. The average cfu of 3 replicate samples were calculated and plotted in a line graph, as shown in Figure 6.
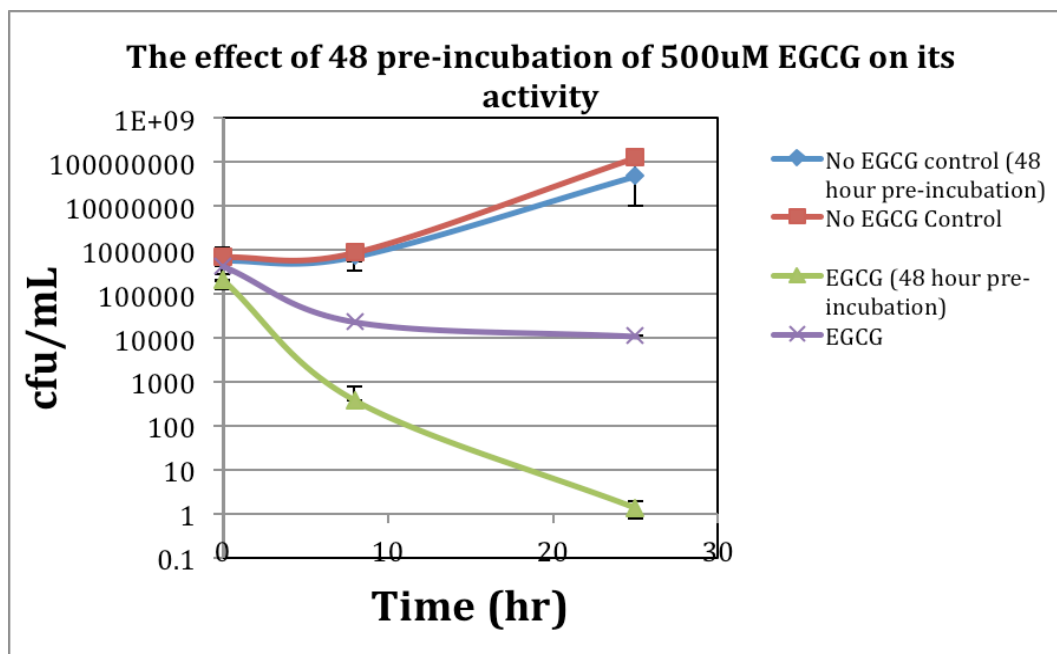
Figure 6. The effect of 48 hour pre-incubation of EGCG in the media on its activity.
The experiment was set up to meausre the activity of 500uM EGCG after a 48 hour pre-incubation. A no EGCG control and a 500uM EGCG sample were set up with no pre-incubation and 48 hour pre-incubation, followed by the addition of 1:1000 dilution of bacterial cells. The red line and the blue line represent the two no EGCG controls, with no pre-incubation and 48 hours pre-incubation, respectively. The purple and green lines depict the 500uM EGCG samples with no pre-incubation and 48 hours pre-incubation, respectively.

Although there was visible decrease in the number of colonies with no pre-incubation, surprisingly there was a much sharper decrease in colony counts with the pre-incubation. This indicated that pre-incubation of EGCG in the medium strengthens its anti-microbial activity.

4.1.4   Measuring 0, 1, 2, 3 and 4-day pre-incubation

Since it was observed in the previous experiment that 48 hour pre-incubation of EGCG in the media increased its activity, an experiment was set up to identify the optimal pre-incubation time to maximize the anti-mycobacterial activity of EGCG. The experiment included at 0, 1, 2, 3, 4, and 5 day pre-incubation of 500uM EGCG. Cell counts were performed at 0 and 24 hours and percent survival rates were calculated. The average rates of 3 replicates were then determined and plotted on a bar graph as depicted in Figure 7.
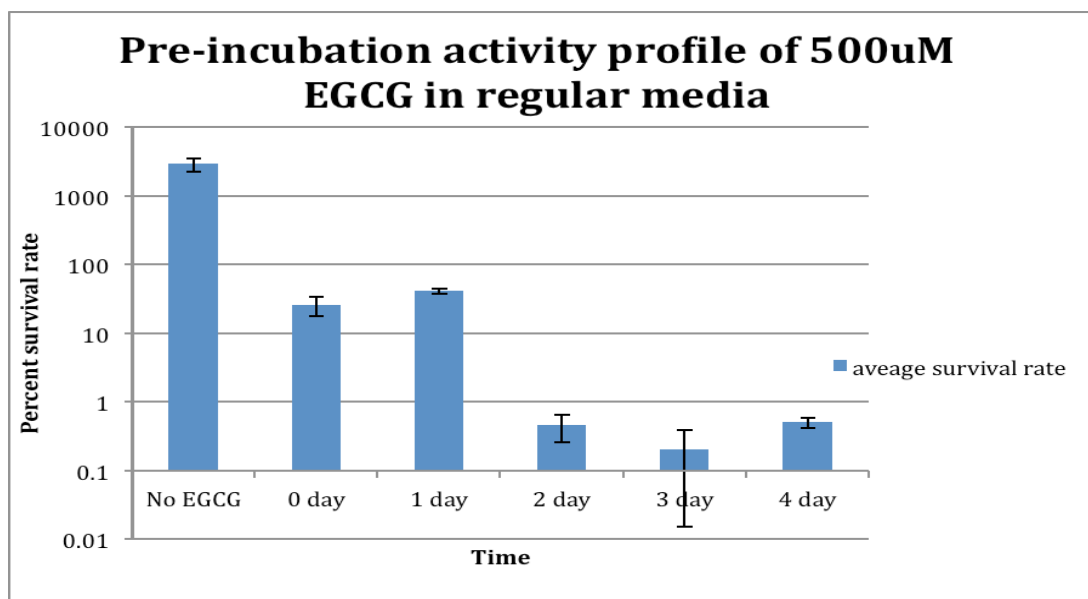
Figure 7. Pre-incubation activity profile of 500uM EGCG in biofilm media.
The experiment was performed to better understand the activity profile of EGCG in pre-incubated biofilm media.

According to the results, 1-5 day pre-incubation showed some anti-mycobacterial activity, as the survival rates are lower than in the 0 day pre-incubation. However, 3-day pre-incubation had the lowest survival rate, indicating that it was the optimal pre-incubation, yielding the strongest antimicrobial activity of EGCG.

4.1.5   High Pressure Liquid Chromatography

Increased activity of EGCG in the medium after a 3-day pre-incubation suggested that a secondary degradant of EGCG has the anti-mycobacterial activity, and therefore the complete degradation profile of EGCG was studied via HPLC. Base biofilm media was to be used in the HPLC experiment in order to reduce the interference of the complex profile of casamino acids and glucose in the chromatographs. However, before HPLC analysis it was necessary to make sure that EGCG has the same activity profile in the base media as it did in the regular biofilm media (Fig 5, 6 and 7).

4.1.5.1 Observing whether EGCG has the same activity profile in base biofilm media

The experiment was performed in order to determine whether EGCG has optimal microbicidal activity after a 3-day pre-incubation in base media as it did in regular biofilm media. Samples were set up with 500uM EGCG and 1:1000 dilution of *M. smegmatis* cells. Serial dilutions were performed at 0 and 24 hours. Growth inhibition was determined to calculate the percent survival rates after a 24 hours exposure to EGCG. Average survival rates of 3 sample replicates were plotted on a bar graph as shown in Figure 8.
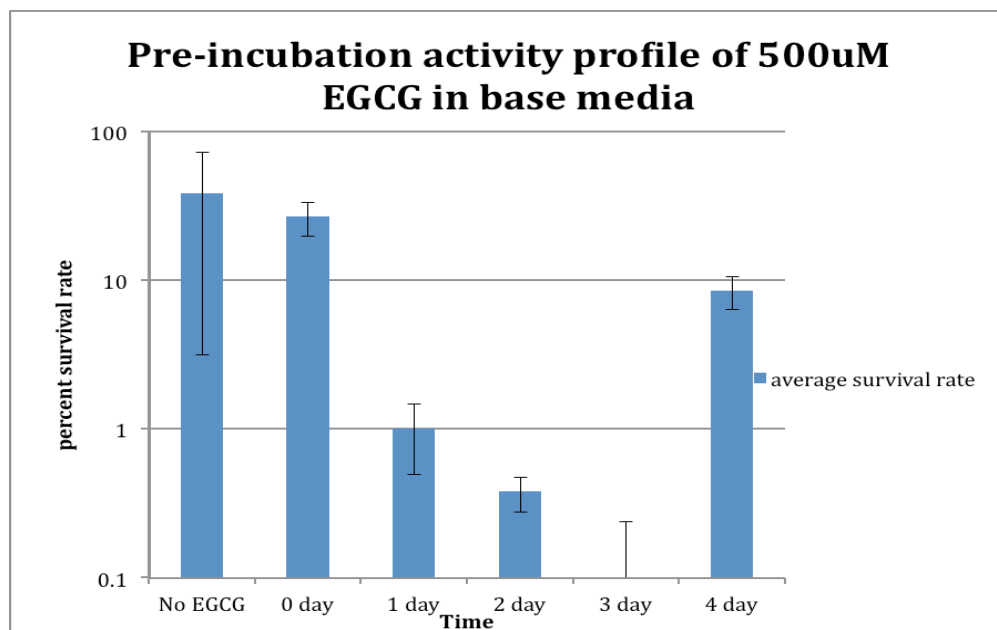
Figure 8. Avergae percent survoval rates of M. smegmatis in 500uM EGCG base medium.
The experiment was set up to determine whther EGCG has the same activity profile in pre-incubated base media as it does in regular biofilm media. Samples were set up with 500uM EGCG and serial dilutions performed at 0 and 24 hours after inoculation with the bacteria.

The results were consistent with what was discovered using regular biofilm medium: 3 day pre-incubation of EGCG in the medium maximizes its anti-mycobacterial activity, even in base biofilm media. Since the EGCG activity profile was the same, base media was shown to be a fitting substitute for regular biofilm media in HPLC experiments.

4.1.6   HPLC of 1mM EGCG at 0, 1, 2, 3, and 4-day pre-incubation

This experiment was performed in order to better understand the behavior of EGCG in the media over a period of 5 days. A 1mM EGCG sample was taken to Mr. Phillip Graebing for 5 consecutive days, so that he could run it through the HPLC. Besides the 1mM EGCG sample, a base media control and an EGCG standard were also fractionated out. The chromatograms depict retention times of the various components found in the sample, with the most polar ones eluting

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the fastest. Results of the HPLC are represented in Figure 9, with the retention times and area represented in Table 1.

A   B

EGCG Standard (Pitt)

EGCG 50μg/mL Control

Blank Mobile Phase with Ascorbic Acid

Standards

EGCG Control

Day 0   EGCG in Media

Day 0   Biofilm Media

Day 0   Immediate appearance of peaks at 4.0, 5.9, and 6.8 minutes

C   D

EGCG Control

Day 1   EGCG in Media

Day 1   Biofilm Media

Day 1   EGCG completely degraded. Increase of Degradants at 3.6, 6.8, and 7.3 minutes.

EGCG Control

Day 2   EGCG in Media

Day 2   Biofilm Media

Day 2   Secondary Degradant forming at 4.1 minutes

E   F

EGCG Control

Day 3   EGCG in Media

Day 3   Biofilm Media

Day 3   Secondary Degradants at 4.0, 4.3, and 5.5 minutes. Primary 6.7 minute Degradant persists.

EGCG Control

Day 4   EGCG in Media

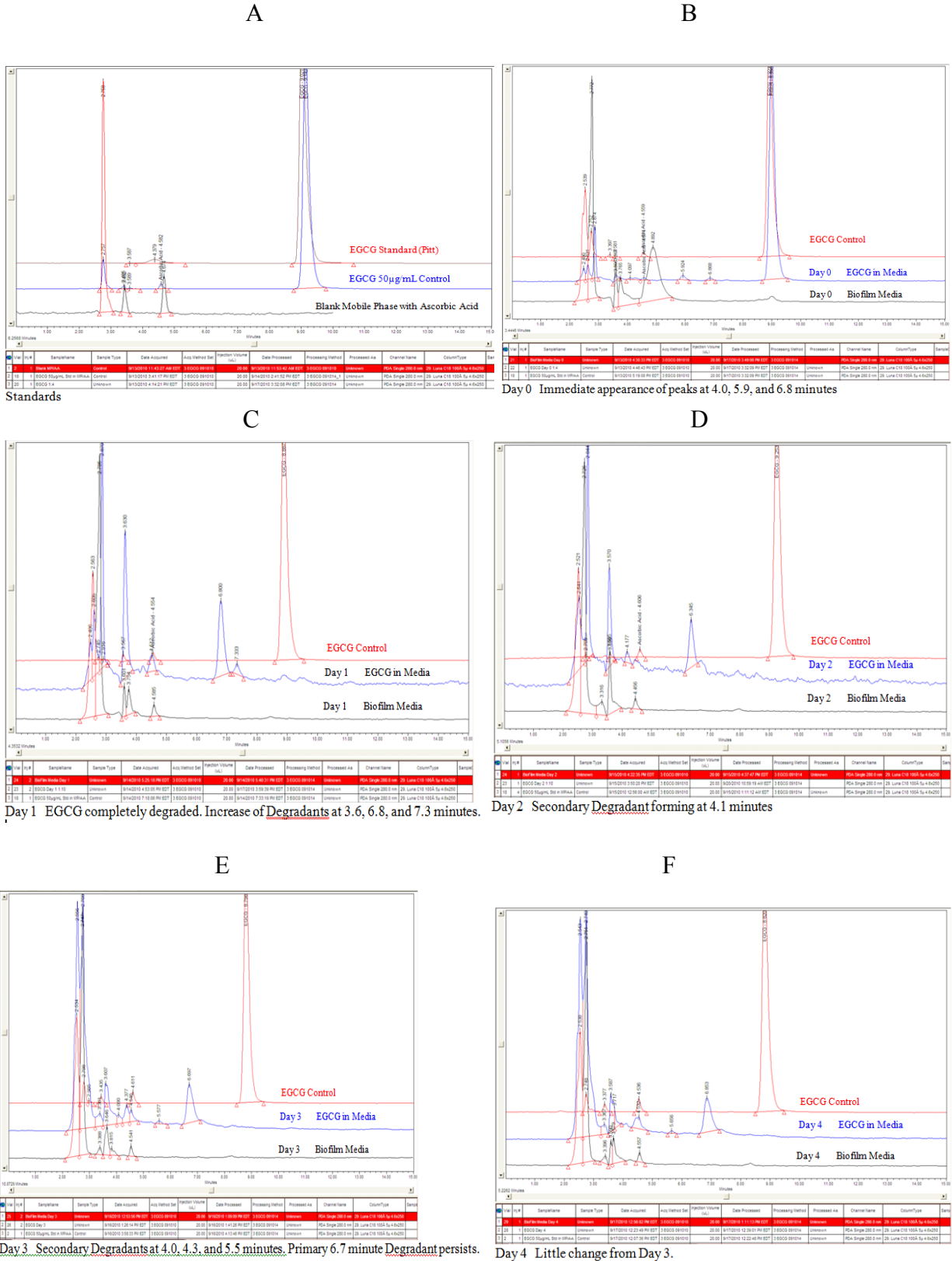Day 4   Biofilm Media

Day 4   Little change from Day 3.

Figure 9. HPLC of 1mM EGCG at 0, 1, 2, 3, and 4 day pre-incubation.

Figure 9. A 1mM EGCG sample was run through an HPLC in order to better understand its behavior in the media over a span of 5 days. A blank base biofilm media control and an EGCG standard were also included. (A) EGCG Standards. (B) No pre-incubation (day 0) EGCG sample. (C) Day 1 of pre-incubation. (D) Day 2. (E) Day 3. (F) Day 4.

Table 1. Retention times and AUCs of the 0, 1, 2, 3, and 4 day pre-incubated EGCG and base biofilm media.

Sample	Day	Retention time	AUC	Sample	Day	Retention time	AUC
Biofilm media	0	2.539	172607	Biofilm Media	3	2.535	189868
		2.772	225071			2.749	232730
		3.597	32583			3.404	7449
		3.765	65122			3.64	37332
		4.892	214410			3.85	4228
1mM EGCG 1:10 dilution	0	2.490	14377			4.539	9360
		2.631	18134	EGCG	3	2.556	314764
		2.874	40968			2.760	401147
		3.596	6925			3.381	24166
		4.097	5230			3.607	87300
		4.574	3274			4.080	12111
		5.924	8059			4.377	24757
		6.868	3557			4.546	14442
		EGCG-8.996	532381			5.577	4061
Biofilm Media	1	2.562	171402			6.697	84767
		2.785	232105	Biofilm media	4	2.538	189589
		3.602	16913			2.751	234121
		3.758	21256			3.396	8722
		4.586	8768			3.59	14657
						3.679	30343
1mM EGCG 1:10 dilution	1	2.487	9980			4.557	8501
		2.608	15048	EGCG	4	2.543	309791
		2.873	33033			2.748	401443
		3.635	36928			3.367	15463
		4.523	5214			3.587	45946
		6.834	29808			3.717	9986
Biofilm media	2	2.521	184978			4.532	24070
		2.726	266726			5.656	3386
		3.316	24132			6.853	82118
		3.585	57374	1mM EGCG control	0	2.757	78719
		4.456	9648			3.405	6740
EGCG 1:10 dilution	2	2.541	20321			3.589	2316
		2.844	23900			4.582	14355
		3.57	15105			EGCG- 9.142	1339613
		6.345	10925				

According to the HPLC, by day 1 EGCG gets completely degraded in the media (Fig. 9C) and the EGCG peak (retention time= 9 minutes) disappears. New degradants started showing up at 3.6, 6.8 and 7 minutes. At day 2 (Fig. 9D), secondary degradants begin to form at around 4.1 minutes. At day 3 of pre-incubation (Fig. 9E), even more secondary degradants get eluted at 4.0, 4.3 and 5.5 minutes, while the primary degradant at 6.7 remains. At day 4 (Fig. 9F) not much changes.

When looking at Table 1, which identifies the retention times (RT) and the area under the curve (AUC) for the peaks, associated with Figure 9, there are a few points that can be deduced. First of all, the biofilm media control remains fairly stable throughout the analysis, which standardizes control. The word relative is used because at Day 1 the RT=4.9 peak shifts to RT=4.5 and remains that way for the rest of the days. Peak shifts may occur since the chemical and physical interactions between the column and the molecules may vary during different runs. Second of all, as seen in Figure 9C, after day 1 the EGCG peak disappears, but its mass cannot do so as known from the Conservation of Mass physics laws. The mass from the EGCG peak shifts to other peaks, which accounts for changes in their AUC. When looking at AUC values in the table, there is a peak that stands out from the rest- RT=3.6. From day 0 through day 4 it's AUC's are as follows: 6925 → 36928 → 15105 → 87300 → 45946. At day 3, the peak shoots to 87300 which may be correlated with the effectiveness of a 3-day pre-incubation. The AUC values of peak RT=6.8 also increase significantly by day 3. Therefore, as the parent peak decreases at day 1, there is an increase in the secondary peak, which potentially is observed at day 3 of incubation.

The results have shown that EGCG gets degraded in the media by day 1. Since the previous experiments have suggested that its optimal anti-microbicidal activity occurs at day 3 of

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pre-incubation, it was hypothesized that it is in fact a secondary degradant of EGCG that is responsible for it anti-mycobacterial activity.

4.1.7   Effect of green tea pre-incubation in water on the growth of *M. smegmatis*

The HPLC experiment showed that EGCG gets completely degraded in the media after day 1 of incubation. Questions arose of whether it was the biofilm media itself that was influencing the degradation process of the EGCG, and if EGCG degradation would similarly occur in water. Questions also arose about whether green tea itself would have anti-mycobacterial activity against the bacteria and would it do so in water. This experiment was performed to figure out if green tea itself would have anti-mycobacterial activity against *M. smegmatis*. The green tea was brewed, both in sterile Millipore water and in base biofilm media, for 5 minutes. The extracts were filtered and cooled before cells were added. Negative controls were set up with water and base biofilm media. Another sample was set up with 1:4 diluted green tea extract sample. A set of samples was set up without any pre-incubation and serial dilutions were performed at 0 and 24 hours. Another set was set up after a 3-day pre-incubation of the green tea extract in the medium and water. Dilutions were once again performed at 0 and 24 hours. Average percent survival rates of 3 sample replicates were calculated after determining the cfu in each sample. The results are depicted in Figure 10 and 11.
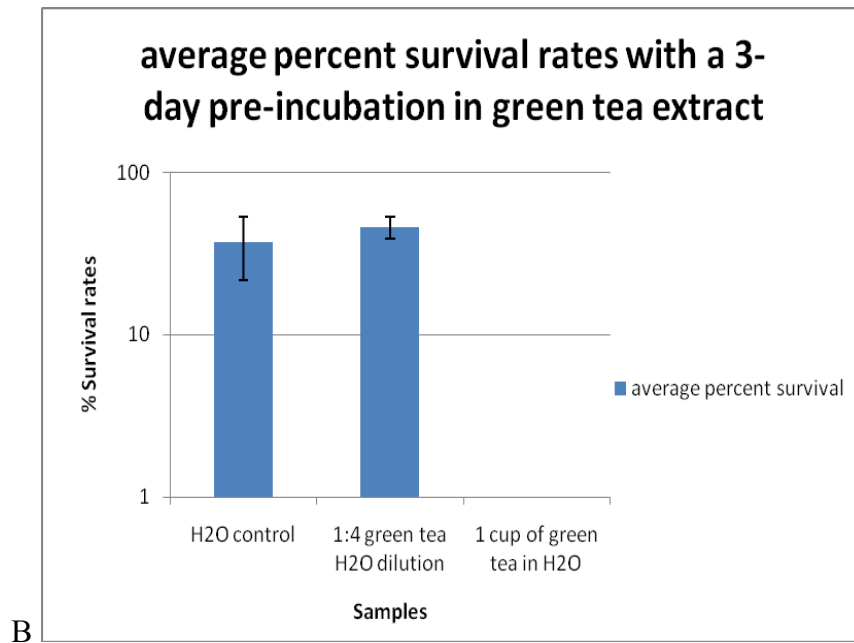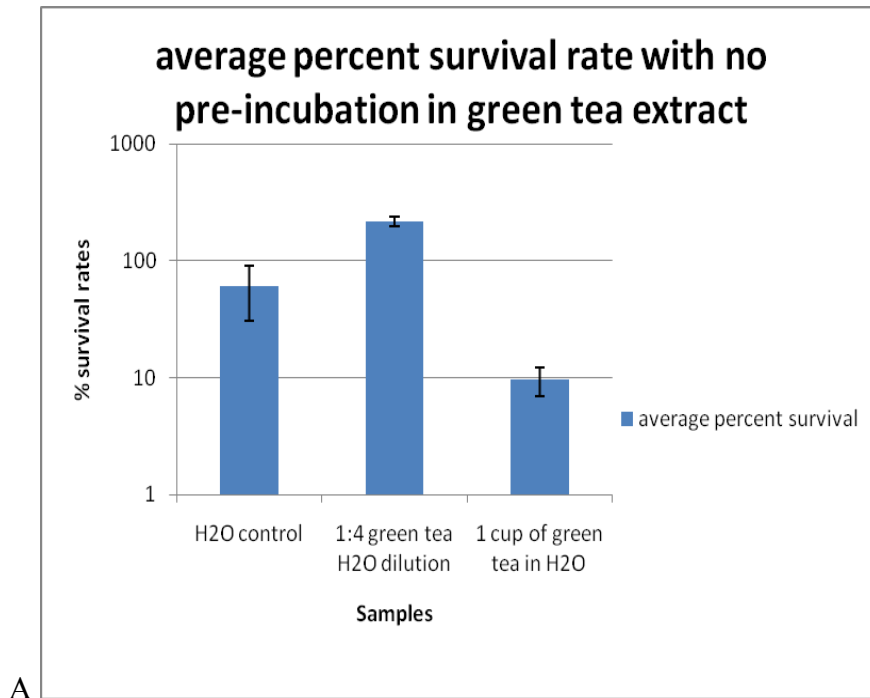
Figure 10. Effect of green tea extract, in water, on the growth of M. smegmatis.
The experiment was performed to observe if green tea itself had anti-mycobacterial activity against *M. smegmatis*. The green tea was brewed in sterile Millipore water. Samples were set up without any pre-incubation and with a 3-day pre-incubation of the extract. The experiment was performed to study a cup of green tea (0.5 g of tea in 40ml), as well as a 1:4 diluted sample of it. (A) Samples with no pre-incubation, (B) Represents results after a 3 day pre-incubation..

33
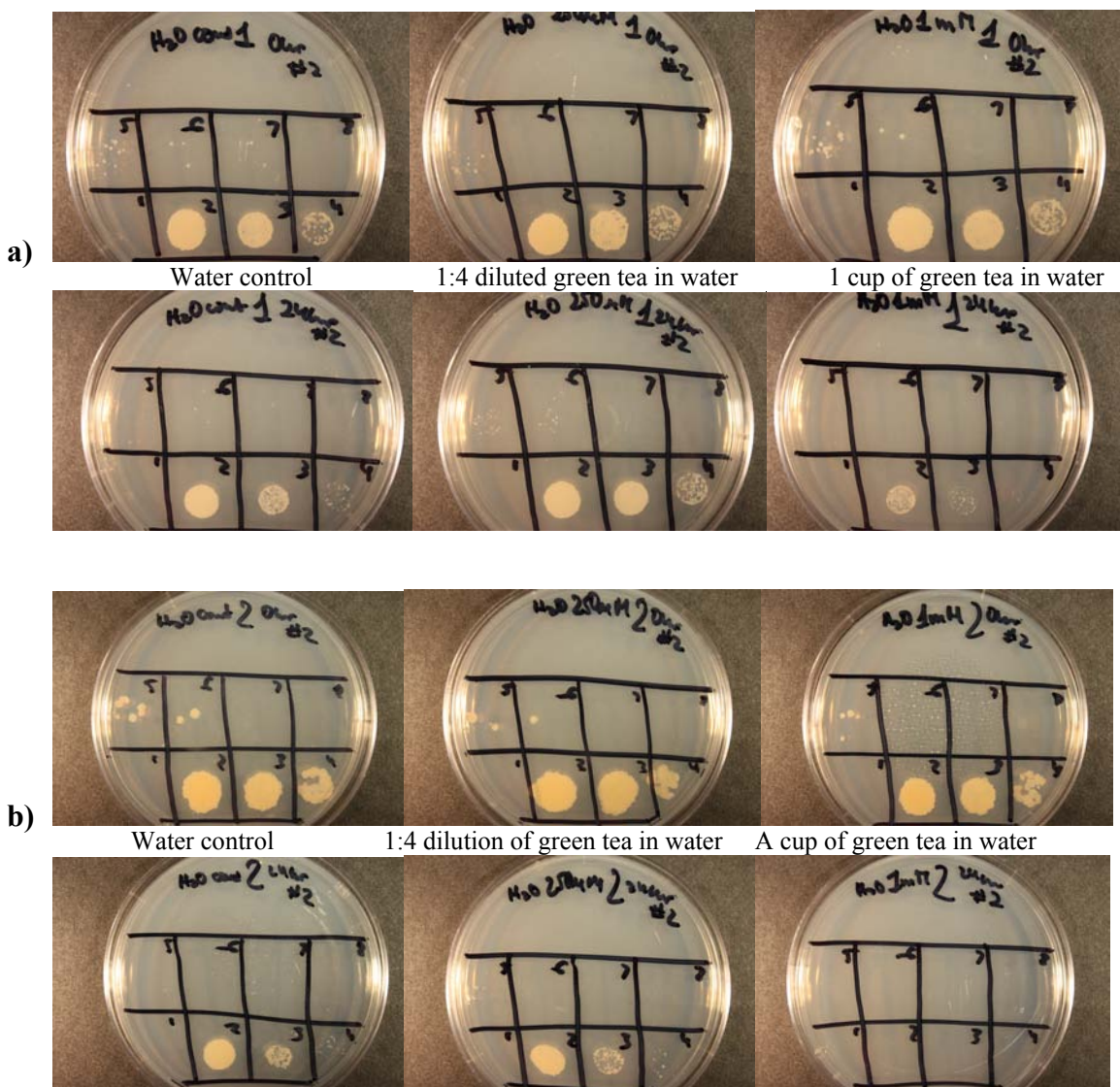
Figure 11. Effect of green tea on the growth of M. smegmatis.
These pictures were taken of the 7H10ADC plates (one of three replicates were chosen randomly for the picture), showing the effect of green tea on the bacteria in water. The first two sets of pictures are those of green tea with no pre-incubation: (A) The first row of pictures shows the water control, 1:4 diluted green tea sample I water and the regular cup of green tea in water at 0 hours of the experiment (left to right respectively). The row below that is the same samples but at 24 hours. Decrease in cell counts is visible in the 'cup of green tea' sample after 24 hours. (B) The last two sets of pictures are the plates after 3 day pre-incubation of green tea in water at 0 and 24 hours. There is complete killing of *M. smegmatis* cells in the cup of green tea sample.

It was clearly observed that a 3-day pre-incubation of the green tea extract in water completely kills 10^5 *M. smegmatis* cells. Although there was a small amount of killing without pre-incubation, it was far less significant. Overall, these results are consistent with the EGCG data from the previous experiments.

4.1.7.1 Repeat of the green tea experiment in water

The experiment performed to study the effects of green tea in water on the growth of *M. smegmatis* was repeated once again, in order to have more statistically significant results. An additional sample was set up of 250uM EGCG in water, to see if EGCG itself behaved the same way in water as it does in biofilm media. Once again, samples were set up with no pre-incubation and with 3 day pre-incubation. Dilutions were carried out at 0 and 24 hours and average survival rates of 3 sample replicates were calculated. Results are depicted in Figure 12.
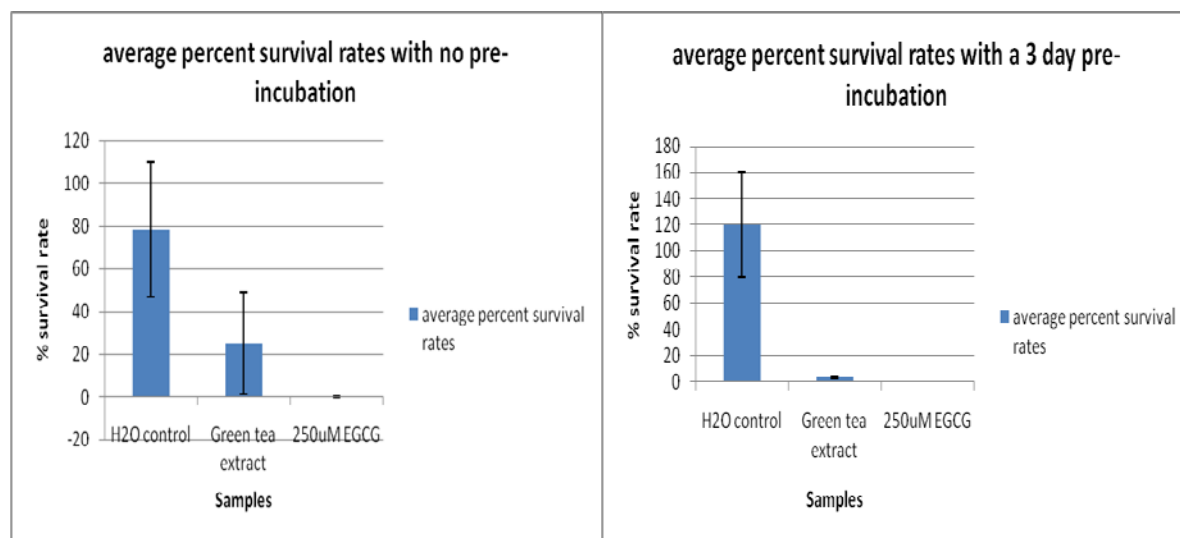
Figure 12. Green tea experiment in water.
The experiment was performed as a repeat of the previous experiment with the addition of the 250uM EGCG sample in water, to observe if EGCG had similar antimicrobial activity in water.

The results of this experiment support the findings of the previous green tea experiment, which showed that a 3 day pre-incubation of the green tea extract significantly strengthens its anti-mycobacterial activity. However, the 250uM EGCG sample in water did not follow the same activity profile as 250uM EGCG in base biofilm media. The anti-microbicidal activity of the EGCG in water seems to be a lot stronger than in the base media. This makes it difficult to use EGCG in water for the HPLC analysis as it may affect the degradation profile of the molecule.

4.2   SPECIFIC AIM#2: *M. TUBERCULOSIS*

4.2.1   The effect of 1mM EGCG on the growth of *M. tuberculosis*

A preliminary experiment was set up to determine if 1mM EGCG had any effect on the growth of *M. tuberculosis* cells. The experiment involved the inoculation of Sauton's media with a 1:1000 dilution of a growing stock of bacterial cells. Since TB is a slow growing organism, the bacilli in EGCG containing media were incubated at 37^0C for 48 hours and then the CFU were measured by plating the serial dilutions. The following figure (Figure 13) represents the effect of 1mM EGCG on *M. tuberculosis* growth.
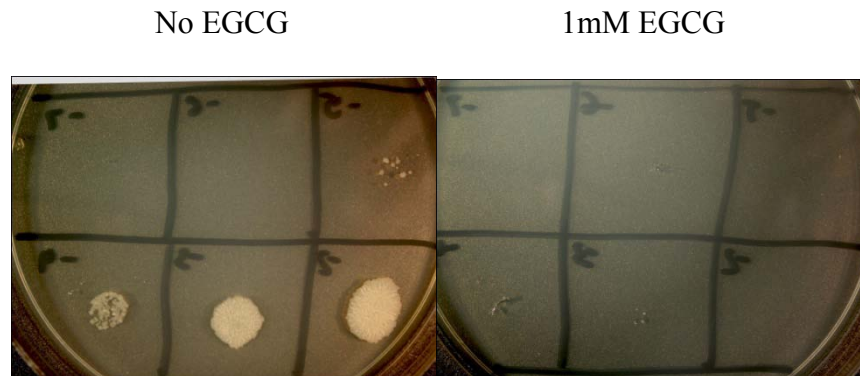
<div align="center">No EGCG   1mM EGCG</div>

Figure 13. The effect of 1mM EGCG on M. tuberculosis.
The experiment was set up to observe if EGCG has any effect on TB cells. (A) no EGCG control; (B) 1mM EGCG sample.

As seen in Figure 10, 1mM EGCG has strong microbicidal activity against *M. tuberculosis*. Figure 10A represents bacterial colonies resulting from the no EGCG control, whereas Figure 10B is the 1mM EGCG sample, clear of any bacterial growth.

5.0   DISCUSSION

Despite the use of anti-Tb drugs for about five decades, tuberculosis remains an important public health issue worldwide.[23] According to the World Health Organization, over a third of the world's population is infected with tuberculosis. They have estimated that the largest number of new TB cases in 2008 occurred in the South-East Asia region, accounting for approximately 35% of all incident cases worldwide. However, the estimated incidence rate of Sub-Saharan Africa is almost twice that of South-East Asia. Approximately 1.7 million individuals died from TB in 2009, with the highest number of casualties being in the Africa Region.[26]

There are many difficulties in dealing with tuberculosis. Individuals who have latent TB often go undiagnosed, as the latent form of the disease is asymptomatic, and have a higher risk of progressing to the disease stage if they are not treated. There is a scarsity of diagnostic capabilities in thirld world countries, which adds to the problem. In many places, even if a patient is diagnosed with TB, there is a lack of treatment available. The treatment for tuberculosis involves a combination of many various drugs. These drugs are expensive and people in the poor regions of the world may not be able to afford them. If they can afford them, they need to carefully adhere to the therapy for a long six months in order to sucessfully be treated. If they do not adhere, then they are at risk of developing MDR-TB or XDR-TB, which would require second line drugs to treat. [20] To add to the problem, over 30 million individuals

are infected with HIV globally. Individuals infected with HIV are at a higher risk of acquiring tuberculosis and at a higher risk of acquiring the drug resistant strains.

In terms of treatment, there have been new second line drugs that have been developed in the past few years and there was an increase in drug distributors. However, major challenges still remain in that persepctive. That is why scientific research into alternative treatment and prophylactic methods is very important.

The goal of this research study was to examine whether green tea, and specifically EGCG, had any mycobactericidal activity. Since green tea is a common drink, especially in South-East Asia, and its polyphenols including EGCG are known to have antimicrobial activity, it could possibly be used as an effective prophylaxis agent, would be very cost-effective, and many people would have access to it. However, one might also wonder then why the TB incidence rates are so high in that region if a lot of people consume green tea. Does that mean it is not effective?

In this research study, it was discovered that green tea extract as well as a solution of EGCG did in fact have potent anti-mycobacterial activity. A 3-day pre-incubation of both resulted in significantly stronger activity. This suggests that regular preparation of the green tea is not enough to achieve its optimal health benefits. Another explanation for why the TB incidence rates are high in South-East Asia could be the fact that not everyone drinks green tea as oolong tea is also widely popular in that region, or they may not drink the right dosage (which still has not been determined).

Since not everyone drinks green tea, it may also be beneficial to turn it into a pill form- either as a supplement or as an actual antibiotic drug. This study has shown that green tea does

have antimycobacterial activity, therefore it could eventually be developed into an effective anti-Tb drug.

In this study, it was further discovered, via HPLC analysis, that the EGCG is fully degraded in the media after day 1. This suggests that the activity in fact lies in a secondary degradant of the catechin. Several attempts have been made to isolate the degradant molecule and identify it using mass spectroscopy. However, the results have been inconclusive as of yet.

5.1   QUESTIONS TO BE ANSWERED AND FUTURE PLANS

There are many more questions that need to be answered before green tea could ever be thought off as a prophylactic agent, or a drug, against *M. tuberculosis*. Due to the fact that there is a lack of literature on the effects of EGCG on mycobacteria, the majority of the research project was focused on using *M. smegmatis* as it is a fast growing bacterium and would allow for the research to go further. Therefore, additional studies need to be performed with the emphasis being on the effects of EGCG and green tea on *M. tuberculosis.* At this point, the research has shown that there is in fact an effect of the catechin on *M. tuberculosis,* but the extent of it needs to be studied more.

Next, it is important to isolate and identify the degradant molecule of EGCG which appears to contain the anti-mycobacterial activity against the bacteria. As shown in Table 1, the peaks eluting at 3.6 and 6.8 minutes may be a good location to start. If the degradant molecule gets successfully located and isolated, however, the next step will be to determine if it is stable in physiological conditions and, if it is not, could it be stabilized? It is also important to make sure that this molecule is not toxic to the host. In vivo mice experiments could provide such answers.

Additionally, it is critical to make sure that a 3-day pre-incubation of the green tea extract is not toxic to individuals as it becomes a very concentrated solution at that point. Can all of this information be used to better process the green tea to achieve maximum prophylactic potential?

For the isoltion experimemts, water will be used as the solvent for the EGCG instead of the biofilm media. This will yield a cleaner HPLC analysis and aid in isolating the molecule of interest. This will also allow for Mass Spectroscopy analysis to be performed in order to indentify the degradant molecule. Future studies may also include the isolation of a EGCG resistant mutant organism.

In conclusion, as shown in the vast amounts of literature, EGCG is a very beneficial molecule.[5]Although a lot of research has been done with it, many unanswered questions remain, especially in terms of its antimicrobicidal activity against organisms such as *Mycobacterium tuberculosis.*

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