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# The Use of Natural Products as Potential Anti-Pseudomonas Agents

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**THE USE OF NATURAL PRODUCTS AS POTENTIAL  
ANTI-PSEUDOMONAS AGENTS**

**By**

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Submitted in partial fulfillment of the requirements for the  
degree of Master of Science in Microbiology from the  
Department of Biological Sciences of Seton Hall University

May 2014

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## Abstract

*Pseudomonas* is a genus of gram-negative gammaproteobacteria with a large range of diversity. Because of its ability to grow at low temperature, *Pseudomonas* is a cause of food spoilage. This bacterium is also a very common nosocomial infection of hospital patients. In this study, we investigated the effects of different natural products on *Pseudomonas*. The products included a polysaccharide, two flavonoids tangeretin and nobiletin and their derivatives, 5'OH-tangeretin and 5'OH-nobiletin, black tea polyphenol – theaflavins (TFs), as well as an herb named *Fallopia multiflora* (Chinese Knotweed). *P. fluorescens* and *P. aeruginosa* were used as model organisms. Antimicrobial effects were evaluated at various concentrations by using a microtiter plate assay or culture tube assay. The inhibition of biofilm formation was also determined using crystal violet assay. The results indicated that the selected natural products have antibacterial effect and anti-biofilm formation on *Pseudomonas*. Of the seven compounds studied 2.5% theaflavin, 2.5% Chinese Knotweed, and 500  $\mu$ M of tangeretin showed the greatest antimicrobial effect.

## Introduction

*Pseudomonas* is a genus of gram-negative bacteria that is the source of many acute and chronic infections. The bacteria can be isolated from environmental sources such as freshwater and soil.

*Pseudomonas* has a unique resistance to many microbicides. This makes these bacteria a problem in its removal from equipment and surfaces in hospitals (Lavoie et al 2011). *Pseudomonas aeruginosa* is a primary concern in healthcare. The bacteria are the major causing agents in immunocompromised patients (Martin-Espada et al 2013). *P. aeruginosa* can cause ventilator-associated pneumonia in intubated patients. Chronic infections are common in patients in intensive care units. These chronic infections occur in the respiratory tracts of 80% of adults with cystic fibrosis. Chronic *Pseudomonas* infection also occurs in heart, middle ears, and para-nasal sinuses (Alhede et al 2014).

The *Pseudomonas* genus is a very common source of food spoilage after harvest. The bacteria are so harmful because they can infect almost all types of vegetables. The bacteria can enter the plant tissue after mechanical injuries or the barrier being broken by other organisms. The contamination rate is very high in vegetables due to frequent contact with the soil during the growing process. The high infection rate of food after harvest not only causes an economic loss, but can be very harmful to humans.

*Pseudomonas* infections can be seen in cabbage, lettuce, beans, tomatoes, and soybeans (Tournas 2005).

The water systems act as a source of *P. aeruginosa* infection in healthcare. Although the direct mode of transmission is unclear, the correlation between *P. aeruginosa* and the water system is strong. Compared to other waterborne pathogens, *P. aeruginosa* excels because of its ability to adapt to a wide range of temperatures. *P. aeruginosa* also has the ability to grow in poor nutrient environments. Water treatments remove most microbial contamination from the water but *P. aeruginosa* problems remain in large buildings such as hospitals. Issues with *P. aeruginosa* arise because the plumbing installation allows the bacteria to proliferate and form a biofilm. This is seen in the areas of the plumbing where there are high

levels of nutrients for bacteria such as infrequently used outlets. *P. aeruginosa* growth is best seen near the distal ends of the plumbing like the sink because of the aerobic conditions (Loveday et al 2013).

*P. aeruginosa* infection is a serious healthcare issue as not only it can grow aerobically, but it also has the ability to grow in the microaerobic and anaerobic environment. One major example is the chronic lung infection in cystic fibrosis. With cystic fibrosis, *P. aeruginosa* is the dominant pathogen that cannot be treated with antibiotics. *P. aeruginosa* has the ability to adapt to different conditions because of its complex enzyme system. The enzyme systems are used to generate enzymes needed under oxygen-restricted or anaerobic conditions. Because of the heterogeneous environment of the cystic fibrosis produced by the mucus in the lungs, *P. aeruginosa* has the ability to produce enzymes for microaerobic growth, de-nitrification, and fermentation. The stress response system and universal stress proteins produced by the *P. aeruginosa* allow the bacteria to survive during anaerobic energy starvation conditions (Schobert and Jahn 2010).

When *P. aeruginosa* is grown under hypoxic conditions, causing the stress proteins secretion, physiological changes occur such as alterations to the outer membrane. These alterations can lead to increased tolerance to antibiotics (Schobert and Jahn 2010). Because the bacterium is gram-negative, it has an outer membrane, lipopolysaccharide (LPS), which protects it from the outside environment. The membrane also works as a sieve using specific and general pre-forming proteins. The pores allow for small molecules to cross the membrane, but larger molecules that are needed to be transported as well. Many known soluble exoproteins are secreted from *P. aeruginosa*. Of these exoproteins, most of them are virulence factors. *P. aeruginosa* is also known as one of the most actively secreting gram-negative bacteria (Bitter 2003). In addition to the chaperone/usher pathway, there are five more protein secretion systems including Type I (T1SS), II (T2SS), III (T3SS), IV (T4SS) and V (T5SS) secretion systems in Gram-negative bacteria. An example of the complexity of the protein secretion system was observed in the Type III secretion system (T3SS). *P. aeruginosa* can have one or more of the four genes with encode

for four cytotoxins. The cytotoxins being produced can lead to migration of the bacteria from the site of infection and inhibition of host cell DNA synthesis (Jabalameli et al 2012). Another example of the cytotoxins being produced is seen in the production of autotransporters mainly by *P. fluorescens*. This autotransporter PfaI is a part of the type V protein secretion system (T5SS). This system is unique because it has all of the components necessary for protein translocation within a single polypeptide. PfaI also has a virulence factor involved in the interaction with the host cell and modulating the immune response (Hu et al 2009). Along with the cytotoxins being produced, *P. aeruginosa* produces biosurfactants. One of the most studied biosurfactants is rhamnolipids produced by *P. aeruginosa*. Rhamnolipids have potential industry and environmental applications because of the antimicrobial properties (Samadi et al 2012). *Pseudomonas* has the ability to produce the gamma-aminobutyric acid (GABA) and the GABA-binding proteins. In the presence of GABA, *P. fluorescens* does not produce the biosurfactants (Dagorn et al 2013). *Pseudomonas* also produces extracellular proteases which typically help to provide nutrient for the bacteria by hydrolyzing large proteins. The proteases can also play a role in plant pathogenesis (Anderson et al 2004).

Along with the cases previously mentioned, *P. aeruginosa* is the source of many other infections including urinary tract infections, upper and lower respiratory tract infections, and bloodstream infection (Lavoie et al 2011). *P. aeruginosa* is also a major pathogen in infections of burn patients (Jabalameli et al 2012). There are certain innate immune responses that primarily control *P. aeruginosa*. These responses are very important to recognize and clear the pathogen. In respiratory tract infections, this has to be done while maintaining minimum inflammation-mediated damage. *P. aeruginosa* has the ability to alter the host immune response after infection. It also has many variability factors that affect the host immune response to the bacteria. In the mammalian respiratory tract, the bacteria have the ability to change the expression and structure of ligands that normally are known to be immutable. These changes cause the innate immune response to the *P. aeruginosa* to vary (Lavoie et al 2011). The acquired differences were seen in a study observing the difference between *P. aeruginosa* from an infected wound and an isolated

colony (Ramos et al 2008). The variability was also seen in *P. fluorescens* with its ability to change its metabolism to adjust to the dangers of toxic compounds. To combat oxidative stress, *P. fluorescens* decreases the formation of the pro-oxidant NADH and increase in the anti-oxidant NADPH (Mailloux et al 2011).

Bacteria have different abilities to adapt to rapid changes of environmental conditions (Baumgarten et al 2012). In addition to the metabolic effects of *Pseudomonas*, the genus of bacteria is notoriously harmful because of its ability to grow a biofilm. Biofilm is the accumulation of the microorganism in an exopolysaccharide matrix. *P. aeruginosa* biofilm is usually present in infections in acute burn wounds. Because of this biofilm formation, the survival rate in hostile environments increases. Biofilm formation protects the bacteria from the host immune response (Jabalameli et al 2012) so the immune response cannot eradicate the biofilm (Alhede 2014). The growth of this biofilm leads to a more persistent infection. It also increases the bacteria's tolerance to antibiotics by creating a physical barrier to the host defense system (Schobert and Jahn 2010). Biofilm formation can promote horizontal gene transfer. This can lead to genetic diversity (Ramos et al 2010). Biofilm formation can also lead to sharing of metabolic by-products within the biofilm community (Baumgarten et al 2012).

The physical barrier created by *Pseudomonas* becomes problematic because it has a reduced susceptibility to antimicrobial agents. The formation of a biofilm occurs by coordinating different cells to participate in certain surface-association behaviors. The formation occurs in a five-stage process including (1) Initial attachment, (2) Irreversible attachment, (3) Maturation I, (4) Maturation II and (5) Dispersion (Sauer et al 2002). The cells first enter a transitional state of reversible surface attachment, which must be stabilized for biofilm formation. As the biofilm matures, it forms microcolonies and macrocolonies. These colonies are encased in an extracellular matrix. This matrix is to organize and structure the bacteria community. The biofilm matrix consists of proteinaceous material, membrane vesicles, DNA, and exopolysaccharides (Merritt et al 2007). Biofilm formation is in part regulated by flagellum-based motility. The two-

component system SadC/B influences motility and biofilm formation. Mutations in flagellar regulation or biogenesis in *Pseudomonas* effects the formation of the biofilm (Mastropaolo et al 2012).

Biofilm formation plays a major role in *Pseudomonas* infections in a clinical setting. Patients with cystic fibrosis and chronic *P. aeruginosa* infections show biofilm formation in the conductive airways within the mucus in the lungs. Patients with diabetes and cardiovascular disease are showing an increase in chronic wounds. The intense accumulation of biofilm found in cystic fibrosis patients is also seen in chronic wounds. Another location of the intense accumulation of biofilm is in otitis media in the middle ear. Biofilm formation is detrimental in healthcare because of medical device related infections. These infections may occur from contamination from a patient's skin or mucous membrane. The infection can also occur from the surgical or clinical staff. *P. aeruginosa* biofilm infections are almost unavoidable in prolonged catheterization (Alhede et al 2014).

There have been many approaches to inhibit growth of this dangerous *Pseudomonas* genus. A novel approach would be to use natural products. Of the drugs in clinical use, approximately half of them are derived natural products. Even though this statistic is fairly high, chemists do not usually embrace the use of natural products. A major downfall of the natural products is the compounds are usually found in small quantities in comparison to synthesized compounds. This leads to the need for drugs with a greater quality than quantity. Many treatments, such as cancer chemotherapy, are ideal opportunities for natural product. Natural products are beneficial in comparison to artificially designed molecules because they naturally have a high specify and potency. Natural products also have well defined three dimensional structures with many functional groups. The targets of the natural products are well conserved (Paterson and Anderson 2005).

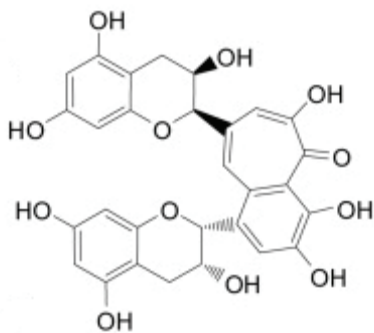
One major group of natural products is commonly seen in herb and spices called essential oils. The essential oils of thyme, oregano, clove, and cinnamon have been shown to have strong antimicrobial

effects. The complexity and synergy of essential oils make them very useful against multidrug resistant bacterial strains such as *Pseudomonas* (Sienkiewicz et al 2012). The use of essential oils is seen in mouthwash. The active ingredients in commercial mouthwashes are menthol, eucalyptol, thymol, and methyl salicylate (Erriu et al 2013). The use of natural products is also seen in other extracts from various microbes. Crude extracts from endophytic fungi of mangrove plants were analyzed for antimicrobial activity. It was found that the extracts had an antimicrobial effect over a wide range of bacteria, including *Pseudomonas* (Buatong et al 2011).

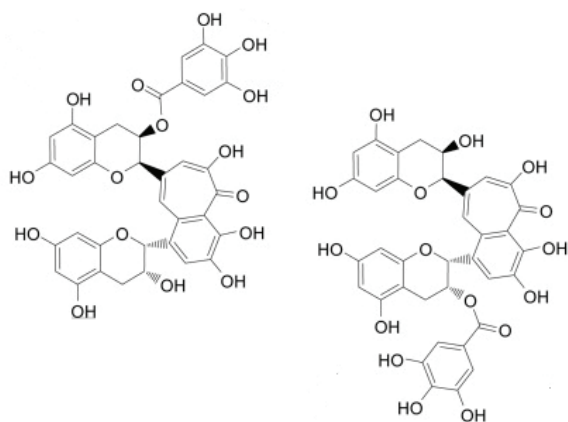
In this study, a variety of natural products were studied to observe any antimicrobial effect. One of the products used was theaflavin. Theaflavin is a type of polyphenol found in black tea extract. The formation of theaflavins occurs after the fermentation of monomeric flavan-3-ols. This leads to the production of theaflavins along with other oligomers. Theaflavins and its oligomers normally consist of 1-2% of the total dry matter in tea. In this study, the powder was 20% theaflavin. Many studies have been performed to observe the properties and structure of theaflavin and the oligomers (Figure 1). More recently, the pharmacological functions have been studied (Vermeer et al 2008).

The next polyphenol used in this study was *Fallopia multiflora* (*Polygonum multiflorum*). This tuberous root is also known as Chinese Knotweed (Figure 2). Chinese Knotweed grows in Guangxi, Henan, and Guizhou (Wang et al 2010). Chinese Knotweed is known as a tonic and anti-aging agent in traditional Chinese medicine. The herb also has a protective effect on the cardiovascular system (Liu et al 2010). Other tradition uses include nerve injury, constipation, and premature white hair (Wang et al 2010). Figure 3 showed the major constituents of the herb include emodin, rhein, other phenolic compounds and their glycosides (Yao et al 2006).

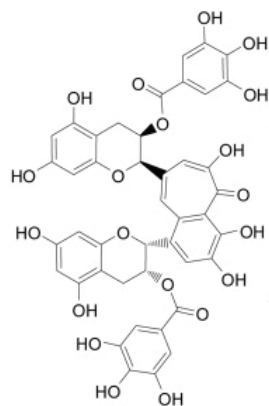




Theaflavin (TF1)



Theaflavin-3-Gallate and Theaflavin-3'-Gallate (TF2a and TF2b)

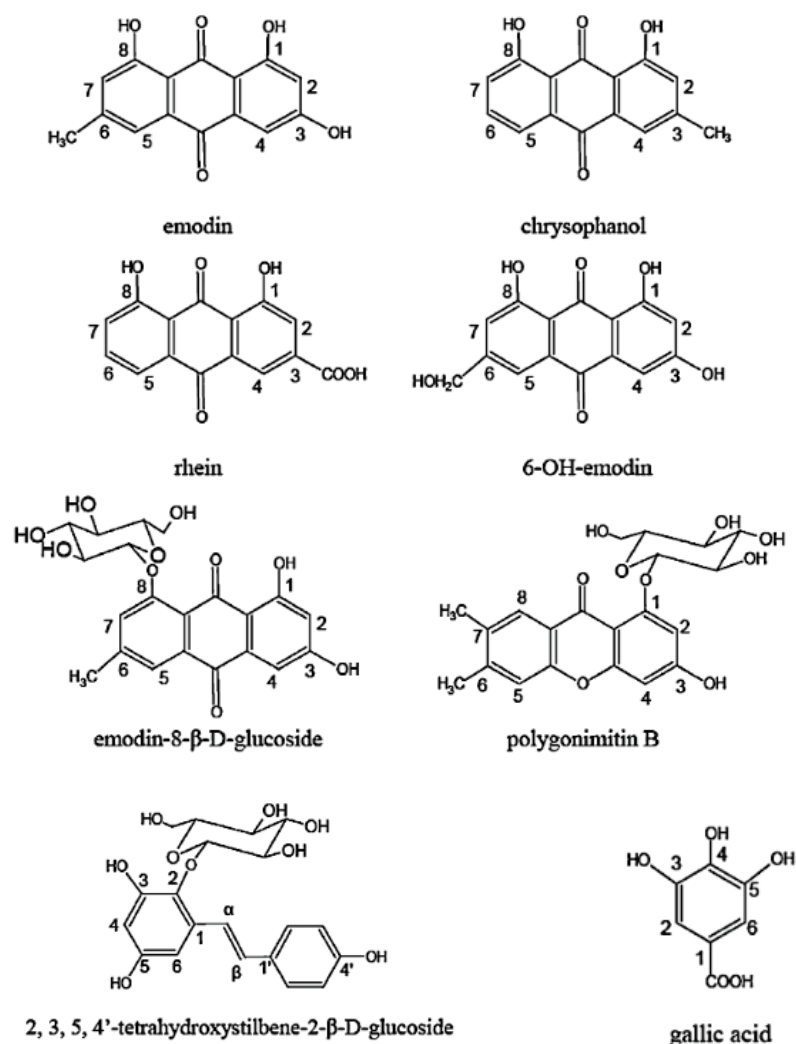


Theaflavin-3-3'-digallate (TF3)

**Figure 1:** Molecular structure of major Theaflavins (TF1, TF2 & TF3) found in black tea.

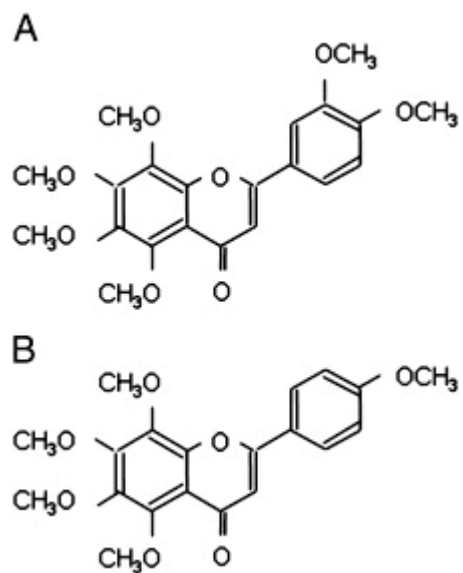


**Figure 2:** *Fallopia multiflora* root.



**Figure 3:** Active components of *Fallopia multiflora* root.

The next natural product studied were two polymethoxyflavones (PMFs) (Figure 4), a subgroup of flavonoids, and two derivatives. Aside from black tea, flavonoids are present in fruits, vegetables, nuts, and seeds. About 800 varieties of flavonoids have been identified (Mohan and Nandhakumar 2013). Flavonoids are polyphenol compounds involved in plant secondary metabolite processing. Flavonoids have the ability to modulate protein kinases. They also modulate epidermal growth factor receptors, cyclin-dependent kinases, vascular endothelial growth factor receptors, and platelet derived growth factor receptors. Flavonoids also have the ability to inhibit multiple enzymes involved in inflammation and cancer pathology (Ravishankar et al 2013). Nobiletin and tangeretin are PMFs derived from orange and tangerine peel. PMFs play a role in numerous biological functions. They also have neuroprotective, anticancer, and antimetastatic properties. More recent studies show that nobiletin and tangeretin can modulate adipocytokine secretion balance leading to treatment of insulin resistance (Miyata et al 2011). Antimicrobial effects of nobiletin and tangeretin against *Pseudomonas* have been previously studied at 3.6 mg/mL (Yao et al 2011). This is why derivatives of the two products were also studied. Structural modifications can be acquired by adding a functional group or using genetic engineering to reconstruct the pathways. In this study, the derivatives of nobiletin and tangeretin had an addition 5'-hydroxyl group. A new alternative in medicine is using combinational chemistry and diversity goaled synthesis. This offers a range of structural differences that are based on a common core. This provides a larger screening library in a shorter amount of time (Paterson and Anderson 2005).



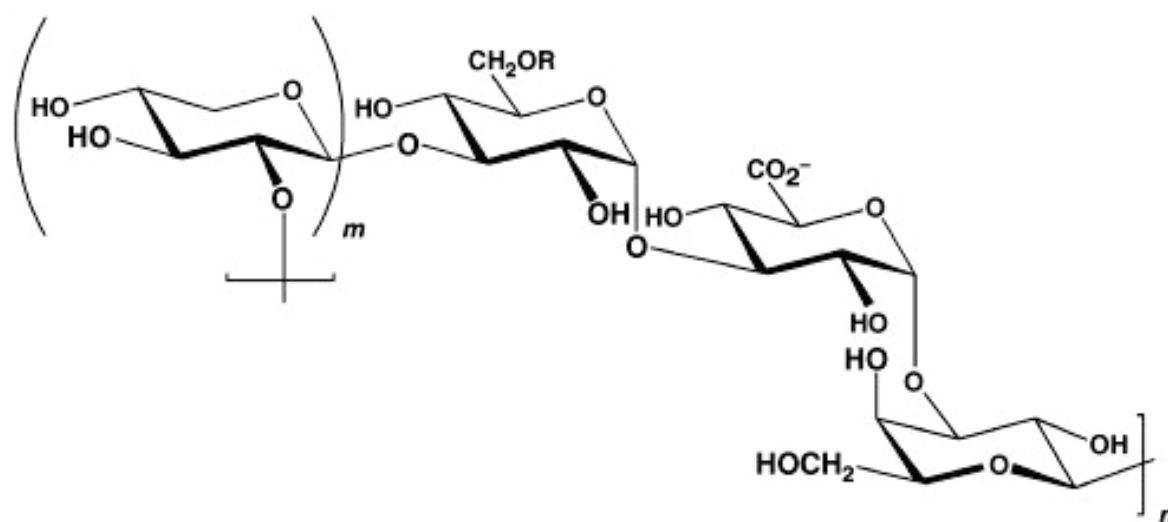
**Figure 4:** Molecular structure of the polymethoxyflavones nobiletin (A) and tangeretin (B).

A sulfated polysaccharide extracted from red microalgae *Porphyridium* was used to observe any antimicrobial effects. The red microalgae used were a habitat was fresh water, sea water, and brackish water. The red microalgae were cultivated in a large scale algae cultures (Figure 5). This improved the light availability by increasing the surface volume ratio. The red microalgae were encapsulated with sulfated polysaccharide (Figure 6). The polysaccharide was sulfated at the bioactive groups. These polysaccharides are believed to have a wide range of uses. The uses include antibacterial, antifungal, and anti-inflammatory. The sulfated polysaccharide has shown antiviral effects against herpes simplex virus 1 and 2. It also has shown antiviral effects against a variety of other viruses (Huheihel et al 2001). The polysaccharide also has a buffer layer to protect against extreme environments such as temperature, pH, and salinity. The polysaccharides in the cell wall have different compositions, but similar functions (Geresh and Arad 1991).

In this study, the mentioned seven natural products were used to observe any antimicrobial effects on the very harmful bacteria *Pseudomonas*. Biofilm inhibitory effects by natural products were also determined.



**Figure 5:** Large scale cultivation of red microalgae *Porphyridium*.



**Figure 6:** Proposed structure of the polysaccharide extracted from the microalgae species *Porphyridium*.

## Materials and Methods

### *Bacterial culture*

*P. aeruginosa* and *P. fluorescens* were obtained from Carolina Biological Supply Company. The bacteria were cultured using Difco™ LB Broth (LB), Difco™ Tryptic Soy Broth (TSB), and Carolina Nutrient Broth (NB) dehydrated media. The bacteria was cultured at 37° C and constantly shaken at 250 rpm.

### *Preparation of compound solutions*

The red algae polysaccharide was obtained from Dr. Shoshana Arad of the Institutes of Applied Research at Ben-Gurion University of the Negev in Be'er-Sheva, Israel. Because of its high viscosity, the polysaccharide was diluted to 1% using diH<sub>2</sub>O. The pure flavonoids tangeretin and nobiletin, as well as their derivatives, were received from the laboratory of Dr. Chih-Yu Lo of National Chiayi University in Chiayi, Taiwan. 3mM stock solutions were made by dissolving the compounds in DMSO. The solutions were then filtered using Acrodisc® 13 mm sterilized syringe filters with 0.2 µm Supor® membranes. The black tea polyphenol was purchased from Jiangyin Dehe Biotechnology Company in Edison, NJ. The 20% theaflavin powder was dissolved in DMSO to prepare a 5% stock solution. The solutions were then filtered using Acrodisc® 13 mm sterilized syringe filters with 0.2 µm Supor® membranes. The *Fallopia multiflora* (Chinese Knotweed) was obtained from Dr. Lo as well. All compounds were stored at 4°C. For the experiments, a vehicle control was used to observe any toxic effects of the solvents.

**Table 1:** Complete list of the natural products used as potential anti-*Pseudomonas* agents.

Natural Product	Stock Concentration	Working Concentration
Theaflavin	5%	5%
Chinese Knotweed	5%	5%
Polysaccharide	5%	1%
Nobiletin	3 mM	1 mM
Tangeretin	3 mM	1 mM
5'OH-Nobiletin	3 mM	1 mM
5'OH-Tangeretin	3 mM	1 mM

***Microtiter plate antimicrobial inhibition assay***

A microtiter plate assay was used to observe any antimicrobial effects of the natural compounds using *P. fluorescens*. The *P. fluorescens* was inoculated in a 5-7 mL culture overnight to grow to stationary phase. The appropriate amount of NB or LB was added to the wells in a clear Corning 96-well microtiter plate so that each well had a total volume of 120  $\mu$ L. The desired concentration of the natural product was then added to the well. The same amount of *P. fluorescens* (5  $\mu$ L) was added to the wells. The growth of the bacteria in the presence and absence of the natural products was then observed. The growth was monitored over a 24-hour period obtaining the OD<sub>600</sub> at 0, 1, 2, 3, 4, 5, 6, 7, 8, 12, and 24 hours. The absorbance was measured at 600 nm wavelength using a SpectraMax M5 from Molecular Devices. Over the 24 hours, the microtiter plate was maintained at 37° C and constantly shaken at 250 rpm. Any antimicrobial effects of the natural product were then observed.



### ***Culture tube antimicrobial inhibition assay***

A culture tube assay was used to observe any antimicrobial effects of the natural compounds using *P. aeruginosa*. The *P. aeruginosa* was inoculated in a 5-7 mL culture overnight to grow to stationary phase. The appropriate amount of NB or LB was added to the VWR 17x100 mm culture tubes with closures so that each tube had a total volume of 6 mL. The desired concentration of the natural product was then added to the culture tubes. The same amount of *P. aeruginosa* (250  $\mu$ L) was added to the culture tubes. The growth of the bacteria in the presence and absence of the natural products was then observed. The growth was monitored over a 24-hour period obtaining the OD<sub>600</sub> at 0, 1, 2, 3, 4, 5, 6, 7, 8, 12, and 24 hours. Using proper aseptic technique, 1mL of the bacteria culture was removed to gather the absorbance using a Pharmacia LKB Ultraspec III and returned to the culture tube. Over the 24 hours, the culture tubes were maintained at 37°C and constantly shaken at 250 rpm. Any antimicrobial effects of the natural product were then observed.

### ***Biofilm inhibition using crystal violet assay***

A crystal violet assay was performed to observe any biofilm inhibition properties of the natural products. The *P. aeruginosa* and *P. fluorescens* were inoculated in a 5-7 mL culture overnight to grow to stationary phase. The appropriate amount TSB was added to the non-treated Cellstar® 24-well suspension culture plate so that the total volume was 2 mL. The desired treatment concentration of the natural product was then added to the appropriate well. The bacteria were grown to stationary phase (OD<sub>600</sub>≈1) was then diluted to 1:5 using TSB, and 1mL of the diluted bacteria was added to the wells. The plates were then covered and incubated at 37° C and 250 rpm for 5 days. After the incubation period, a crystal violet staining was performed. Four small

trays were set up in a series adding 1-2 inches of tap water to the last three. The planktonic bacteria were removed by shaking it over the first dish. The plate was then submerged in the first water tray to wash the wells. Two hundred microliters of a 0.1% crystal violet/water solution was then added to each well. The plates were incubated at room temperature for 10 minutes to stain the biofilm. The crystal violet solution was then removed by shaking it over the waste tray. The plates were then washed in the remaining 2 trays. As much liquid as possible was removed after each wash. After the washes, the plate was inverted and tapped on paper towels to remove excess liquid. The plate was then allowed to air dry. The dye was solubilized by adding 500  $\mu$ L of 95% ethanol. The plate was incubated at room temperature for 10 minutes. The mixture was resuspended using a pipette. The absorbance was then read at 570 nm wavelength.

### ***Microscopic observation of biofilms***

A crystal violet assay with cover slips was performed to visualize any biofilm inhibition properties of the natural products. The *P. aeruginosa* and *P. fluorescens* were inoculated in a 5-7 mL culture overnight to grow to stationary phase. The non-treated Cellstar® 24-well suspension culture plate was prepared by adding 500  $\mu$ L of TSA and allowing it to solidify. A glass microscope cover slip (12 mm) was then added to the agar in each well at a 90° angle. The appropriate amount TSB was added to the culture plate so that the total liquid volume was 1.5 mL. The desired treatment concentration of the natural product was then added to the appropriate well. The bacteria grown to stationary phase ( $OD_{600} \approx 1$ ) was then diluted to 1:5 using TSB, and 1 mL was added to the wells. The plates were then covered and incubated at 37°C and 250 rpm for 5 days. After the incubation period, a crystal violet staining was

performed on the cover slips. The cover slips were carefully removed from the wells. A sterile pipette was used to add 3-4 drops of a 0.1% crystal violet/water solution to the cover slip. The cover slip was incubated at room temperature for 10 minutes. At the end of the incubation, the cover slip was washed by adding drops of  $\text{dH}_2\text{O}$  using a sterile pipette. The cover slip was then allowed to air dry. Images were then obtained of the cover slip using the bright field on an Olympus Fluoview FV1000 microscope.

### ***Statistical Analysis***

Each experiment was performed in triplicate. The p value was obtained to determine the statistical significance ( $p < 0.05$ ) using student's t-test. Standard deviation error bars were added to the growth curves.

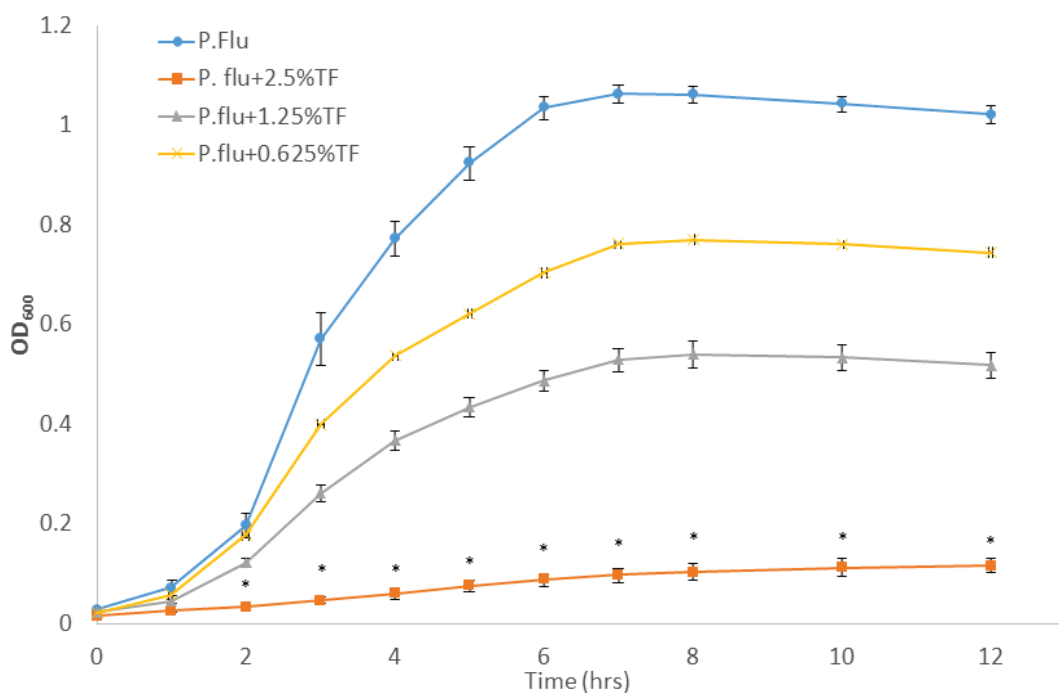
## Results

### *Antimicrobial Inhibition Assay*

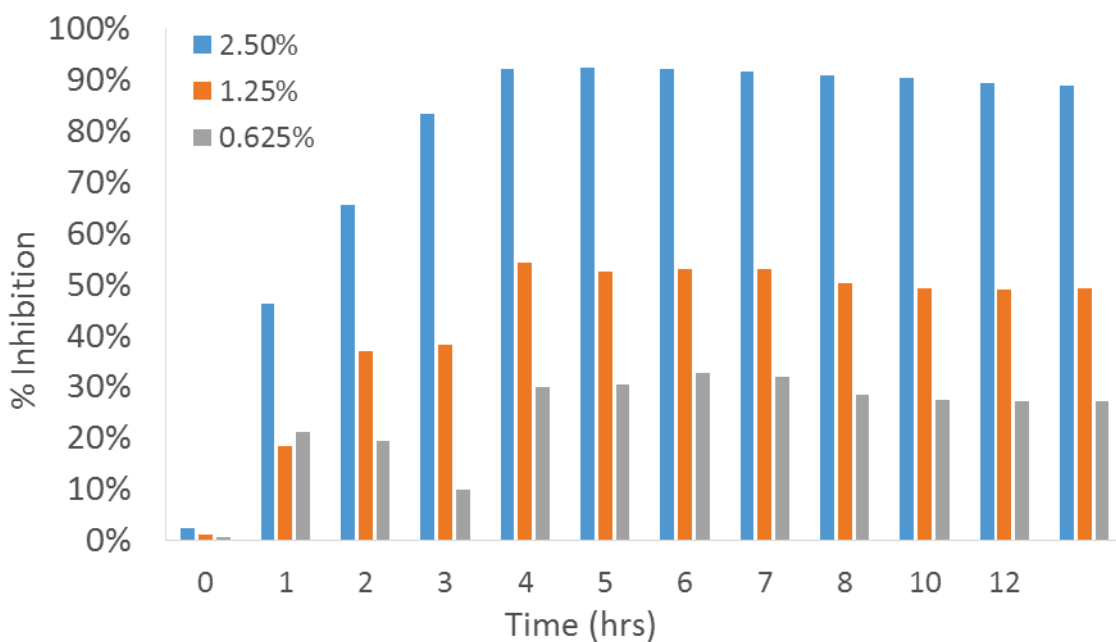
Antimicrobial effects of the seven natural products on *P. fluorescens* and *P. aeruginosa* were determined. The growth curves of the bacteria were monitored by turbidity study at 600 nm wavelength. The percent inhibition of the natural products on the bacteria was also determined. The first graph for each compound shows the growth monitoring, followed by the percentage of inhibition. The percentages reported are from the 8 hour time point because this is the time point the bacteria were found to reach stationary phase.

#### 1. Theaflavin

The concentration of *P. fluorescens* treated with the theaflavin compared to the untreated bacteria was lower, and demonstrated the antimicrobial effect of theaflavin. *P. fluorescens* treated with 2.5% theaflavin showed completely inhibition of growth. *P. fluorescens* treated with 1.25% theaflavin showed the growth was reduced by half of the control. *P. fluorescens* treated with 0.625% theaflavin showed slight inhibitory effect. There was a statistically significant difference ( $p < 0.05$ ) between the control and treated group (with 2.5% TF) from the second hour and on (Figure 7). *P. fluorescens* treated with 2.5% theaflavin showed 90.89% growth inhibition. *P. fluorescens* treated with 1.25% theaflavin showed 50.24% growth inhibition. *P. fluorescens* treated with 0.625% theaflavin showed 28.37% growth inhibition (Figure 8).

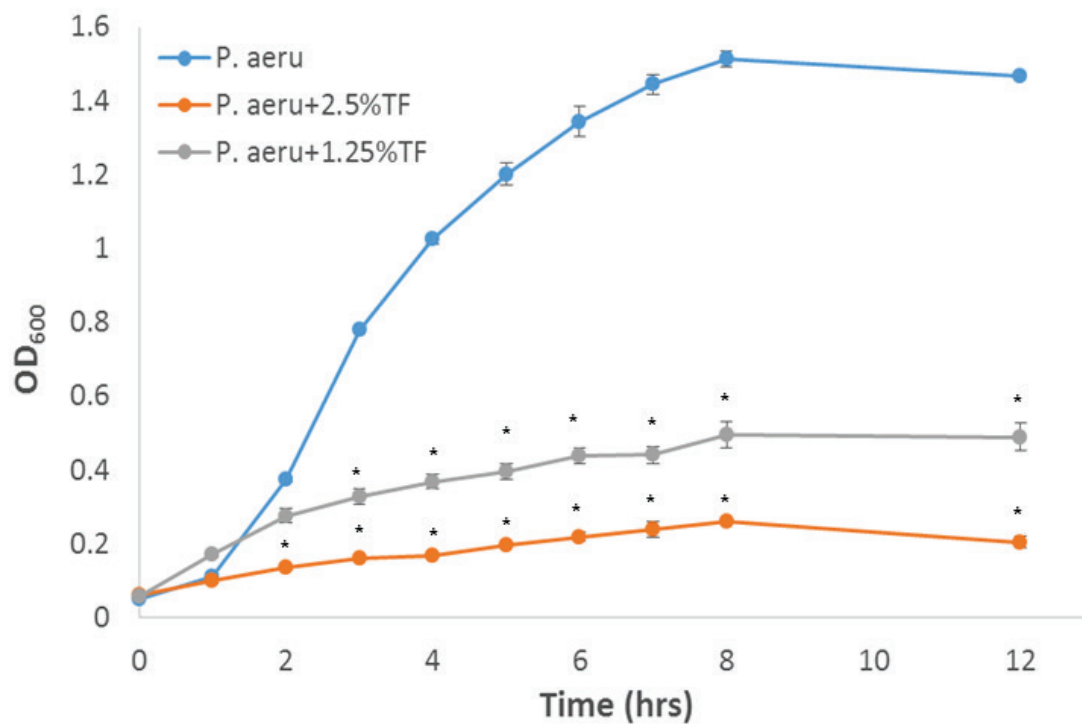


**Figure 7:** Growth curve of *Pseudomonas fluorescens* treated with theaflavin. \* indicates statistically significant.

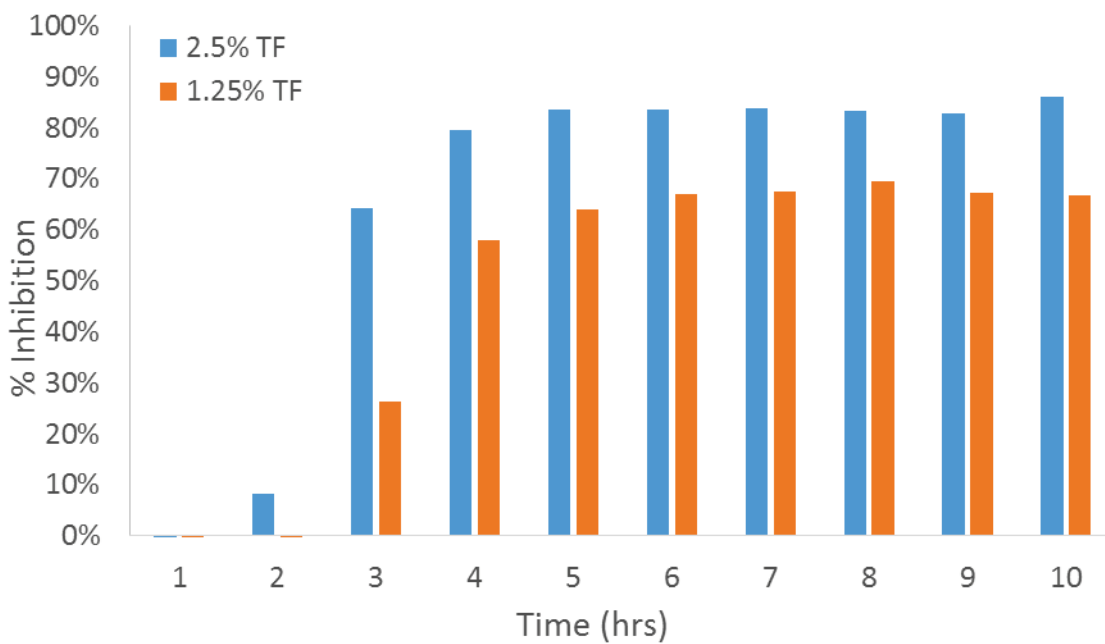


**Figure 8:** Percent inhibition of *Pseudomonas fluorescens* treated with theaflavin.

The concentration of *P. aeruginosa* treated with the theaflavin compared to the bacteria not treated was lower and demonstrated the antimicrobial effect of theaflavin. *P. aeruginosa* treated with 2.5% theaflavin showed the growth was completely inhibited. *P. aeruginosa* treated with 1.25% theaflavin showed strong inhibition of growth, but not to the effect as 2.5% theaflavin. There was a statistically significant difference ( $p < 0.05$ ) between control and treatment group (1.25% TF) since the third hour and 2.5% TF from the second hour and on (Figure 9). *P. aeruginosa* treated with 2.5% theaflavin showed 82.76% growth inhibition. *P. aeruginosa* treated with 1.25% theaflavin showed 67.14% growth inhibition (Figure 10).



**Figure 9:** Growth curve of *Pseudomonas aeruginosa* treated with theaflavin. \* indicates statistically significant.

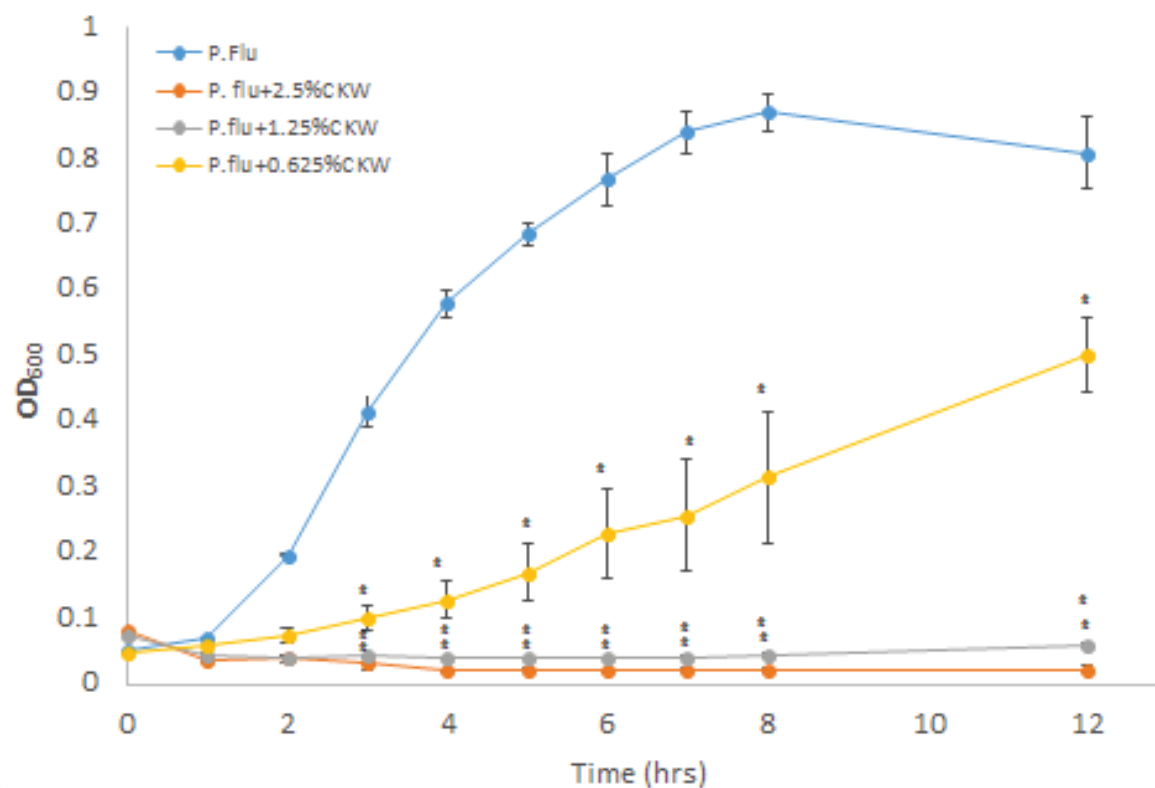


**Figure 10:** Percent inhibition of *Pseudomonas aeruginosa* treated with theaflavin.

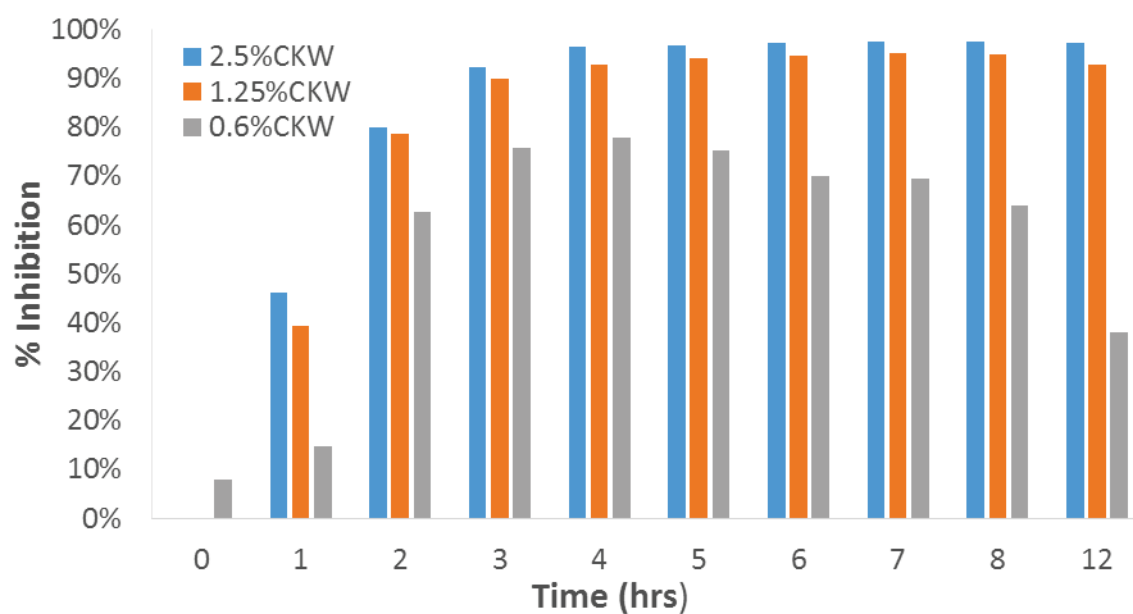
## 2. Chinese Knotweed

The concentration of *P. fluorescens* treated with the Chinese knotweed compared to the untreated bacteria was lower and demonstrates the Chinese knotweed antimicrobial effect. *P. fluorescens* treated with 2.5% and 1.25% Chinese knotweed showed completely inhibition of its growth. *P. fluorescens* treated with 0.625% Chinese knotweed showed some inhibitory effect on the growth. The difference between control group and the treatment groups (all three concentrations: 2.5%, 1.25% and 0.625%) are statistically significant ( $p < 0.05$ ) from the third hour (Figure 11). *P. fluorescens* treated with 2.5% Chinese knotweed showed 97.54% growth inhibition. *P. fluorescens* treated with 1.25% Chinese knotweed showed 94.95% growth inhibition. *P. fluorescens* treated with 0.6% Chinese knotweed showed 63.91% growth inhibition (Figure 12).





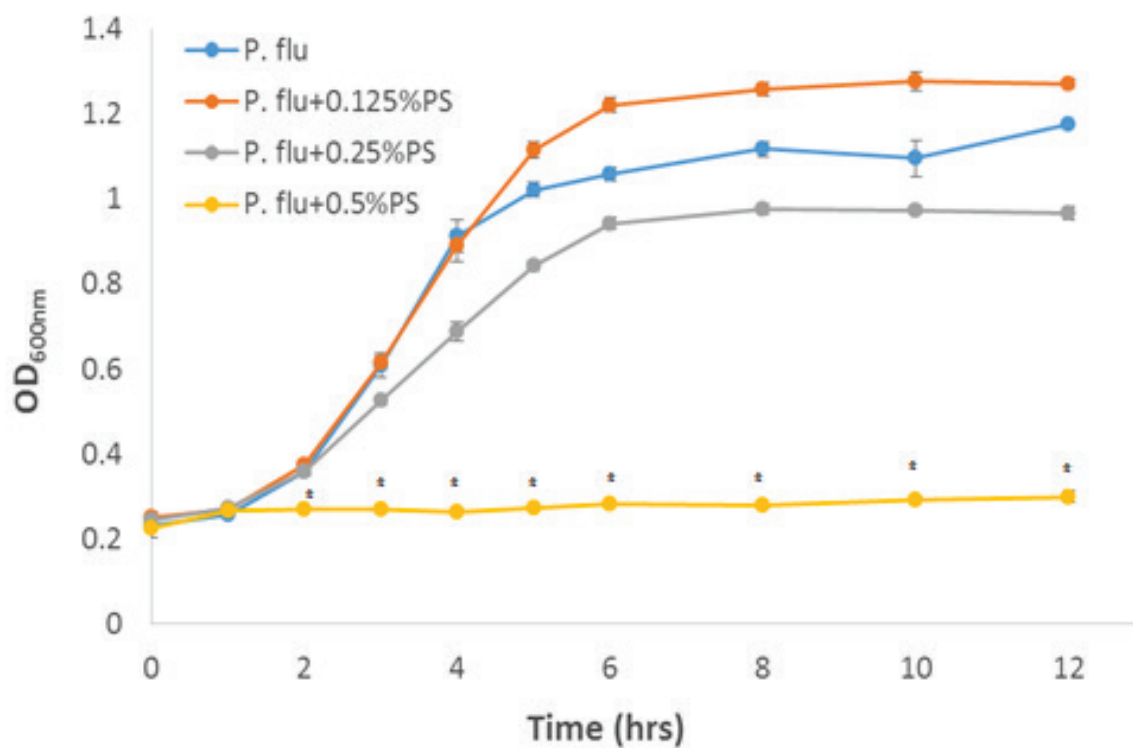
**Figure 11:** Growth curve of *Pseudomonas fluorescens* treated with Chinese Knotweed. \* indicates statistically significant.



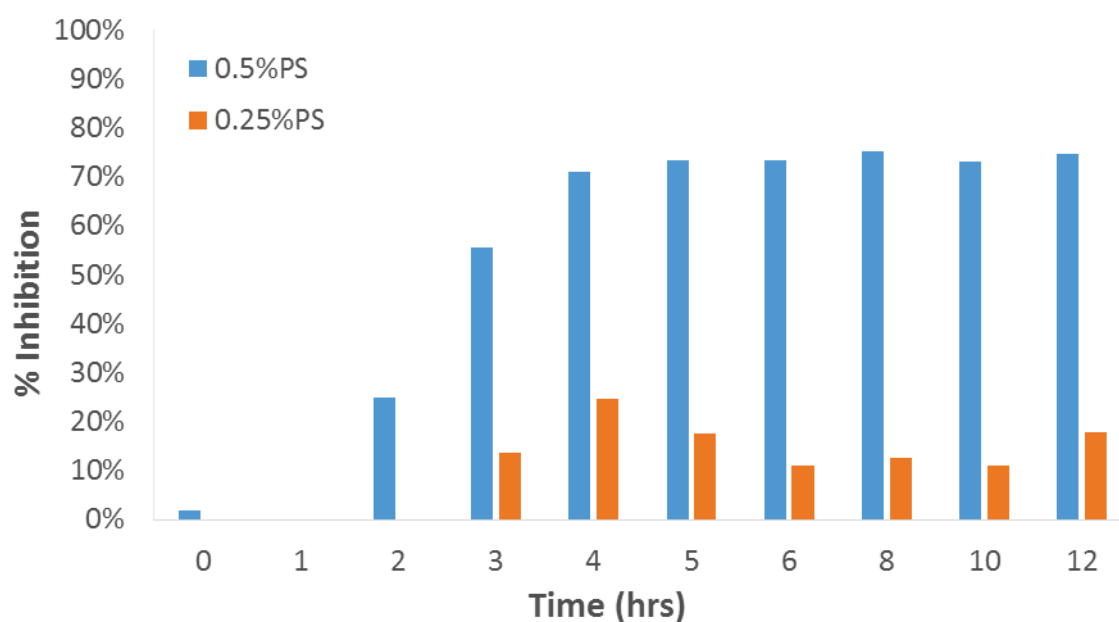
**Figure 12:** Percent inhibition of *Pseudomonas fluorescens* treated with Chinese knotweed.

### 3. Red Algae Polysaccharide

The concentration of *P. fluorescens* treated with the red algae polysaccharide compared to the bacteria not treated was lower demonstrating the polysaccharides antimicrobial effect. *P. fluorescens* treated with 1.0% polysaccharide showed completely inhibition of growth. *P. fluorescens* treated with 0.5% polysaccharide showed slight inhibition of its growth. *P. fluorescens* treated with 0.1% polysaccharide showed very little inhibitory effect. For polysaccharide, 0.5% showed statistically significant difference ( $p < 0.05$ ) when compared with control (Figure 13). *P. fluorescens* treated with 1% of the red algae polysaccharide showed 75.09% growth inhibition. *P. fluorescens* treated with 0.5% of the polysaccharide showed 12.66% growth inhibition (Figure 14).



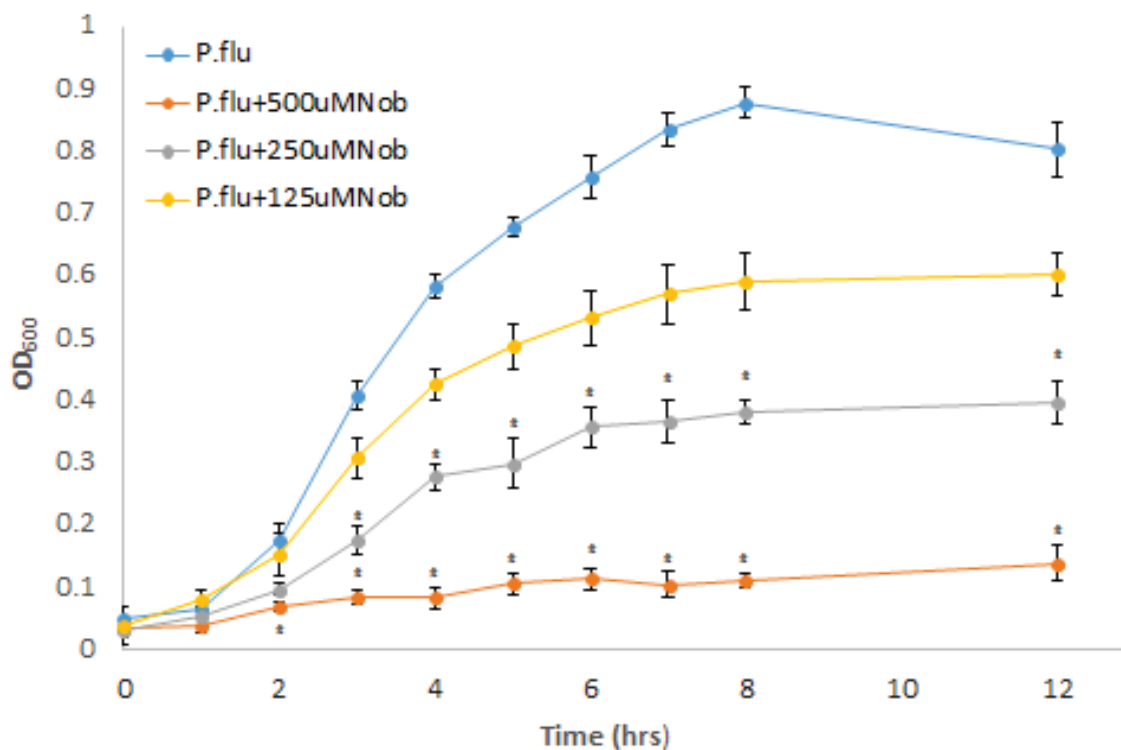
**Figure 13:** Growth curve of *P. fluorescens* treated with the red microalgae polysaccharide. \* indicates statistically significant.



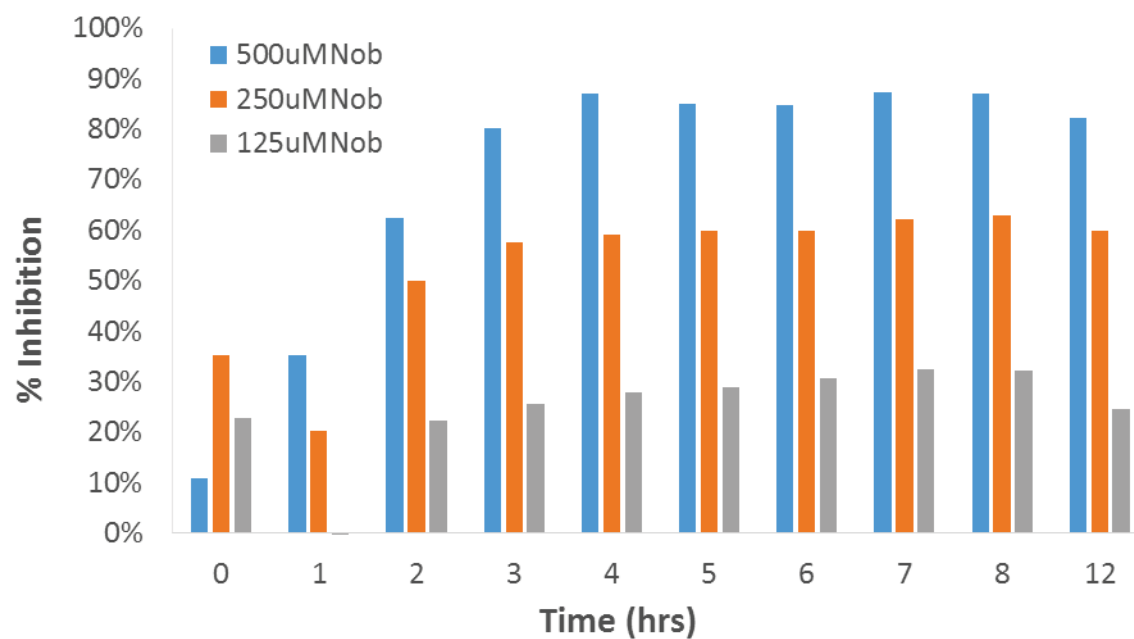
**Figure 14:** Percent inhibition of *P. fluorescens* treated with the red microalgae polysaccharide

#### 4. Nobiletin

The concentration of *P. fluorescens* treated with the nobiletin compared to the bacteria not treated was lower demonstrating the nobiletin antimicrobial effect. *P. fluorescens* treated with 500  $\mu$ M nobiletin showed completely inhibition of growth. *P. fluorescens* treated with 250  $\mu$ M nobiletin showed 63% inhibition. *P. fluorescens* treated with 125  $\mu$ M nobiletin showed little inhibition or the growth was slightly inhibited. The bacteria treated with 500  $\mu$ M nobiletin were shown almost complete inhibition from the second hour with statistically significant difference ( $p < 0.05$ ) while 250  $\mu$ M nobiletin showed significant inhibition ( $p < 0.05$ ) from the third hour and on compared with the control (Figure 15). *P. fluorescens* treated with 500  $\mu$ M nobiletin showed 87.18% growth inhibition. *P. fluorescens* treated with 250  $\mu$ M nobiletin showed 62.90% growth inhibition. *P. fluorescens* treated with 125  $\mu$ M nobiletin showed 32.04% growth inhibition (Figure 16).



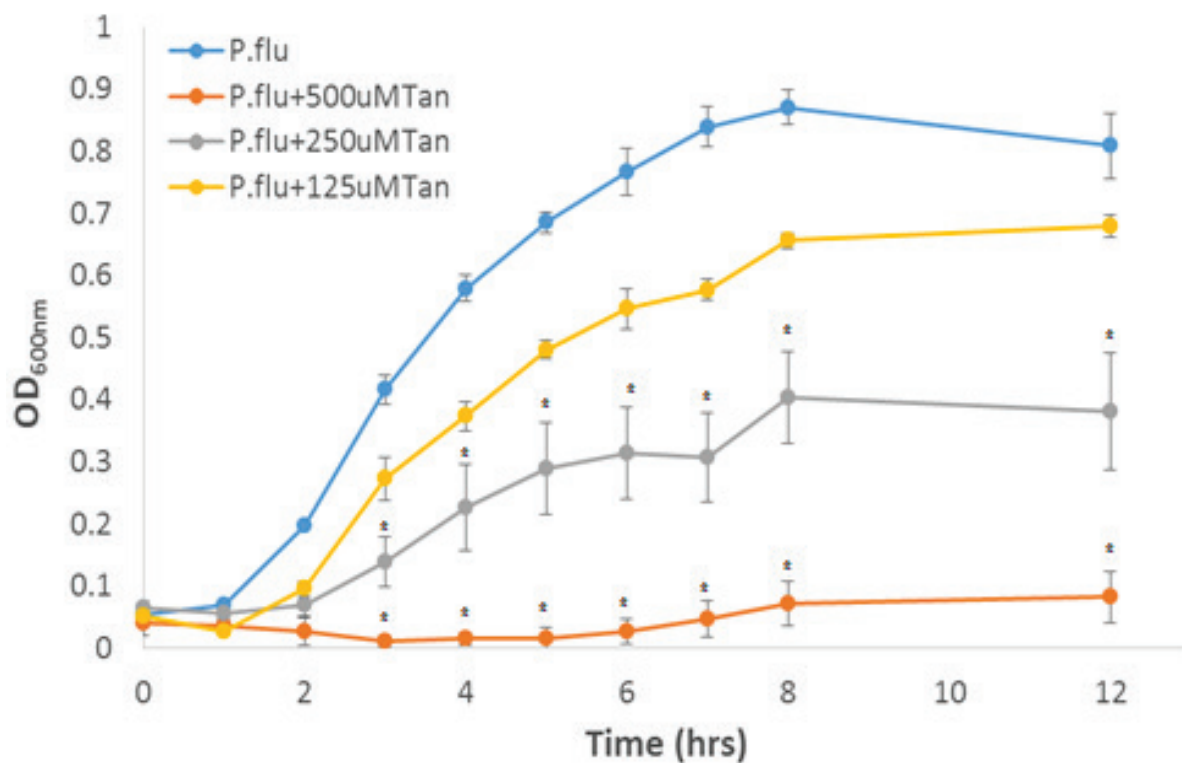
**Figure 15:** Growth curve of *Pseudomonas fluorescens* treated with nobiletin. \* indicates statistically significant.



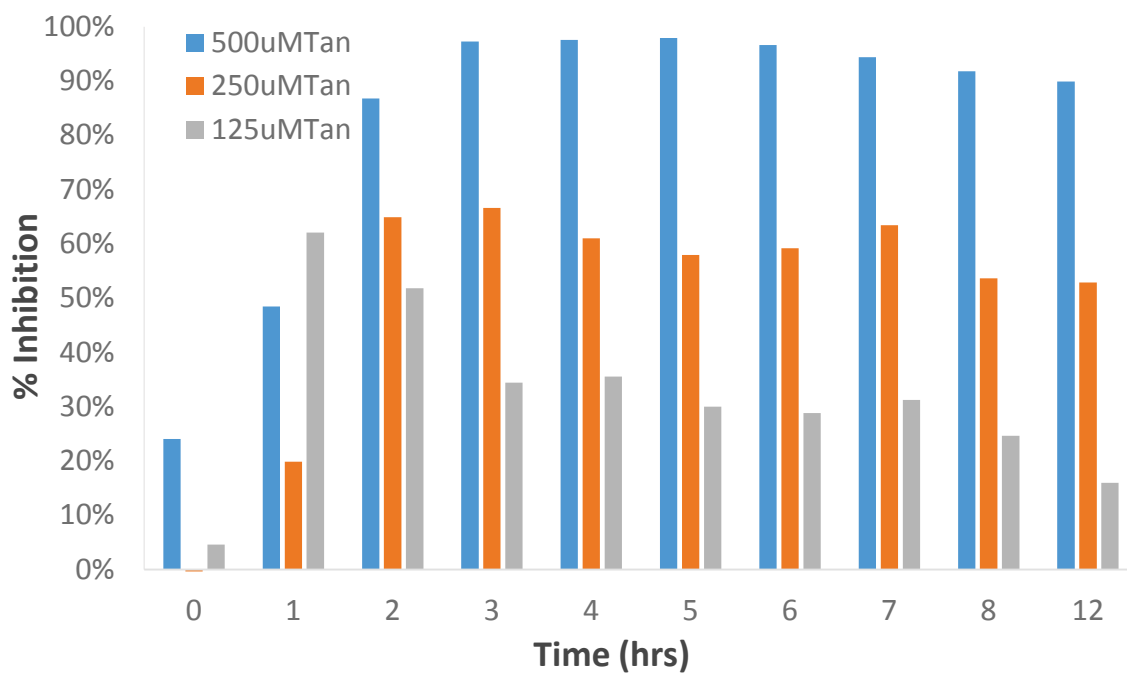
**Figure 16:** Percent inhibition of *Pseudomonas fluorescens* treated with nobiletin.

## 5. Tangeretin

The concentration of *P. fluorescens* treated with the tangeretin compared to the bacteria not treated was lower demonstrating the tangeretin antimicrobial effect. *P. fluorescens* treated with 500  $\mu\text{M}$  tangeretin showed completely inhibition of its growth. *P. fluorescens* treated with 250  $\mu\text{M}$  tangeretin showed inhibition of growth by half. *P. fluorescens* treated with 125  $\mu\text{M}$  tangeretin showed little inhibition of growth. Both 250 and 500  $\mu\text{M}$  tangeretin showed statistically significant difference ( $p < 0.05$ ) compared with control groups (Figure 17). *P. fluorescens* treated with 500  $\mu\text{M}$  tangeretin showed 91.80% growth inhibition. *P. fluorescens* treated with 250  $\mu\text{M}$  tangeretin showed 53.66% growth inhibition. *P. fluorescens* treated with 125  $\mu\text{M}$  tangeretin showed 24.63% growth inhibition (Figure 18).



**Figure 17:** Growth curve of *Pseudomonas fluorescens* treated with tangeretin. \* indicates statistically significant.

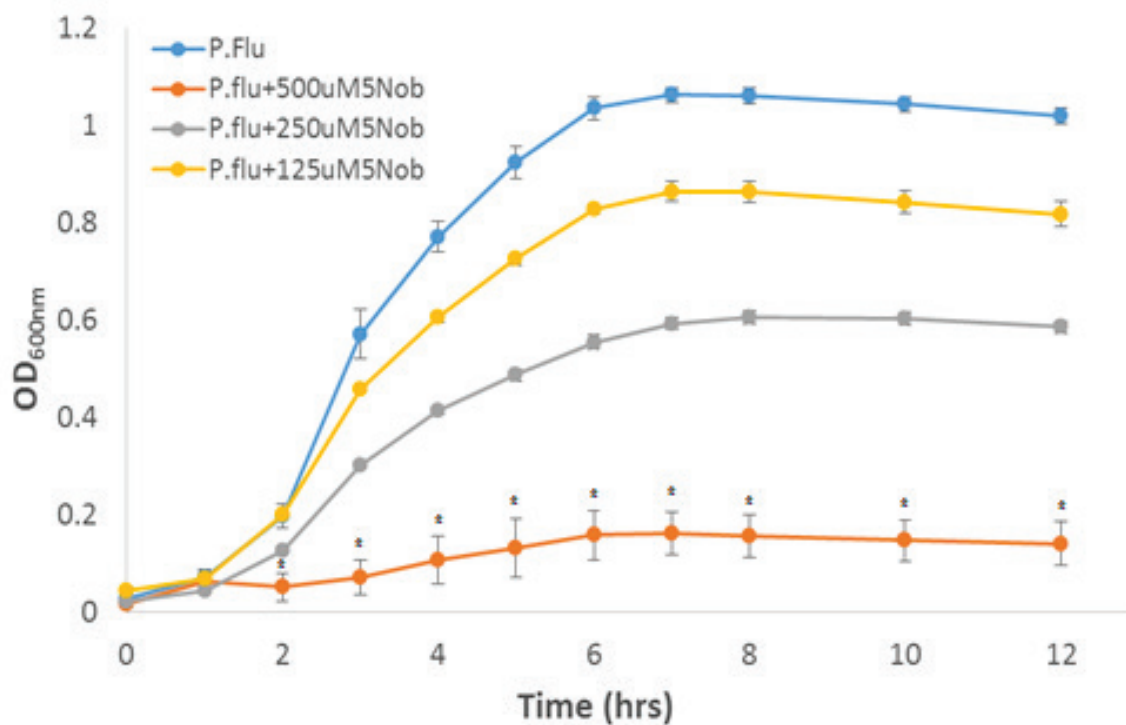


**Figure 18:** Percent inhibition of *Pseudomonas fluorescens* treated with tangeretin.

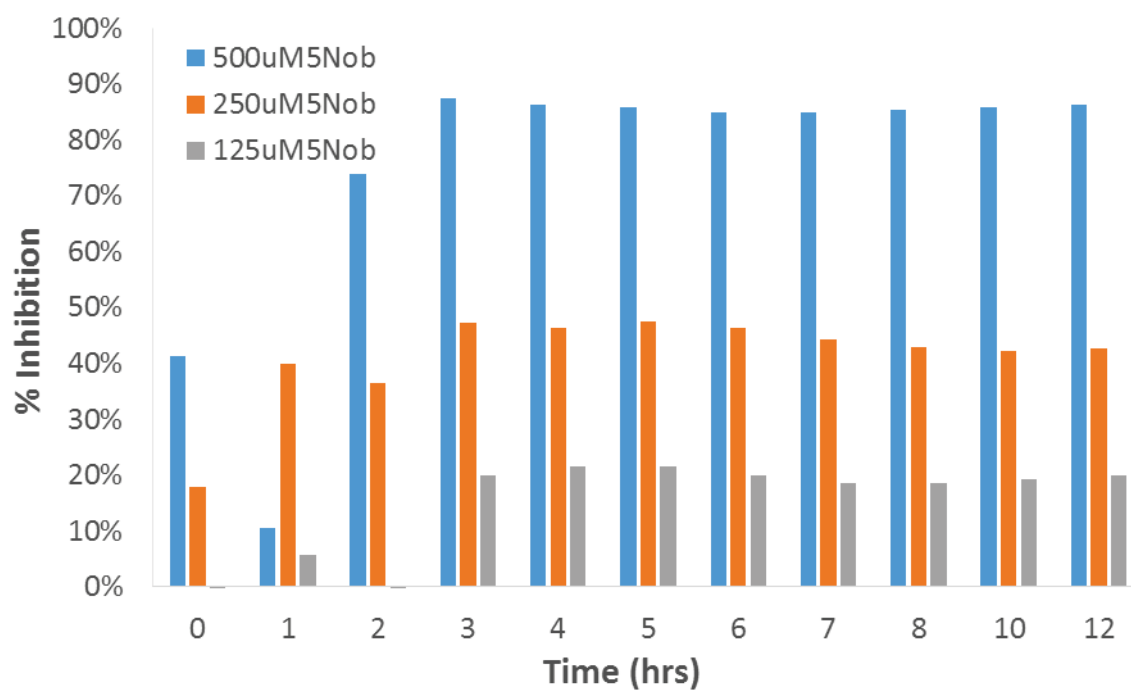
## 6. 5'OH-nobiletin

The concentration of *P. fluorescens* treated with the 5'OH-nobiletin compared to the bacteria not treated was lower demonstrating the 5'OH-nobiletin antimicrobial effect. *P. fluorescens* treated with 500  $\mu\text{M}$  5'OH-nobiletin showed highest inhibition ( $p < 0.05$ ). *P. fluorescens* treated with 250  $\mu\text{M}$  5'OH-nobiletin showed the growth was inhibited severely. *P. fluorescens* treated with 125  $\mu\text{M}$  5'OH-nobiletin showed little inhibition of growth (Figure 19). *P. fluorescens* treated with 500  $\mu\text{M}$  5'OH-nobiletin showed 85.27% growth inhibition. *P. fluorescens* treated with 250  $\mu\text{M}$  5'OH-nobiletin showed 42.92% growth inhibition. *P. fluorescens* treated with 125  $\mu\text{M}$  5'OH-nobiletin showed 18.67% growth inhibition (Figure 20).





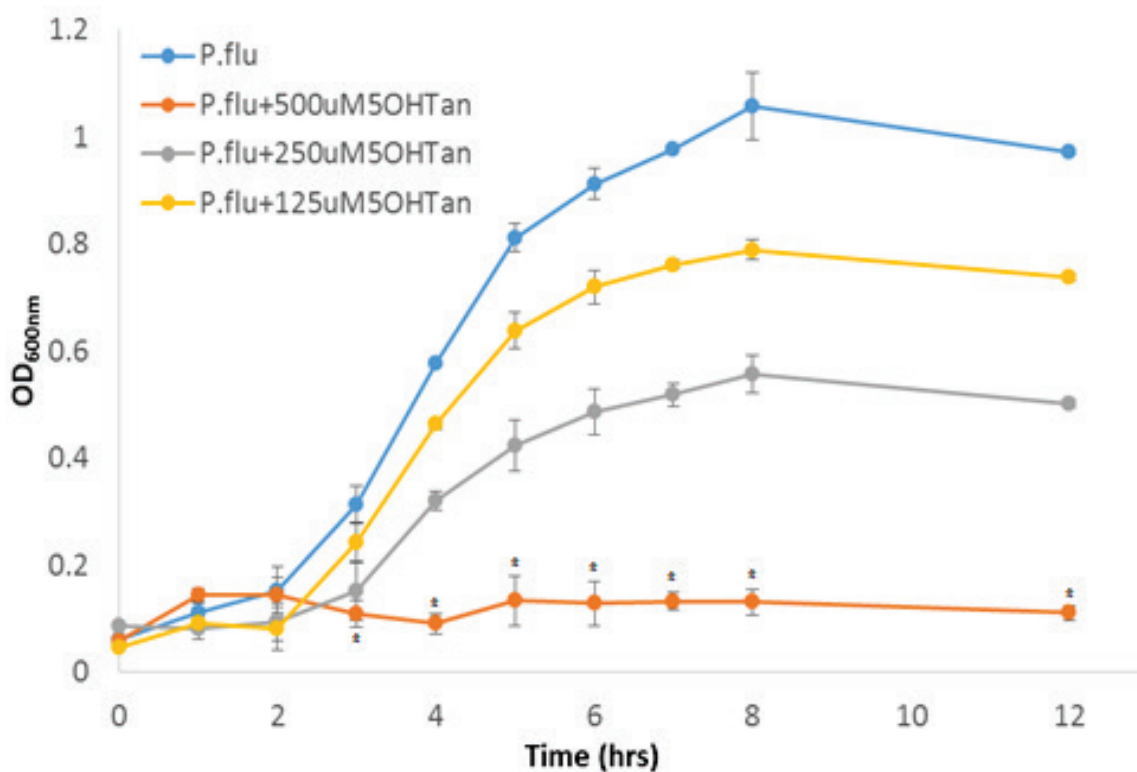
**Figure 19:** Growth curve of *Pseudomonas fluorescens* treated with 5'-OH nobiletin. \* indicates statistically significant.



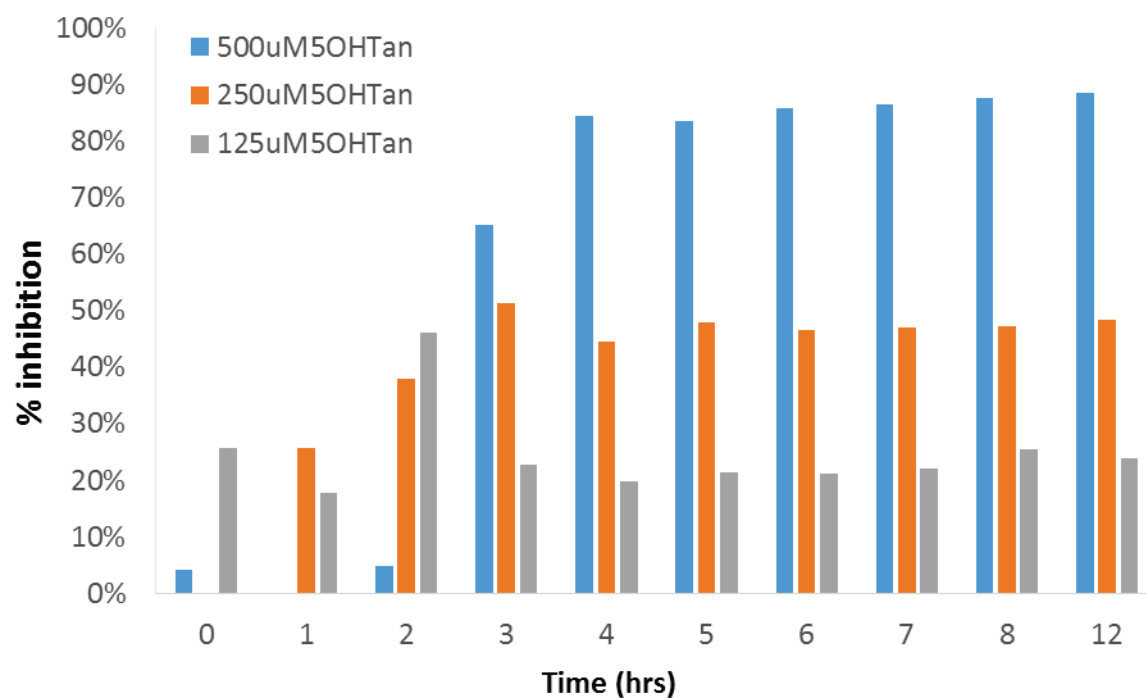
**Figure 20:** Percent inhibition of *Pseudomonas fluorescens* treated with 5'-OH nobiletin.

## 7. 5'OH-tangeretin

The concentration of *P. fluorescens* treated with the 5'OH-tangeretin compared to the bacteria not treated was lower demonstrating the 5'OH-tangeretin antimicrobial effect. *P. fluorescens* treated with 500  $\mu\text{M}$  5'OH-tangeretin the growth was completely inhibited from the third hour and on ( $p < 0.05$ ). *P. fluorescens* treated with 250  $\mu\text{M}$  5'OH-tangeretin showed the growth severely inhibited. *P. fluorescens* treated with 125  $\mu\text{M}$  5'OH-tangeretin showed little inhibition of growth (Figure 21). *P. fluorescens* treated with 500  $\mu\text{M}$  5'OH-tangeretin showed 87.64% growth inhibition. *P. fluorescens* treated with 250  $\mu\text{M}$  5'OH-tangeretin showed 47.37% growth inhibition. *P. fluorescens* treated with 125  $\mu\text{M}$  5'OH-tangeretin showed 25.40% growth inhibition (Figure 22).

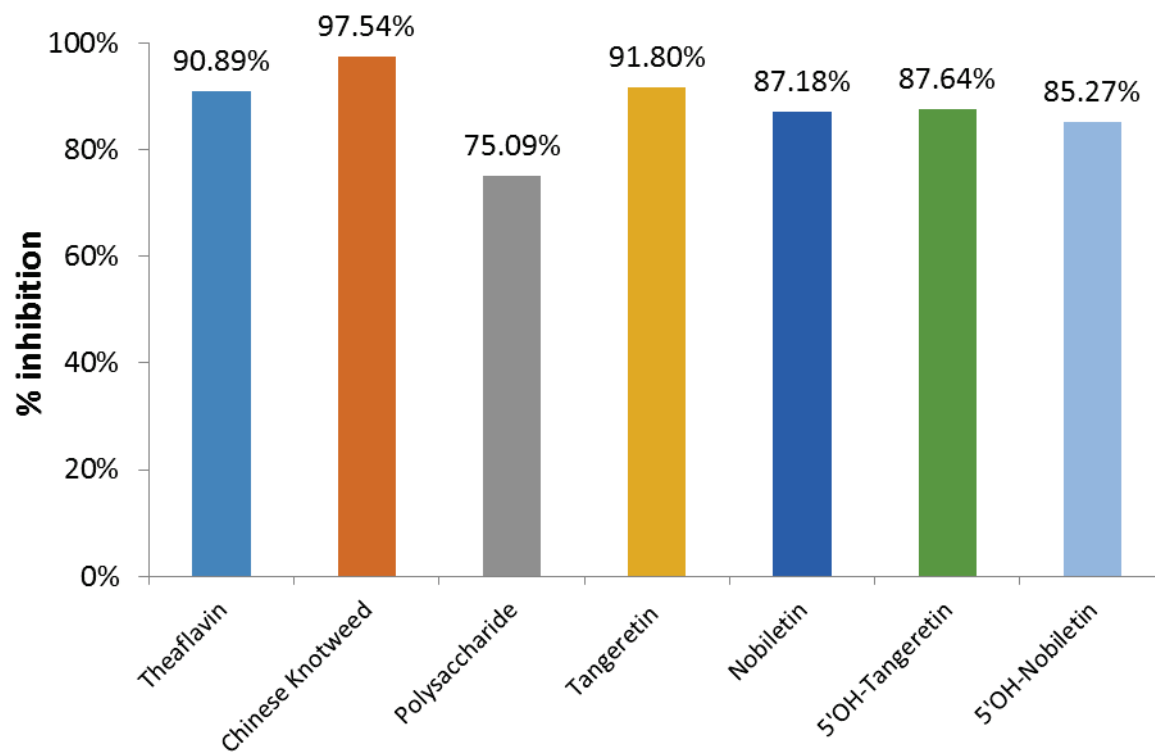


**Figure 21:** Growth curve of *Pseudomonas fluorescens* treated with 5'OH-tangeretin. \* indicates statistically significant.



**Figure 22:** Percent inhibition of *Pseudomonas fluorescens* treated with 5'OH-tangeretin.

The percent inhibition of *P. fluorescens* treated with the natural products at the highest concentration was observed at the 8 hour time point (Figure 23). At 8 hours the bacteria reached stationary phase. The noteworthy antimicrobial effects with a growth inhibition above 50% were compiled to compare the effects on *Pseudomonas* (Table 2).



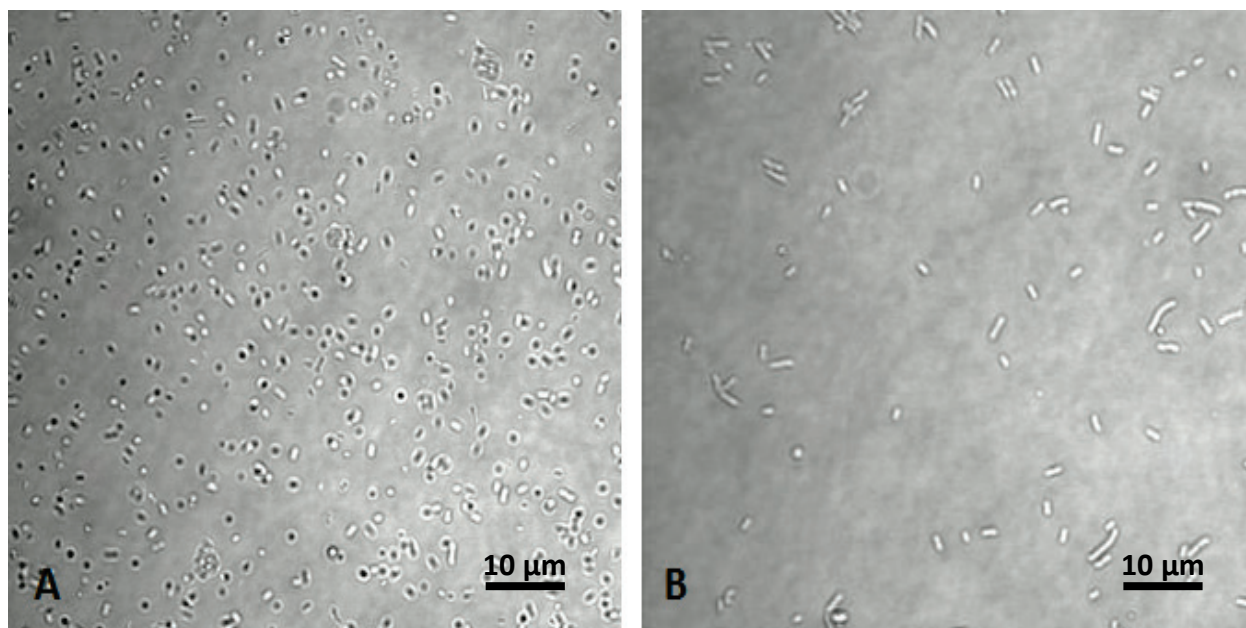
**Figure 23:** Percent inhibition at 8 hours of *P. fluorescens* treated with all seven natural products.

**Table 2:** Summary of the antimicrobial effects of natural products on *Pseudomonas fluorescens*.

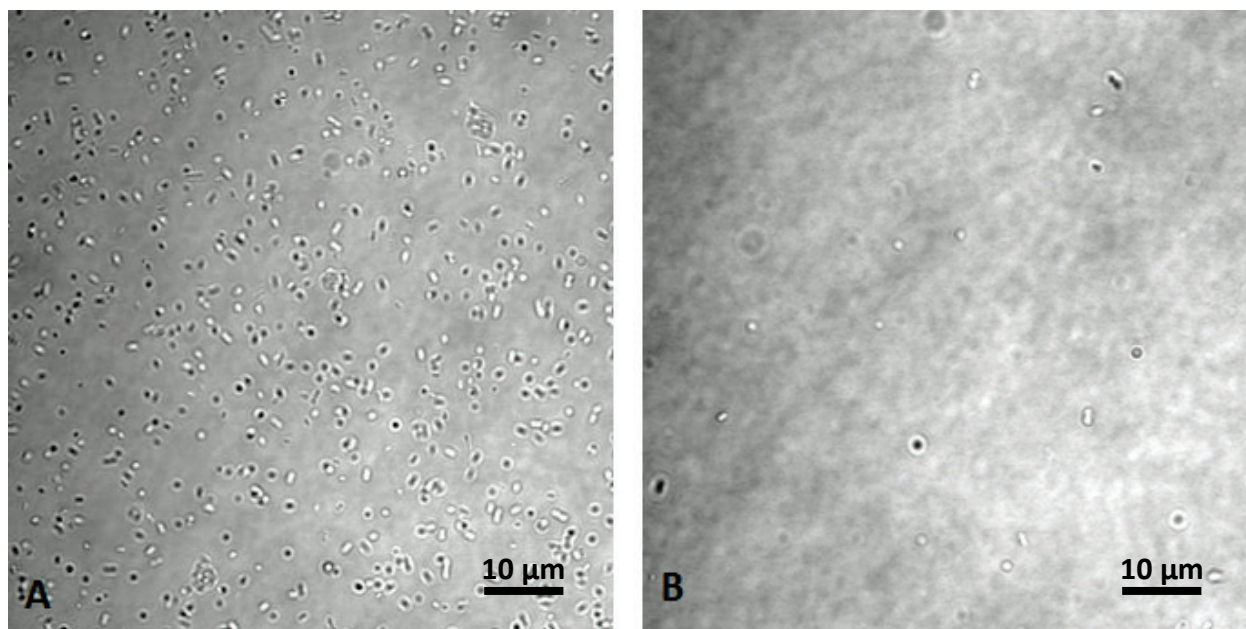
<b>Natural Product</b>	<b>Percent Inhibition</b>	<b>Concentration</b>
Theaflavin ( <i>P. flu</i> )	50.24%	1.25%
	90.89%	2.50%
Theaflavin ( <i>P. aeru</i> )	67.14%	1.25%
	82.76%	2.50%
Chinese Knotweed	63.91%	0.625%
	94.95%	1.25%
	97.54%	2.50%
Red Microalgae Polysaccharide	75.09%	0.5%
Nobiletin	62.90%	250µM
	87.18%	500µM
Tangeretin	53.66%	250µM
	91.8%	500µM
5'OH-Nobiletin	85.27%	500µM
5'OH-Tangeretin	87.64%	500µM

### ***Microscopic Observations***

The antimicrobial effects of the natural products on *P. aeruginosa* were observed using the bright field on an Olympus Fluoview FV1000 microscope. The images were analyzed looked at characteristics such as morphology and cell population (Figure 24-30). *P. aeruginosa* treated with the theaflavin showed a lower cell population in comparison to the untreated bacteria. The morphology of the bacteria also appeared to be elongated. *P. aeruginosa* treated with the Chinese knotweed did not show a morphology change, however the cell population was greatly reduced. *P. aeruginosa* treated with the polysaccharide show a slightly lower cell population. *P. aeruginosa* treated with tangeretin showed a lower cell population in comparison to the untreated. The morphology of the bacteria appeared more elongated. *P. aeruginosa* treated with nobiletin showed a lower cell population in comparison to the untreated. The morphology of the bacteria appeared more elongated. *P. aeruginosa* treated with 5'OH-tangeretin showed a slightly lower cell population in comparison to the untreated. The morphology of the bacteria appeared to be a mixture of the wild type and elongated cells. *P. aeruginosa* treated with 5'OH-nobiletin showed a lower cell population in comparison to the untreated. The morphology of the bacteria appeared more elongated. All images are shown in a total magnification of 600X.

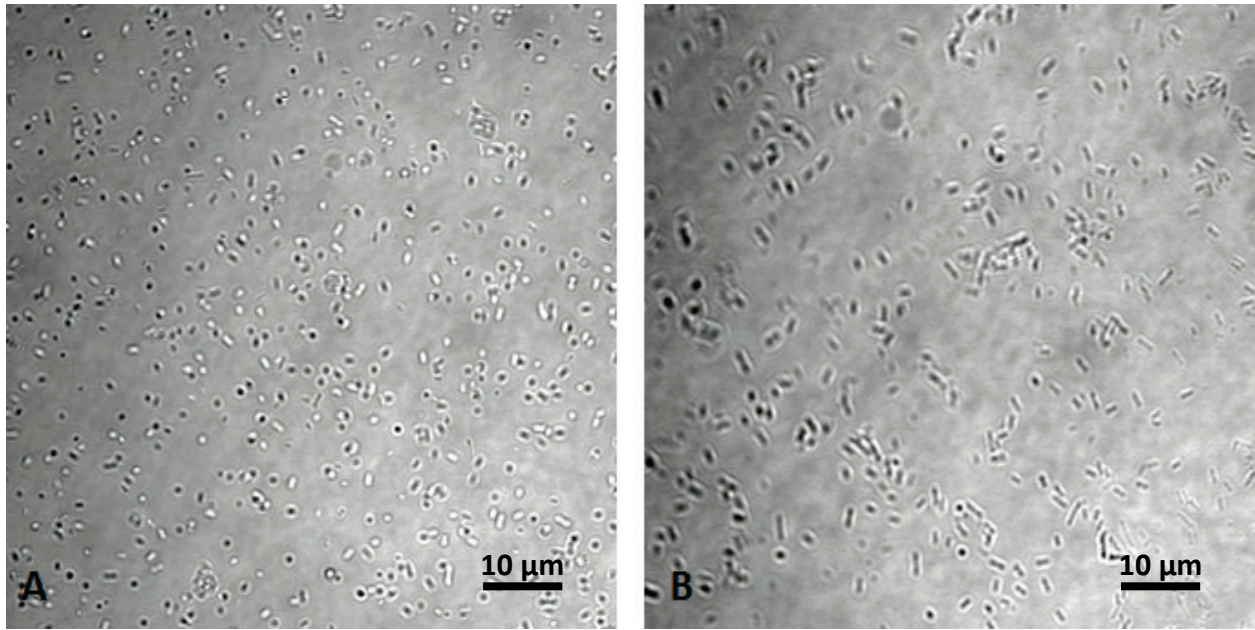


**Figure 24:** *Pseudomonas aeruginosa* untreated (A) and treated with 2.5% theaflavin (B).

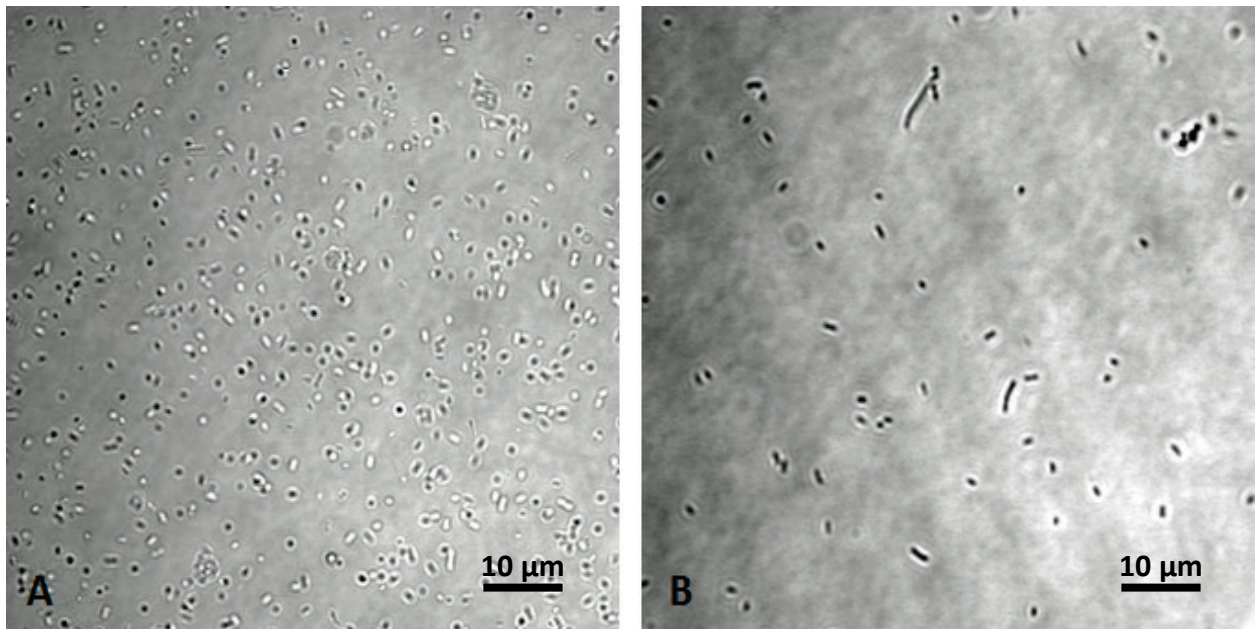


**Figure 25:** *Pseudomonas aeruginosa* untreated (A) and treated with 2.5% Chinese Knotweed (B).

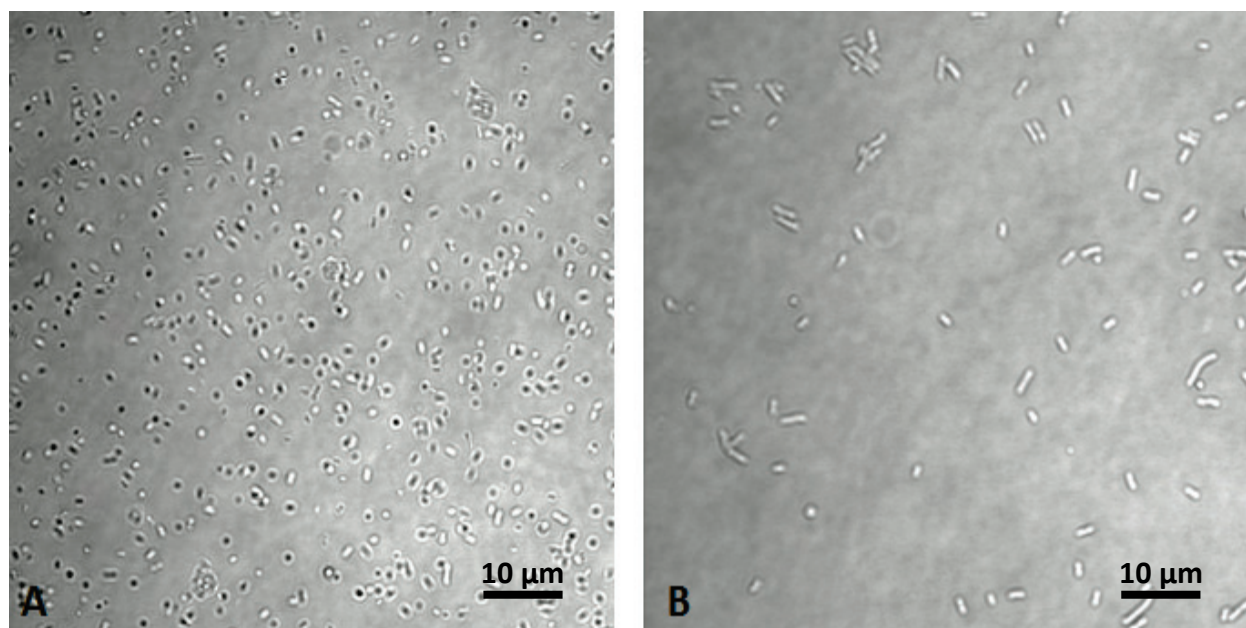




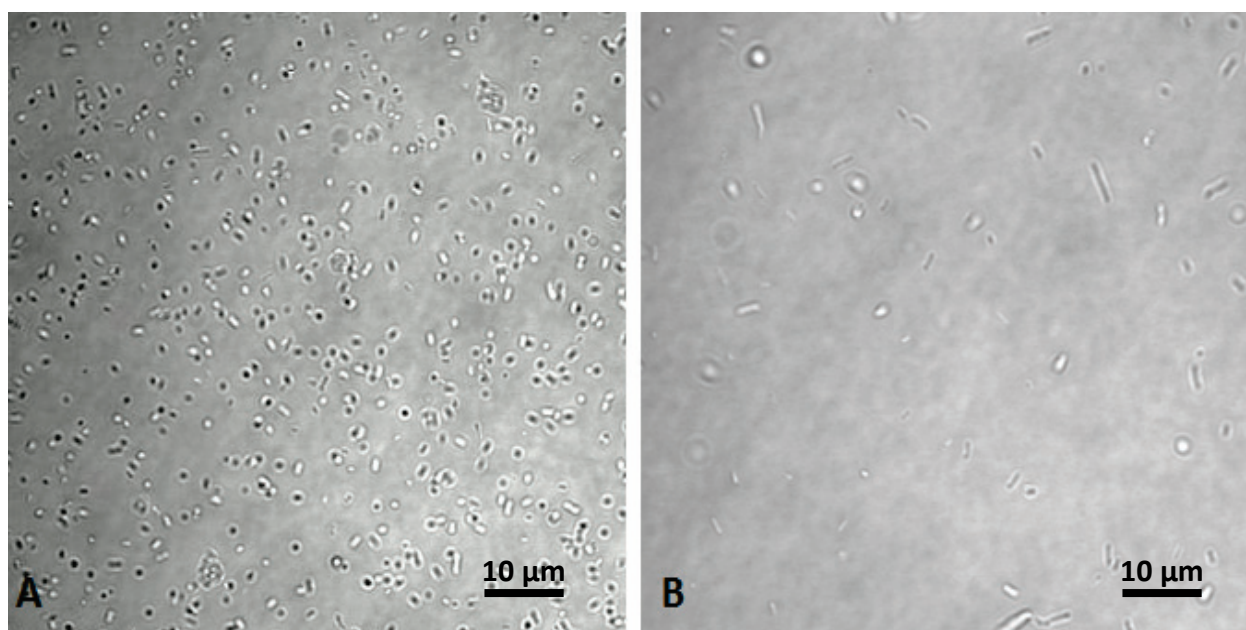
**Figure 26:** *Pseudomonas aeruginosa* untreated (A) and treated with the 0.5% red microalgae polysaccharide (B).



**Figure 27:** *Pseudomonas aeruginosa* untreated (A) and treated with 500  $\mu$ M nobiletin (B).

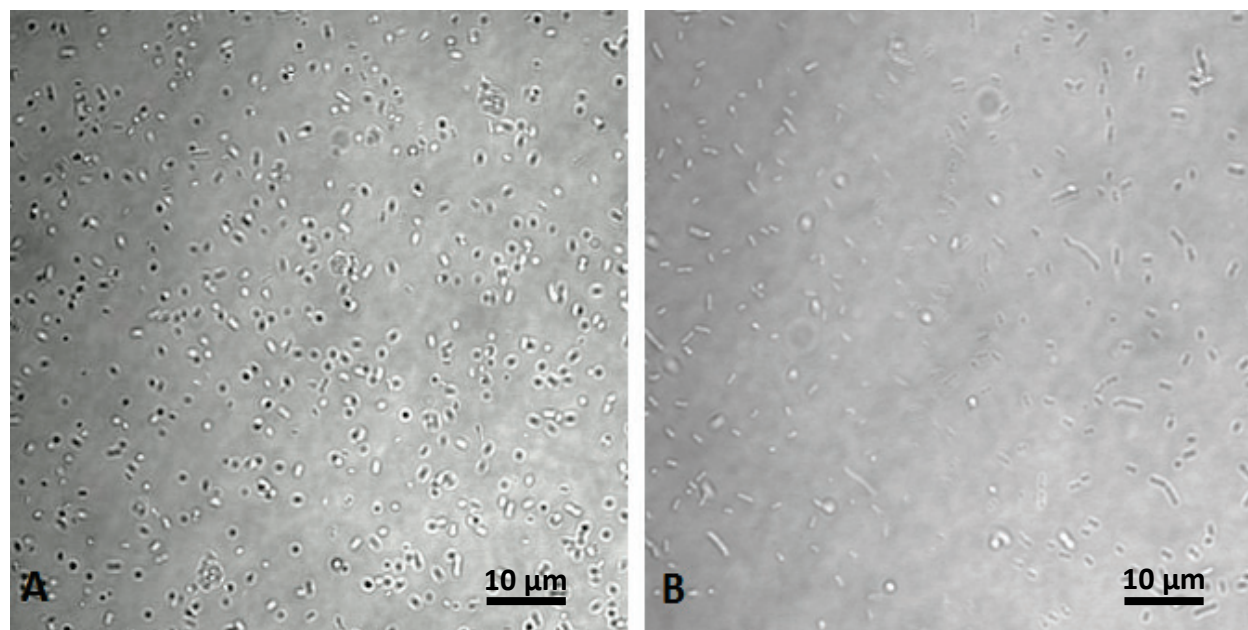


**Figure 28:** *Pseudomonas aeruginosa* untreated (A) and treated with 500  $\mu$ M tangeretin (B).



**Figure 29:** *Pseudomonas aeruginosa* untreated (A) and treated with 500  $\mu$ M 5'OH-nobiletin (B).

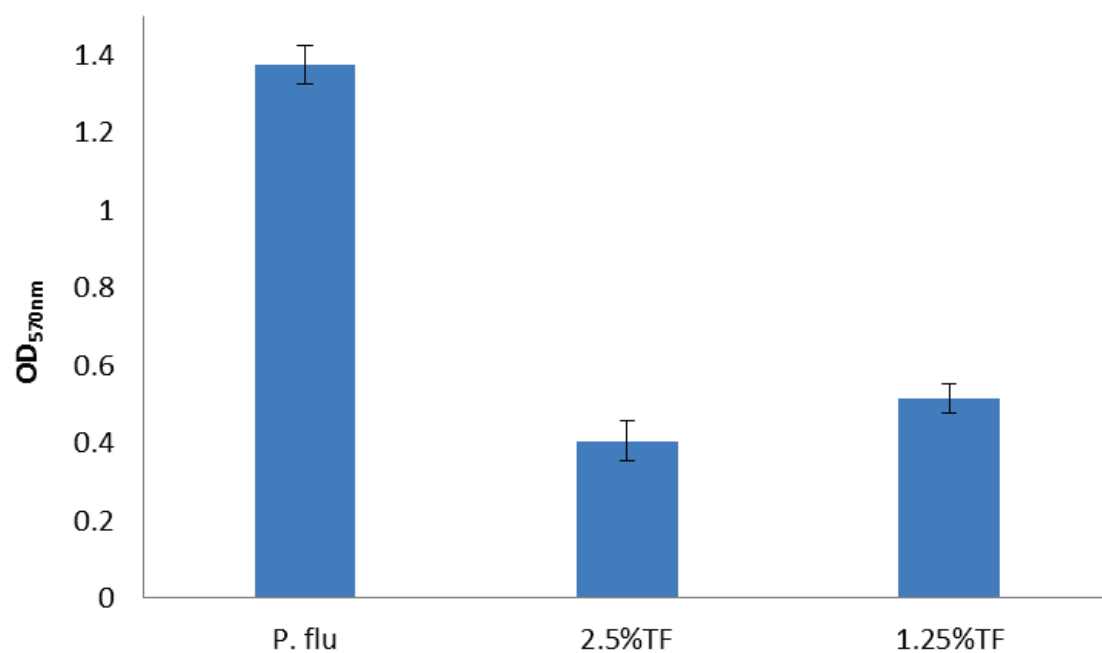




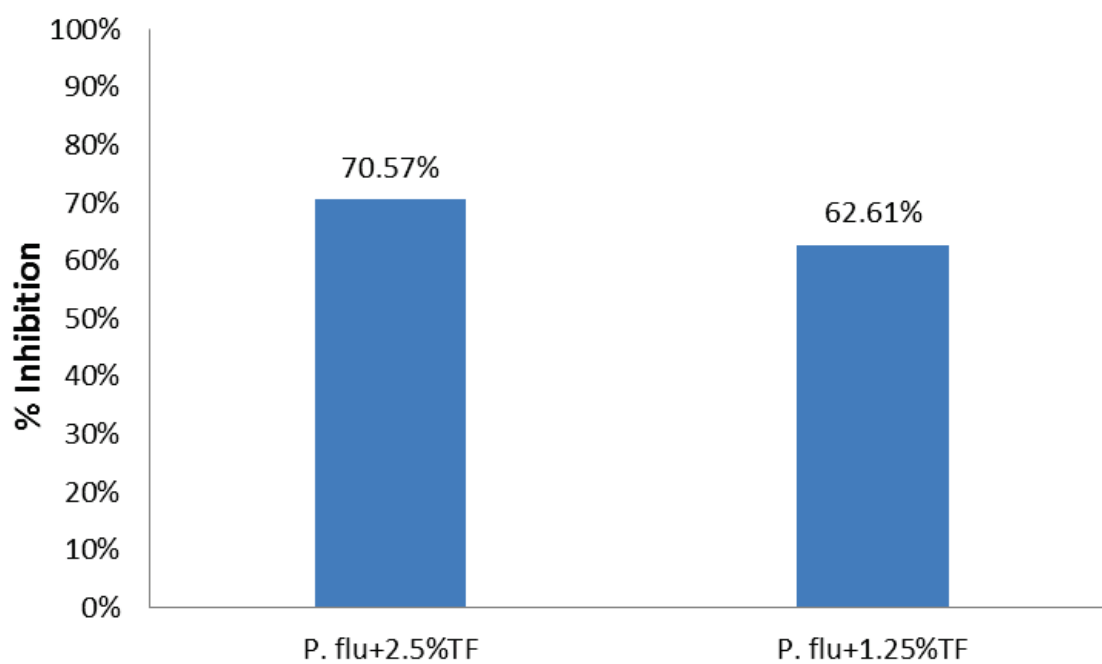
**Figure 30:** *Pseudomonas aeruginosa* untreated (A) and treated with 500 μM 5'OH-tangeretin (B).

### ***Biofilm Inhibition Assay***

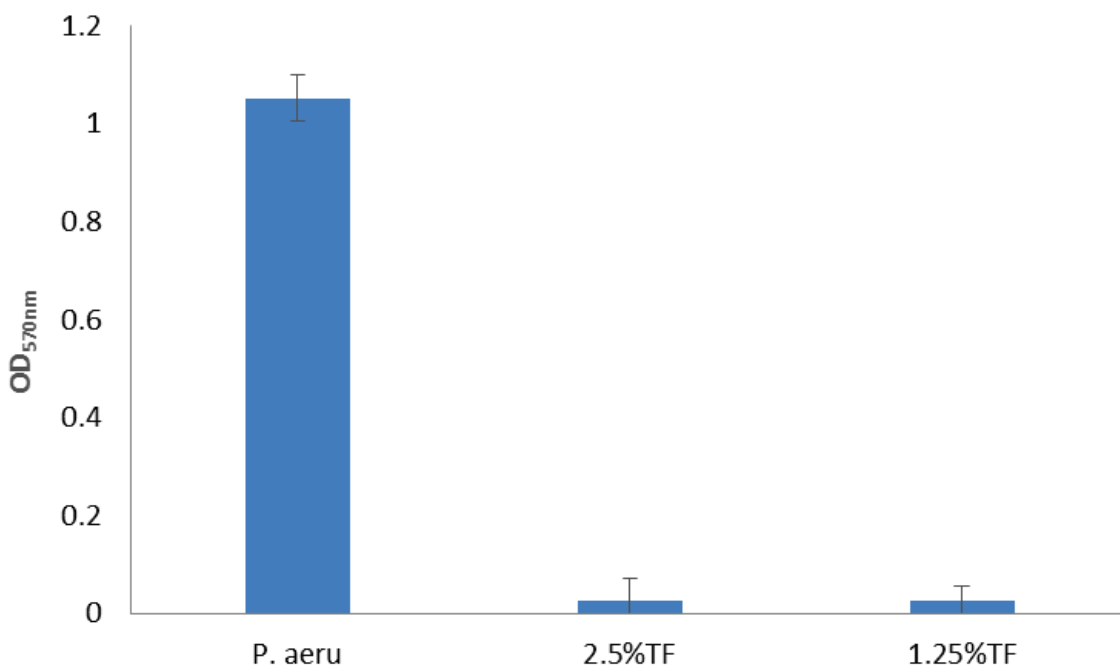
The inhibitory effects of the natural products on the biofilm formation of *P. fluorescens* and *P. aeruginosa* were observed. A crystal violet assay was performed on the 24-well plate to detect the biofilm and obtain visual and quantitative results. The absorbance at 570nm was used to quantitatively analyze biofilm. *P. fluorescens* treated with both 1.25% and 2.5% theaflavin showed a lower amount of biofilm present demonstrating biofilm inhibition. Each concentration had a  $p < 0.05$  meaning there was a statistically significant difference among the groups (Figure 31). *P. fluorescens* treated with 2.5% theaflavin inhibited biofilm formation by 70.57%. *P. fluorescens* treated with 1.25% theaflavin inhibited biofilm formation by 62.61%. *P. aeruginosa* treated with two different concentrations of theaflavin demonstrated a very strong biofilm inhibition. . Each concentration had a  $p < 0.05$  meaning there was a statistically significant difference among the groups (Figure 33). *P. aeruginosa* treated with 2.5% theaflavin inhibited biofilm formation by 97.72%. *P. aeruginosa* treated with 1.25% theaflavin inhibited biofilm formation by 97.50% (Figure 34).



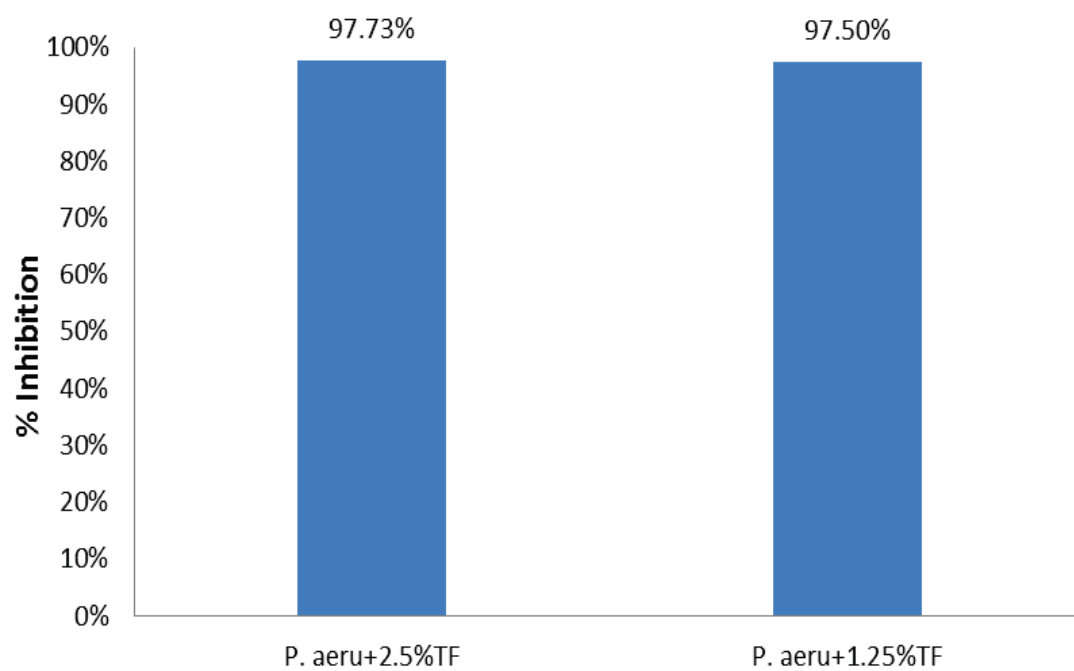
**Figure 31:** Crystal violet assay of *Pseudomonas fluorescens* biofilm treated with theaflavin.



**Figure 32:** Biofilm inhibition of *Pseudomonas fluorescens* treated with theaflavin.

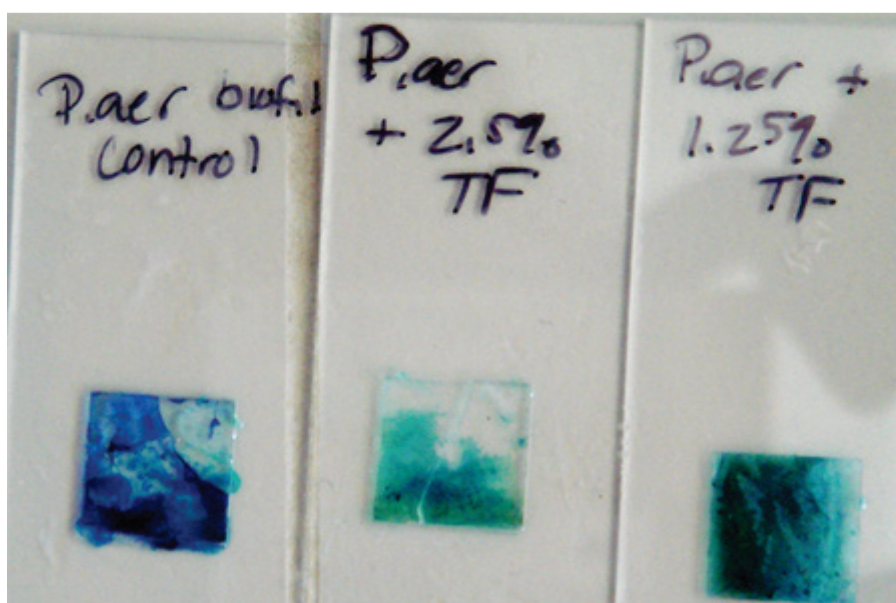


**Figure 33:** Crystal violet assay of *Pseudomonas aeruginosa* biofilm treated with theaflavin.



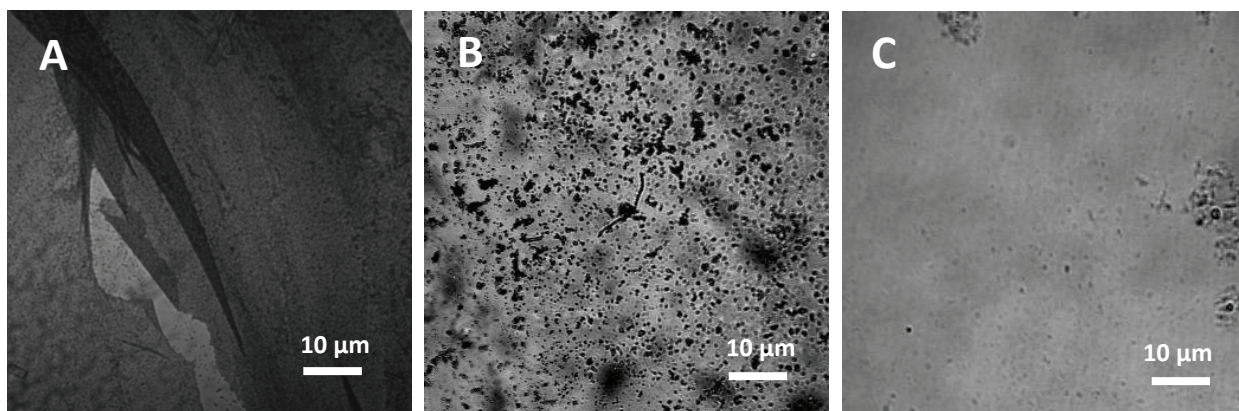
**Figure 34:** Biofilm inhibition of *Pseudomonas aeruginosa* treated with theaflavin.

The inhibitory effects of theaflavin on the biofilm formation of *P. aeruginosa* were observed by using microscopic analysis. An example of the experimental setup is shown in Figure 35. A simple stain with methylene blue was performed on the bacteria biofilm grown on a cover slip and the images were obtained by using the bright field on an Olympus Fluoview FV1000 microscope. *P. aeruginosa* treated with theaflavin showed a lower amount of biofilm present demonstrating biofilm inhibition (Figure 36).



**Figure 35:** *Pseudomonas aeruginosa* biofilm formation experimental setup. (Top) The 24-well plate with glass cover slip positioned perpendicular to the bottom of the well in each well. (Bottom) The coverslips were removed and stained with methylene blue for microscopic observations under total magnification of 600X.





**Figure 36:** Microscope images of *Pseudomonas aeruginosa* biofilm untreated (A) and treated with 1.25% (B) and 2.5% (C) of theaflavin using a cover slip.

## Conclusions and Discussion

In this study, seven natural products were studied to observe the antimicrobial effects on the harmful *Pseudomonas*. The biofilm inhibitory effects were also observed. The products included polyphenolic compounds found in black tea theaflavins, tuberous root *Fallopia multiflorum* (Chinese Knotweed), polysaccharide found in red microalgae, flavonoids found in citrus pith and peels: nobiletin, tangeretin, and their derivatives, 5'-OH-nobiletin and 5'-OH-tangeretin.

The results from antimicrobial assays showed that all selected natural products contain anti-*Pseudomonas* activities as the bacterial growth was severely (if not completely) inhibited. The top three natural products, theaflavins, Chinese Knotweed and Tangeretin, displayed a growth inhibition above 90%. *P. fluorescens* treated with 2.5% theaflavin showed 90.89% growth inhibition. *P. fluorescens* treated with 2.5% Chinese knotweed showed 97.54% growth inhibition. *P. fluorescens* treated with 1.25% Chinese knotweed showed 94.95% growth inhibition. *P. fluorescens* treated with 500  $\mu$ M tangeretin showed 91.80% growth inhibition. This is very interesting because theaflavin and Chinese Knotweed are both polyphenols. From findings, it may be significant to explore the antimicrobial effects of other phenolic constituents in the flavones. *Fallopia multiflorum* (Chinese Knotweed) showed the greatest effect as it completely inhibited *Pseudomonas* growth at both 2.5% and 1.25%. The results encourage us to investigate more natural products used in traditional Chinese medicine. From the microscopic observations, the bacteria population significantly reduced when treated with the compounds in comparison to the control. This correlates with the results obtained from other assays shown above.

Biofilm formation has been significantly reduced or completely inhibited when the bacteria were treated with the natural compounds. Theaflavin showed a greater biofilm inhibition in *P. aeruginosa* than in *P. fluorescens* treated with the theaflavin. Microscopic observations of the biofilms confirmed the results. The biofilm was clearly present for untreated bacteria. With 2.5% theaflavin, *P. fluorescens* showed significant biofilm reduction while *P. aeruginosa* showed no biofilm with a few isolated cells remained. This suggests that theaflavin and other polyphenols may serve as potential anti-*Pseudomonas* biofilm agent.

The infections of gram-negative bacteria *Pseudomonas* has been an ongoing problem in healthcare, especially in the intensive care unit. This study provides an initial profile for several different types of natural products that could be good antibacterial agents for *Pseudomonas* and its biofilms. The synergistic antibacterial activity of natural products and antiseptics should be explored so we may be able to find a natural way to eradicate *Pseudomonas* infection. The results also lead to the need for the discovery of the anti-*Pseudomonas* mechanism of the natural products. The natural products should be tested on other biofilm forming bacteria. We should also explore the possibilities of using the natural products in therapeutic application. To perform this, the natural products should be used on mammalian cells.

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