Fall 12-14-2010

Quorum Sensing Inhibitory Activities of Various Folk-Medicinal Plants and the Thyme-tetracycline Effect.

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QUORUM SENSING INHIBITORY ACTIVITIES OF VARIOUS FOLK MEDICINAL PLANTS AND THE ELUCIDATION OF THE THYME-TETRACYCLINE EFFECT

by

MARIA M. NAGY

Under the Direction of Dr. Sidney A. Crow

ABSTRACT

Pseudomonas aeruginosa is an opportunistic, nosocomial pathogen for which antibiotic resistance and biofilm development is common. Quorum sensing communication is known to be a major controlling factor in virulence gene expression, biofilm development, antibiotic resistance factors, and specifically MexAB-OprM multi-drug efflux pump expression in *P.aeruginosa*. MexAB-OprM efflux pumps contribute to antibiotic resistance of tetracycline and other antibiotics in pseudomonads and other organisms. *P.aeruginosa* infections are problematic in cystic fibrosis and burn patients; it is also the number one causative agent of respiratory infections for intensive care unit patients. Present day antibiotics are losing the battle against these infections. In theory, quorum sensing inhibitors (QSI) reduce pathogencity of the organism; making it less virulent, thus allowing either the host immune system to clear the infection or use of a QSI in combination with an antibiotic to clear more persistent pathogens. For these reasons

two alternative modes of treatment were explored in this study: quorum sensing inhibition by folk-medicinal plant extracts and an example of combination drug therapy, the "thyme-tetracycline effect".

Fifty folk-medicinal plant extracts were screened for potential anti-quorum sensing activity using two quorum sensing inhibition (QSI) reporter strains, *Pseudomonas aeruginosa* QSIS2 and *Chromobacterium violaceum* 12725. These were used to test specifically for C_4-C_6 and C_{12} HSL quorum sensing inhibition. Of the fifty plants tested, thirty plant families were represented. Eleven plant extracts (basil, chaparral, clove, cranberry, oregano, pomegranate, rosemary, sage, sassafras, thyme and witch hazel) showed C_4 HSL quorum sensing inhibition as determined by both assays. Interestingly, five of the plants were from the Lamiaceae family. *Thymus vulgaris* (thyme), also from the Lamiaceae family, was chosen for further assessment.

Previous research has shown that thyme extract can synergistically augment tetracycline activity against tetracycline-resistant *Pseudomonas aeruginosa,* creating the "thyme-tetracycline effect." Disc diffusion assay, thin layer chromatography (TLC), and TLC bioassay techniques were used to show that thymol is the active component in the thyme extract that augments tetracycline activity against resistant *Pseudomonas*. This study also showed that thymol is a potent C_4 HSL quorum sensing inhibitor. The collective data suggests a potential mode of action for the thyme-tetracycline effect: thymol appears to prevent MexAB-OprM efflux pump gene expression. By blocking MexAB-OprM expression, tetracycline antibiotic accumulation can occur within the cell, thus allowing cellular damage.

INDEX WORDS: *Thymus vulgaris*, Quorum sensing inhibition, Medicinal plants, *Pseudomonas aeruginosa*

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A Dissertation Presented in Partial Fulfillment of Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2010

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Electronic Version Approved:

Office of Graduate Studies

College of Arts and Science

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December 2010

ACKNOWLEDGEMENTS

Thank you God!

Thank you! Dr. Crow and Dr. Pascoe. I'd like to thank Dr. Crow for forever being the stern disciplinarian in this process, and Dr. Pascoe for believing in me since day one. I'd also like to thank Dr. Gilbert and Dr. Peirce for participating on my committee.

Thanks of course to my family Mom, Dad, Kati, Peti, Paul, and everyone else that stood by me and supported me emotionally and intellectually on this long arduous journey. Thank you so much for never giving up on me, especially during those times I wanted to give up on myself. I am very blessed.

Thank you!

***

I'd also like to acknowledge others that have been critical to my success. First, the 4905 Biology classes at Georgia State University that helped pick, prepare and test many of the medicinal plant extracts discussed in this study. Without them I wouldn't have the samples. I'd also like to thank Brandi and Jeannie for proof reading my work, and Bryan Stubblefield and Amro for constantly giving me great ideas at various wee hours of the night.

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LIST OF ABBREVIATIONS

Acyl homoserine lactone	AHL
Disc diffusion assay	DDA
Infectious Disease Society of America	IDSA
Luria-Bertani	LB
Methicillin resistant *Staphylococcus aureus*	MRSA
Mueller Hinton II	MH
N-(butanoyl)--L-homoserine lactone	C_4 HSL
N-(3-oxododecanyoyl)-L-homoserine lactone	C_{12} HSL
N-hexanoyl-L-homoserine lactone	C_6 HSL
Nutrient broth	NB
Office of Technical Assessment	OTA
Quorum sensing	QS
Quorum-sensing inhibition	QSI
QSIS2 reporter strain	QSIS2
Reference factor	RF
Rosmarinic acid	RA
Thin-layer chromatography	TLC
TLC bioassay	TSA
Tetracycline	TET
Tryptic soy agar	TSA
World Health Organization	WHO

INTRODUCTION

Infectious Disease

Infectious diseases are defined by the World Health Organization (WHO) as diseases caused by microbes; these microbes may include bacteria, fungi, protozoa, and viruses (WHO, 2010). These organisms may be found in either the environment or participate in normal commensal flora for humans, plants, or animals. When in their natural environment, these microbes are typically kept in balance by the surrounding flora. Thus, many of these organisms can be beneficial to their environment by providing nutrient turnover; but when certain bacteria are introduced into a foreign niche within the human body, they may cause disease. Diseases caused by bacterial infections can range from severe to mild and may include wound infections, pneumonia, septicemia, endocarditis, colds, and eye and ear infections (Todar, 2008).

Prior to the discovery of penicillin antibiotics, doctors were unable to treat even relatively simple bacterial infections such as otitis media (inner ear infection) and streptococcal infection of the throat (Office of Technical Assessment [OTA], 1995). Doctors were instructed to keep the infected patient clean and comfortable until the immune system could clear the infection; more serious infections, however, were incurable (OTA, 1995). The treatment of bacterial infections was revolutionized in 1929, when Alexander Fleming discovered penicillin. For several years, antibiotics were thought to be the "end-all" curative agent for many bacterial infections and were considered to be the wonder drug of the era. Penicillin was used to treat infections caused by *Staphylococci, Streptococci* and other Gram-positive organisms (OTA, 1995). On August 14, 1944, Schenley Laboratories unveiled an advertisement campaign in *Life* magazine (Figure 1) that suggested the revolutionary usefulness of penicillin on the battle field (Levy, 2002).

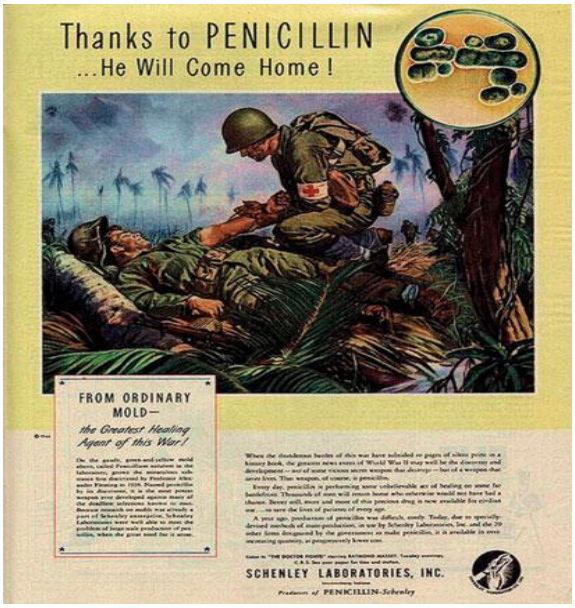


Figure 1: *Life* magazine--Advertisement for penicillin (1944).
ncmuseumofhistory.org/.../topic/16/

However, by 1945 penicillin-resistant strains of *Staphylococcus aureus* were being isolated in hospitals. Methicillin, a semi-synthetic version of penicillin, was introduced in 1959 in hopes of combating these resistant strains. Only one year later, the first isolates of methicillin-resistant strains of *S. aureus* (MRSA) were being found in hospitals in the United States. Vancomycin was introduced in 1956 to treat MRSA (OTA, 1995). The first case of vancomycin resistance was seen in Japan in 1996 (CDC, 2002).

In 2001, WHO officially deemed antibiotic resistance the number three public health concern of the 21st century (Levy, 2002). In April of 2010, the "10 x'20" initiative was launched by the Infectious Disease Society of America (IDSA) to assist in the development of 10 new antibiotic drugs by the end of 2020. The focus of this drug development initiative is to target specific infectious agents deemed "ESKAPE" pathogens. These organisms include: ***E****nterococcus faecium,* ***S****taphylococcus aureus,* ***K****lebsiella pneumoniae,* ***A****cinetobacter baumamnii,* ***P****seudomonas aeruginosa* and ***E****nterobacter species,* and represent the most

prominent causes of hospital-acquired infections today. "ESKAPE" also refers to the ability of these organisms to "escape" present-day antimicrobial treatments (IDSA, 2010). Clearly, exploration of new and alternative drug treatments for infectious diseases is vital.

One particularly troubling organism is *Pseudomonas aeruginosa;* it is a nosocomial, or hospital-acquired, opportunistic pathogen. It is the number one infectious agent causing respiratory infections in people with extended hospital stays (Driscoll et al., 2007).

Pseudomonas aeruginosa

Pseudomonas aeruginosa (Figure 2) is a Gram-negative rod that is propelled by a single flagellum (Todar, 2008). *Pseudomonas* is a genus within the medically-relevant class of Gammaproteobacteria, and belongs to the family Pseudomonadaceae. There are eight genera in the family Pseudomonadaceae and twelve species in the genus *Pseudomonas*. *P. aeruginosa,* native to both soil and water, is also a well-known plant pathogen (Todar, 2008). As a human pathogen, opportunistic infections by this organism can occur when the host immune system is previously weakened due to a prior ailment-such as AIDS, cancer, cystic fibrosis, transplantation, or substantial burns (Driscoll et al., 2007). In these scenarios, pseudomonad's have the ability to infect any tissue where the defenses of the host have been compromised (Todar, 2008). While *P. aeruginosa* infections are most commonly associated with pneumonia in cystic fibrosis patients and wound infections of burn patients, it is also the causative agent of several other illnesses including urinary tract infections, endocarditis, bactermia (Todar, 2008), ulcerative keratitis (in contact lens wearers) and otitis externa (in diabetics) (Driscoll et al., 2007). *P. aeruginosa* is also estimated to be the causative agent in 11%-13% of all nosocomial infections, in 13%-22% of infections in intensive care units (ICUs), and in 16% of all surgical

site infections (Driscoll et al., 2007). Furthermore, it is the second-most common cause of health-associated and ventilator-associated pneumonia (Driscoll et al., 2007).

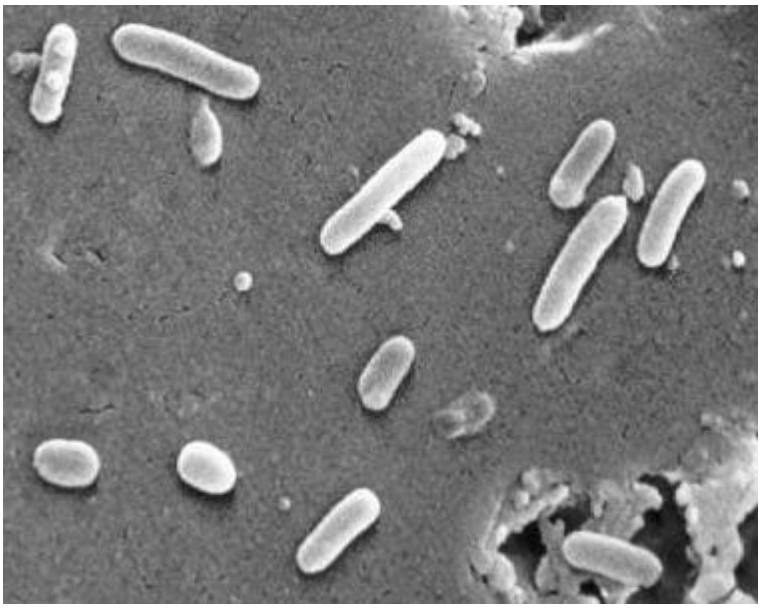


Figure 2: Scanning electron micrograph of *Pseudomonas aeruginosa*-(CDC).
(Todar, 2008)

Virulence

When *P. aeruginosa* infects the host, it expresses a myriad of virulence factors that allow it to attack the host immune system while evading treatment. An intrinsic defensive feature of this organism is its outer phospholipid membrane which has limited permeability for most molecules.

Pseudomonads also secrete a number of compounds that interfere with host defenses including enzymes, exotoxins, and pyocyanin. Enzymes, such as alkaline protease, elastase, and other proteases, aid in degrading host proteins Exotoxins, including Exotoxin A, interfere with

protein synthesis and can even cause cellular death. Pyocyanin is a blue-green pigment which is bactericidal against competing organisms. In the lungs, pyocyanin can also disrupt ciliary action and cause inflammation (Driscoll et al., 2007).

Another virulence factor that can be expressed in *Pseudomonas* is the type III secretion apparatus. Expression of the genes encoding the various parts of the secretion system is seen predominantly in acute or invasive infections more than in chronic infections (Driscoll et al., 2007). This mechanism is a "contact-dependent" system that is triggered by the interaction of *P. aeruginosa* with the host cell. The secretion apparatus allows the cell to inject exoenzymes directly into the host cell. There are four known exoenzymes that are typically expressed by *P. aeruginosa*: Exo S, Exo T, Exo U, and Exo Y (Driscoll et al., 2007). These exoenzymes can damage the host's cellular machinery and even cause cell death (Driscoll et al., 2007).

In addition to the impermeability of the cellular membrane and the secretion apparatus, *Pseudomonas* also has another mechanism of protection. If a damaging molecule does gain entry into the cell, then unique pumps quickly expel the compounds from the cell before any damage can occur. These efflux pumps can remove antibiotics, dyes, detergents, solvents and other compounds from the cell before they can cause harm (Driscoll et al., 2007). There are two types of efflux pumps that are seen in bacteria: those which are encoded in the chromosome and those which are plasmid-borne. For example, the MexAB-OmpM efflux pumps found in *P. aeruginosa* are intrinsic to the genome. Plasmid-borne antibiotic resistance genes that code for efflux pumps can be located on specialty plasmids, such as tetracycline-resistant (Tc) plasmids. These plasmids are typically transferred through conjugation between bacteria and can encode for multi-drug resistance pumps (Chopra and Roberts, 2001).

Biofilms

A final mechanism for protection from the host immune system may be incurred through biofilm formation. It well known that bacteria grow and develop into "tight-knit" communities on biotic and abiotic surfaces. The innate features of a biofilm can protect bacteria from antibiotic damage. Biofilms are so efficient in preventing antibiotic damage that concentrations of antibiotics must be increased by 100-1000--fold to be effective (Costerton et al., 1994).

Biofilms (Figure 3-A) typically consist of microbial communities that secrete and are encased in a thick exopolysaccharide matrix. There are special features associated with most biofilms that allow for increased efficiency of these communities. These particular morphological characteristics include: an attachment surface, mushroom-like clusters of bacterial cells, a thick polysaccharide matrix encasing the bacterial communities; extensive well-hydrated channels that allow for directed liquid flow (providing delivery and removal of nutrients and waste products); and swarmer cells (Figure 3-B). Swarmer cells are cells that easily break off from the mushroom structure, and are thus able to relocate and re-populate a new location (Costerton et al., 1994).

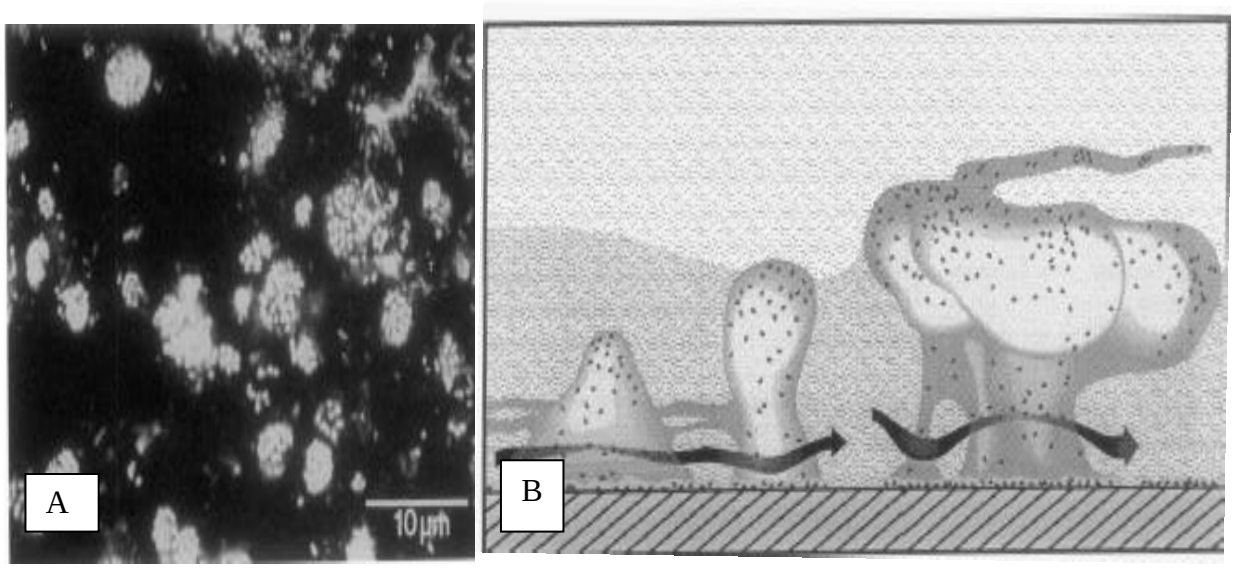


Figure 3: Biofilms. A) Mixed colony biofilm from Bow River Alberta, Canada; B) Diagram of a biofilm: Notice the following features: mushroom structures, thick polysaccharide matrix, excessive channels, well hydrated channels, directed flow (allowing for delivery of nutrients and removal of waste products and swarmer cells. (Costerton et al., 1994)

Medical devices, such as internal catheters, artificial joints, and heart value replacement units, have become commonplace in medicine. While these advances have extended the lives of many, they have also ironically brought about subsequent problems. The number of people that are in extended "immuno-compromised" states has greatly increased. Unfortunately, medical devices provide the perfect breeding ground for the development of opportunistic bacterial and biofilm infections, and for the proliferation of resistant bacteria (Reid, 1999).

As of 1999, catheter and urinary stent usage occurred in over 100 million patients per year (Reid, 1999). Sixty percent of all hospital-acquired microbial infections are caused by biofilms (Lewis, 2001). Without the use of prophylactic antibiotic treatments, the infection rate for urethral stents is 28% and is nearly 100% with catheter usage. For this reason, prophylactic antibiotic treatments are administered to patients to reduce infection rates (Reid, 1999).

There are many features of a catheter that promote bacterial growth. These features include liquid flow, nutrient access, attachment surface, and the occasional planktonic organism, all

of which are readily associated with biofilm development. Because of this, biofilm infections are prominent in catheter-type environments. If a biofilm does develop, then it requires an antibiotic dosage of a 100-1000 times greater concentration of antibiotics than with non-biofilm bacterial infections. The excessive use of these antibiotic treatments increases the development of resistant bacteria, thus reducing the overall effectiveness of available antibiotics (Costerton, 1999).

Many characteristics of biofilms contribute to the difficulty in treating these types of infections with normal antibiotic doses. First, the exopolysaccharide matrix produces a physical barrier, which reduces the amount of antibiotic that can enter into this microbial community. Second, even if antibiotic treatments infiltrate the exopolysaccharide matrix, the antibiotic may still have difficultly accessing the internal cells of the mushroom-like structures. Third, during the formation of a biofilm, specialized virulence gene expression can occur. These virulence factors include the expression of antibiotic denaturing enzymes, efflux pump, and increased plasmid exchange. The development and expression of many of these virulence features is typically under quorum sensing control (Bassler, 1999; Camara et al., 2002; Costerton, 1999; Lewis, 2001).

Quorum Sensing

Quorum sensing (QS) is a population-dependent expression of genes that influences biofilm development, efflux pump expression, toxin production, and many other virulence factors. Quorum sensing occurs through chemical signaling and has been observed in bacteria, fungi, and even plants. Individual cells in an environment constantly express and expel low levels of quorum sensing molecules. It is only when the cell population reaches a certain concentration that the threshold gradient is achieved; these signal molecules diffuse back into the cell and bind to a transcription regulator. This binding triggers the expression of QS-mediated genes, and the entire

population thus responds in a joint expression or repression of a multitude of genes. It has been shown that many organisms have variations on this quorum sensing theme (Bassler, 1999; Camara et al., 2002; Hogan et al., 2004; Otto, 2004). Quorum sensing responses may result from intra-species or interspecies communication. In this way, bacteria within a given area can attack the host as unified army (Bassler, 1999).

While the basics are similar, there are some differences with Gram-positive quorum sensing. First Gram-positive organisms use small peptide units called pheromones as their QS molecules. They also use transmembrane proteins and they utilize a two- component signal transduction system in order to trigger QS-mediated gene expression. For example, in *Staphylococcus aureus* QS communication is encoded on the agrACDB operon. First the A*gr D* gene product produces a nonreactive oligopeptide, which later becomes cleaved and a small peptide unit is exported from the cell by the AgrB ATP binding cassette transmembrane protein. When the concentration of these newly expelled pheromone units reach quorum levels, they eventually bind to a second transmembrane protein, AgrC. Next, the AgrC protein; can autophophorylate the transcription regulator, AgrA. Lastly, the phosphorylated AgrA will bind to the P3 promoter sites of the agrACDB operon triggering the expression of RNA III. RNA III will up regulate or down regulate the expression of many quorum sensing mediated genes such as surface proteins-fibronectin-binding protein, coagulase, toxin expression-enteroxin (TSS), and toxic shock syndrome toxin (TSST-1) (Camara et al., 2002; Otto, 2004).

One of the QS motifs found in Gram-negative bacteria is the Lux I/R system. An autoinducer synthase, Lux I, is responsible for manufacturing QS molecules called acyl homoserine lactone (AHL) derivatives. Acyl homoserine lactones are lactone rings with a carbonyl tail (C_4-C_{16}) with varying functional lengths. QS molecules are excreted into the extra-cellular environment.

When the organisms grow to sufficient density, the concentration of the AHL reaches a critical value; these compounds diffuse back down their concentration gradient and into the cell, binding to an autoinducer regulator compound, Lux R. This triggers the expression of quorum sensing controlled genes. One main difference between the *Lux R/I* system and other QS systems is that transmembrane proteins are not used predominantly because acyl homoserine lactone units tend to be small carbon (C_4-C_6) units (Bassler, 1999).

In *Pseudomonas*, the quorum sensing mechanism consists of two main cascading regulatory systems *Las I/R* and *Rhl I/R* (Figure 4) and varying size acyl-homoserine lactone units (Bassler, 1999). There is a low level constitutive expression of the *Las I* gene product, *N*-(3-oxododecanoyl) homoserine lactone (3-oxo-C_{12}-HSL) (Figure 5). When the 3-oxo-C_{12}-HSL signal molecule binds to the Las R regulator protein, two functions are carried out. First, it triggers modulation of QS-regulated genes including genes associated with exoenzymes- elastase, alkaline and acid proteases, exotoxin A, secretion apparatus (Xcp) and biofilm development. Second, it also triggers the cascading regulatory expression of *Rhl I/R* genes. *Rhl I/R* gene products control the expression of a number of secondary genes including regions that code for: elastase, lectins, hydrogen cyanide, rhamnolipids and siderophores. Expression of these genes occurs when the gene product of *Rhl I*, N-(butanoyl)-L- homoserine lactone (C_4-HSL) (Figure 6) is excreted into the environment; also reaching sufficient levels, and diffusing back into the cell thus binding to the Rhl R regulatory protein (Camara et al., 2002).

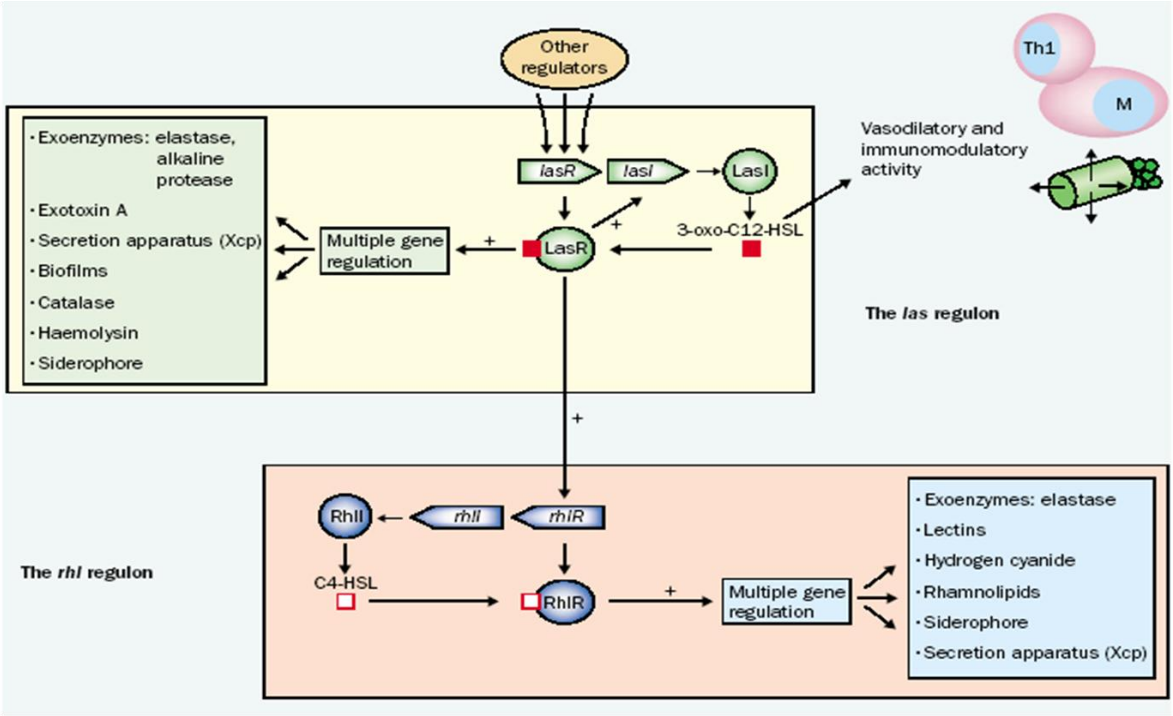


Figure 4: Quorum Sensing cascading system in *Pseudomonas aeruginosa.*
(Camara et al., 2002)

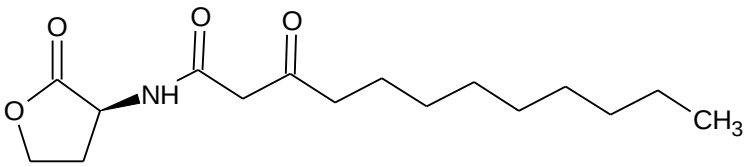


Figure 5: *N*-(3-oxododecanoyl) homoserine lactone. (3-oxo-C_{12}-HSL).

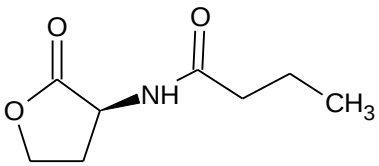


Figure 6: *N*-(butanoyl)-L-homoserine lactone. (C_4-HSL).

Prevention and Treatment

Complete eradication of *P. aeruginosa* in hospitals is likely unattainable, since *Pseudomonas* species can be found growing in every conceivable reservoir in the hospital environment. Reduction is the first line of defense when it comes to infections in hospitals. Reducing nosocomial infection rates can be achieved thru methods such as: proper disinfecting, aseptic techniques and monitoring of patient respirators, ventilators, and other equipment (Driscoll et al., 2007). However if an infection does occur, antibiotic treatment is needed. *P. aeruginosa* infections are typically treated with-aminoglycosides antibiotics such as: gentamicin, tobramycin, monobactams, and some flouroquinolones antibiotics.

Antibiotics

In general there are five major groups of antibiotics used to treat most bacterial infections: β-lactams, sulfonamides, streptomycin, chloramphenicol, and tetracyclines. There are three major sources from which these well known antibiotics are derived. These are the molds *Penicillium* and *Cephalosporium*, the Actinomycetes, such as *Streptomyces spp.*, and the Gram-positive-*Bacillus spp.* *Penicillium* and *Cephalosporium* molds produce the β-lactam antibiotics such as: penicillin, cephalosporin; semi-synthetic versions such as amoxicillin and ampicillin are also available. These drugs interfere with cell wall development.

Actinomycetes, such as *Streptomyces*, are the source for: the aminoglycosides, macrolides, tetracyclines and chloramphenicol. These antibiotics interfere with ribosome or protein synthesis in the target bacteria. *Bacillus spp.* produces polypeptide antibiotics such as polymixins, and bacitracin. Polymixin disrupts phospholipid membrane function, while bacitracin affects cell wall growth (Todar, 2008).

Antibiotic Resistance

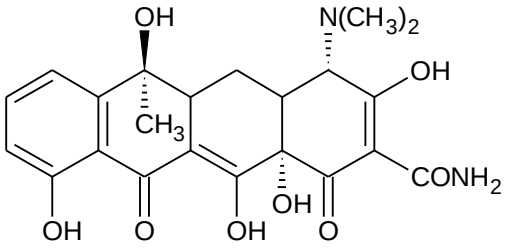
Antibiotic resistance is a major problem encountered by physicians treating bacterial infections. There are some antibiotics to which pseudomonads are naturally resistant. These include: macrolides, β-lactams, tetracyclines and some fluoroquinolones (Driscoll et al., 2007). Pseudomonads, as well as other Gram-negative organisms' posse several mechanisms which promote antimicrobial resistance to common antibiotics. First, they have an outer phospholipid membrane that creates a selective barrier, which limits uptake into the cell. Next, many bacteria can express a number of proteins that can either degrade or expel the antibiotic from the cell. For instance, efflux pumps can quickly expel a number of compounds from the cell before they can cause injury to the cell's machinery. Third, many bacteria quickly develop into biofilm communities. These biofilms are quite resistant to antibiotic treatment. Lastly, resistance by bacteria is also easily gained by conjugation and plasmid transfer of antibiotic resistance genes between organisms. It is through these mechanisms that many bacteria can circumvent antibiotic treatments. For instance, *Pseudomonas aeruginosa* is naturally resistant to tetracycline antibiotics.

Tetracycline

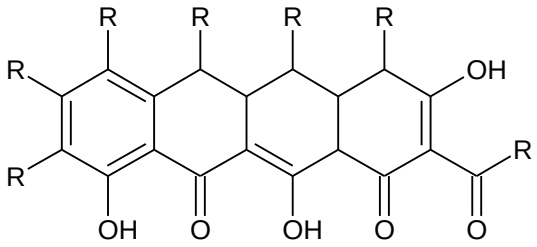
The tetracycline family of antibiotics was first discovered in the 1940's and originally isolated from *Streptomyces.* They are broad-spectrum antibiotics that are effective against both Gram-positive and Gram-negative bacteria. They are also used to treat chlamydia, rickettsia, mycoplasms and some protozoa. Tetracycline (Figure 7-A) disables protein synthesis in a cell by binding to the ribosomal acceptor (A) site of the aminoacyl t-RNA (Chopra and Roberts,

2001). The family of tetracycline antibiotics includes: chlorotetracycline, oxytetracycline, tetracycline, demethylchlorotetracycline, and minocycline.

The tetracycline scaffolding (Figure 7-B) has four 6-carbon rings designated as A, B, C and D, with varying functional groups. 6-deoxy-6-demethyltetracycline (Figure 7-C) is the simplest known tetracycline to show antimicrobial activity. From this scaffolding, functional side groups may be added and removed to augment activity and reduce toxicity (Chopra and Roberts, 2001).



A.



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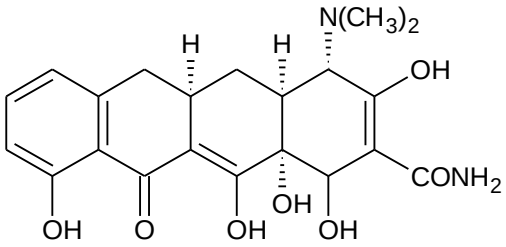


C.

Figure 7: Tetracycline structures. A) Tetracycline B) Tetracycline Scaffolding, R indicates locations where functional groups can be added and removed to augment activity, C) 6-deoxy-6-demethyltetracycline, the minimum tetracycline structure which shows antibiotic activity,

In Gram-negative organisms, tetracyclines are strong chelating agents; prior to entering the cell they bind to positively charged cations such as magnesium (Mg^{+}). This tetracycline complex gains entry into the cell via OmpC porin channels and accumulates in the periplasmic space where it dissociates from the cation. Tetracycline is mildly lipophilic so it can easily diffuse through the bi-lipid membrane and gain entry into the cytoplasm of the cell. In Gram-positive organisms, tetracycline takes advantage of the proton motive force and it is thrust through the cytoplasmic membrane, where it will bind another magnesium ion. The complex then settles into the A site of the of the 30S ribosomal subunit. This binding of tetracycline prevents normal binding of aminoacyl t-RNA to the A site of the ribosome and thus disrupting normal protein synthesis within the cell (Chopra and Roberts, 2001).

Intrinsic Expression of Efflux Pumps

Bacteria can intrinsically express a number of multidrug resistant (MDR) efflux pumps. These efflux pumps are made up of three protein subunits, which each function to bridge different sections of the phospholipid bilayer of the bacterial membrane. In Gram-negative organisms; there are two phospholipid bilayers (outer and inner membranes) that are located on either side of the periplasmic space. The outer membrane factor (OMP) crosses the outer membrane; the periplasmic membrane fusion protein (MFP) covers the periplasmic space. Lastly, proteins called the Resistance–Nodulation-Division proteins (RND); transverse the inner membrane of the cells. Nomenclatures for these complexes are written as: RND-MFP-OMP types.

P. aeruginosa has four well known tripartate efflux systems. These are the MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM (Poole, 2001). While MexAB-OprM

(Figure 8) is heavily involved in tetracycline resistance, it is also involved in the removal of other substrates including: β-lactams, fluoroquinolones, chloramphenicol, novobiocin, macrolides, ethidium bromide, crystal violet, sodium dodecanoyl sulfate, toluene, aromatic hydrocarbons, and homoserine lactones (Poole, 2001).

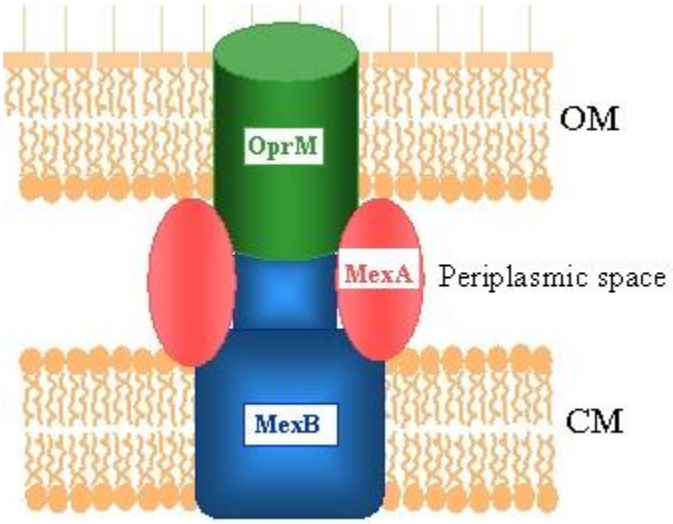


Figure 8: MexAB-OprM multidrug efflux pump. OM: outer membrane, CM: cytoplasmic or inner membrane. © 2009 University of Cambridge Department of Pharmacology

Expressions of these complexes are under multi-level control. For, the MexAB-OprM pump, Mex R is the auto-regulating negative repressor for the MexAB-OprM operon. MexAB-OprM is also under quorum sensing control.

In *Pseudomonas aeruginosa*, the MexAB-OprM pump, releases the C_{12}HSL QS molecule. After quorum levels are reached, this molecule diffuses back into the cell, after which a second QS signal molecule, C_4HSL, is expressed. It is this second signal molecule which greatly up-regulates the expression of MexAB-OprM and other MDR efflux pumps. Maximum transcription expression of these pumps is typically achieved during the mid-stationary growth phase of the cells. Lastly, down-regulation of MexAB-OprM is under the control of Mex T.

Mex T is the positive regulator protein of a second efflux pump system MexEF-OprM. It is this protein which represses further transcription of the *MexAB-OprM* gene transcription (Maseda et al., 2004).

Plasmid Gene Expression Efflux Pumps

Tetracycline resistance genes can be found on Tetracycline-resistant (Tc) plasmids (Chopra and Roberts, 2001). There have been up to 29 tetracycline resistance genes (*tet*) that have been identified and at least 3 oxytetracycline resistance genes (*orp*) that have been characterized in both Gram-positive and Gram-negative organisms. *P. aeruginosa* is known to express four "*tet"* genes: *tet*(A), *tet*(C), *tet*(E) and *tet*(G). These genes code for multi-drug resistant efflux pumps which function to expel the antibiotic from the cell. These "*tet*" genes typically code for two proteins: a repressor and the efflux pump complex. Expression of these genes is partially regulated by the absence or presence of the antibiotic. For instance, in the absence of tetracycline in the cell, a repressor protein prevents the exposure of the "*tet*" gene promoter binding site. At the appearance of even nanomolar concentrations of tetracycline in the cell, the Tetracycline-Mg+ complex binds to the repressor, it to change conformation and release the binding site. This binding of the Tetracycline-Mg+ complex thus allows for transcription of the efflux pump genes (Chopra and Roberts, 2001).

Statistical Data about *P. aeruginosa* Resistance Found in Hospitals

A 2003 study (Driscoll et al., 2007) of *P. aeruginosa* resistance in ICU units showed that approximately 22% percent of isolates were resistant to imipenem. There was almost a 30% resistance to flouroquinolones and a 32% resistance for cephalosporins. Within a 5 year span

there was a 15% surge (from 1% to 16%) of multi-drug resistance pseudomonad strains that are resistant to three or more antibiotics (Driscoll et al., 2007). For these reasons, alternate therapeutic leads are being explored.

Folk-Medicinal Leads

According to the WHO, 70-80% of the world's population still relies on folk-medicinal medicine as part of their main form of medical treatments (WHO, 2008). The pharmaceutical community has taken this knowledge to heart. Ethnopharmacology as this concept is called is the theory of looking at folk medicinal treatments and assessing then as potential leads in drug development. Twenty-five percent of all drugs on the market have at least one compound derived from a plant source. If fungal and animal sources are included, the number jumps to 40% (Houghton, 2001). There are several successful drugs that have been derived from plant or fungal sources. For instance, Taxol isolated from the Pacific yew tree, is one of the most successful anticancer treatments to date, with over 11 billion in revenue the first few years on the market (Stephenson, 2004). Ephedrine, a popular bronchodilator, was derived from the folk-medicinal plant Ma Hung, which demonstrates medicinal properties that were first recorded in China 5,000 years ago (Abourashed et al., 2003). Because of their history of medicinal properties, many folk-medicinal plants have been screened for antibacterial (Cowen, 1999) and anti-quorum sensing activities (Adonizio et al., 2006; Rasmussen et al., 2005b).

Quorum Sensing Inhibitors

Studies have found that one method of defense used by many organisms to protect themselves from invading microbes is the production of compounds called quorum sensing inhibitors (QSI). Bacteria, fungi and plants have all been shown to produce these compounds, which interfere with the QS-regulated gene expression in the invading organism (Hogan et al., 2004; Manefield et al., 2001; Persson et al., 2005; Rasmussen et al., 2005b). Acyl-homo serine lactone analogs and other quorum sensing inhibitors (QSI) have been investigated to determine their ability to prevent expression of quorum sensing controlled genes. QSIs may also reduce microbial virulence by interrupting quorum communication thus preventing microbes to attack the host as a unified army, by prevent the expression of pathogenic and virulence gene expression, and by reducing or preventing the development of biofilm formation. Several compounds have been identified that have the ability to interfere with QS-mediated gene expression (Manefield et al., 2001) through competitive inhibition, thus reducing biofilm thickness (Hentzer et al., 2002).

QSI compounds produced by Fungi

Rasmussen et al. (2005a) studied 100 extracts from 50 *Penicillium* species and found that 33 produced QSI compounds. From these 100 extracts, penicillic acid and patulin (Figure 9–A, B) proved to be inhibitory against *P. aeruginosa* QS-controlled gene expression. Further tests showed that 3-day-old biofilms which were grown on patulin-treated media were more susceptible to tobramycin treatment compared to untreated media, as indicated by a higher degree of cell death. Secondly, studies in a mouse model of chronic pulmonary infections showed that after 3 days of treatment with patulin, there was a 20-fold decrease of bacterial count compared to untreated mice.

These investigators showed that when QS communication is blocked, the host immune system is better able to combat infection. One way that host-derived polymorphonuclear leukocytes neutrophils (PMNs) can clear infection within a host is by production of hydrogen peroxide (H_2O_2). This activity is blocked by rhamnolipids, which are *P. aeruginosa* QS-expressed compounds. Rasmussen's group (2005a) demonstrated that if the QS communication can be blocked by a quorum sensing inhibitor, PMNs could function normally. The compound patulin interrupts pseudomad QS-mediated gene expression; thus PMNs are able to combat biofilm infections by the production of H_2O_2 during oxidative burst.

QSI Compounds Produced by an Alga

Halogenated furanones (Figure 9-C) are QSI molecules that are produced by the micro alga *Delisea pulchra*. These compounds have been shown to prevent quorum sensing gene expression in various organisms. For example, Hentzer et al. (2002) showed that synthesized furanone 56, a halogenated furanone of *Delisea pulchra* is able to block *Las I/R* quorum sensing system of *P. aeruginosa* and prevent expression of elastase and chitinase. Confocal microscopy demonstrated that this furanone helped reduced biofilm thickness (after 7 days) from 61 $\pm$ 6 μm (untreated) to 23 $\pm$ 4 μm (treated), and it lowered QS-mediated gene expression, as measured using fluorescent markers. The appearance of bioluminescence in *Vibrio fischeri* and swarming motility in *Serratia liquefaciens* are also affected (Manefield et al., 2001). The furanone (4-bromo-5-(bromomethylene)-3-(1'-hydroxybutyl)-2-(5H)-furanone) has been shown to inhibit antibiotic production and many extracellular degradative enzymes (pectate lyases, cellulases and proteases) in the plant pathogen *Erwinia carotovora.*

QSI Compounds Produced by Plants

Plant extracts have also been examined for QSI activity (Adonizio et al., 2006; Rasmussen et al., 2005b). Because of its extensive antifungal reputation in medicinal folklore, *Allium sativum L.*, commonly known as garlic, has been examined for this type of activity. Persson et al. (2005) reported that toluene extracts of garlic contained several compounds with varying levels of quorum sensing inhibition against Gram-negative transcriptional regulators Lux R or Las R. Specifically, *N*-heptysulfanylacetyl–L-homoserine lactone, a synthetic derivative, showed QSI activity against both *Lux I/R* and *Las I/R* QS mediated systems. Collectively, the evidence presented here suggests that QSIs may be useful in the treatment and/or prevention of biofilm infections.

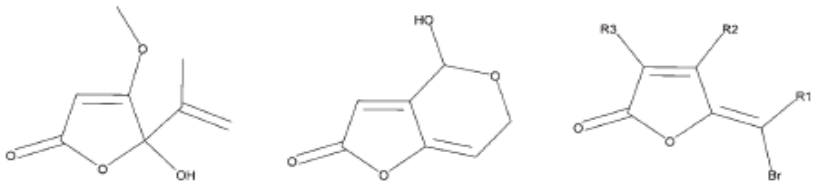


Figure 9: QSI molecules. A) Penicillic acid, B) Patulin and C) Halogenated Furanone (Rasmussen et al., 2005b)

Combination Therapy

Since biofilm infections and antibiotic resistance are on the rise (Lewis, 2001), research has begun to explore the effects of combination therapy to treat these types of infections (Nascimento et al., 2000; Rasmussen et al., 2005b). The term "synergistic activity" is often used to describe various herbal remedies in reference to the fact that their antimicrobial activity is dependent on several compounds working in combination with one another within the whole plant extract. A synergistic effect can be described where the combined resultant activity is greater that

the "sum of its parts," compared to an additive effect that is equal to the "sum of its parts." Many studies have shown that a synergistic effect may be occurring between crude plant extracts and antibiotic compounds against various resistant organisms (Nascimento et al., 2000; Rasmussen et al., 2005b).

Betoni et al. (2006) looked at the effects of sub-inhibitory levels of eight plant extracts (1/4 of MIC $_{90\%}$) combined with 13 antibiotics against 32 strains of *Staphylococcus aureus.* Each extract was able to produce a synergistic effect (synergy was defined as values $p<0.05$ of the Wilcox nonparametric test) with at least two antibiotics. The highest level of synergy occurred with antibiotics that worked by inhibiting protein synthesis (with five extracts/drug). Tetracycline produced a synergistic activity with all eight of the extracts tested. Finally, the weakest and the most potent extracts, from *Cymbopogon citratus*-lemongrass (MIC $_{90\%}$ --17.84 mg/mL) and *Syzygium aromaticum*-clove (MIC $_{90\%}$ --0.36 mg/mL) both showed synergistic activity with 11 of the 13 antibiotics tested. This suggests that synergy maybe an important aspect in antimicrobial treatment against resistant *S. aureus*.

Aburjai et al. (2001) surveyed nineteen methanolic extracts of Jordanian plants. Extracts were combined with seven antibiotics to determine effectiveness against both an antibiotic-resistant strain and a non-resistant strain of *P. aeruginosa.* Individual extracts increased activity of some antibiotics while decreasing the activity of others. For instance, the methanolic plant extracts from *Euphorbia macroclada* antagonized penicillin G and nalidixic acid activity, allowing over 100% percent bacterial growth. In contrast, tetracycline inhibition was augmented and only 26.9 % bacterial growth was observed. The extract of *Mentha piperita L.* prevented gentamicin and erythromycin activity allowing over 100% growth activity compared to the control, but in converse it augmented tetracycline inhibition, allowing only 26.1 % overall

bacterial growth. *Thea sinensis L.* extract had the most significant effect increasing tetracycline inhibitory activity thus only allowing 13.0 % percentage growth. Of the seven antibiotics tested, tetracycline was the most easily augmented, with five of the nineteen plants that were tested against the resistant *Pseudomonas* strain.

Nascimento et al. (2000) demonstrated that a number of crude plant extracts, including thyme, showed an increased killing effect against *Pseudomonas* species when combined with ineffective dosages of commercial antibiotics. For example, the plant extract of *Thymus vulgaris* can inhibit *Pseudomonas* growth at 70 μg/mL; but when combined with ineffective doses (50 μg/mL) of tetracycline, the amount of thyme needed to produce growth inhibition was reduced to 10 μg/mL.

Thymus vulgaris

Thymus vulgaris, (Figure 10) is a well-known plant that has been regarded as a potent medicinal herb for thousands of years. Thyme belongs to the family Lamiaceae, and is an aromatic perennial, that grows to 20-30 cm in height. The stems are long-slender, woody and quadrangular, and they grow from a basal center. The leaves are typically grayish-green in color, smooth oblong-lanceolate in shape, and with little or no petioles which connect the leaves to the woody stems. Set in pairs, the flowers grow at the top of these slightly twisting foot-stalks in a whorl-like pattern. The petals grow in together in a form that creates a closed-corolla tube, and they are typically pinkish-white in color with green sepals at the base of the corolla (Grieves, 1995; WHO, 1999)

Figure 10: ***Thymus vulgaris.***
(Grieve, 1995)

The word “thyme” originated from the Greek word thymus, which means brave or courageous. During ancient times it was used a fumigant, as it's aroma was meant to inspire courage and bravery, and to invigorate and energize those who smelled it. During the middle ages, people believed that the scent of thyme could keep away venomous creatures (Grieve, 1995). The flower and leaves were also used to treat a number of ailments. More recently, thyme is a well-known and potent antiseptic, and useful for oral hygiene (WHO, 1999). The plant has often been dried and made into an infusion, sweetened with honey and used to treat sore throats. Syrup made out of the fresh herb was used to treat whooping coughs; it has also been dried and added to tobacco "to promote good digestion" (Grieve, 1995). Other folk-medicinal uses include the treatment of coughs which stem from bronchitis, laryngitis, pertussis, or tonsillitis (WHO, 1999). Present day uses include cooking, cosmetics, food preservation, and antiseptic (Torras et al., 2007).

Many of thyme's medicinal activities can be attributed to its essential oil. Essential oils of plants are typically extracted from the plant leaves and flowers through steam distillation. Constituents of essential oils include terpenes, monoterpenes, sequesterpines and phenols (Torras et al., 2007). The major constituents of the essential oil thyme include: phenols, thymol and carvacrol, which make up about 20-25% of the essential oils. The other constituents include linaalool, p-cymol, cymeme, thymeme, pinene, apigenin, leteolin and geraniol (Grieves, 1995; Torras et al., 2007; WHO, 1999). Fabio et al. (2007) found potent antibacterial activity of the essential oil against seven various bacteria at a minimum concentration of 0.0002 mL/mL. Four major constituents were chosen for further assessment due to strong scientific evidence of their effectiveness against resistant bacteria and biofilm formations.

Four Main Constituents of Thyme

Baicalein (Figure 11) is a flavone compound that can be isolated from the methanolic extract of *Thymus vulgaris* leaves; it has been shown to produce synergistic activities with tetracycline and various β-lactam antibiotics against methicillin-resistant *Staphylococcus aureus* (MRSA). It appears that the mode of action for this synergistic antibiotic effect varies, dependent upon the resistant features of the organism, such as whether or not the MRSA possesses the tetracycline-resistant efflux pump *tet(K)* (Fujita, 2005).

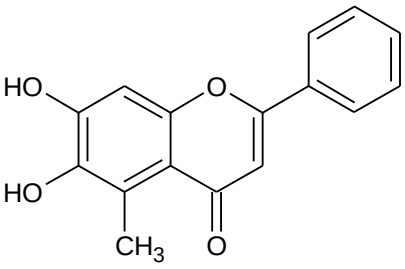


Figure 11: Baicalein.

Rosmarinic acid (Figure 12) is a caffeic acid ester that is excreted naturally by roots of the plant sweet basil when challenged by *Pseudomonas aeruginosa* (Walker et al., 2004). It is also produced by several other aromatic herbs including *Thymus vulgaris* (Wang et al., 2004). Rosmarinic acid has been shown to inhibit planktonic growth of some *Pseudomonas spp*., and interferes with quorum sensing activities and biofilm development (Walker et al., 2004).

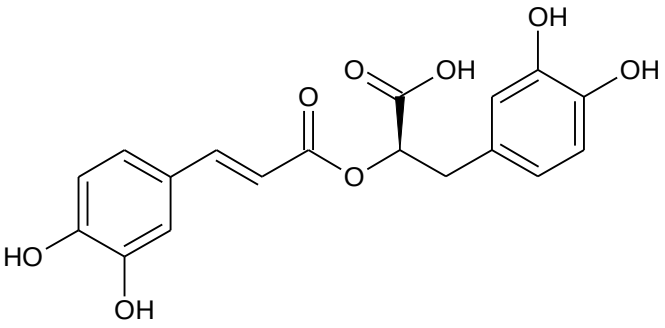


Figure 12: Rosmarinic acid.

Thymol (2-isopropyl-5-methylphenol) (Figure 13.A) and Carvacrol (2 methyl-5-(1-methylethylphenol) (Figure 13.B) are phenolic compounds both isolated from thyme and are

known for their antimicrobial activity against many organisms, including MRSA. These compounds have also been shown to interfere with staphylococcal biofilm growth (Nostro et al., 2007).

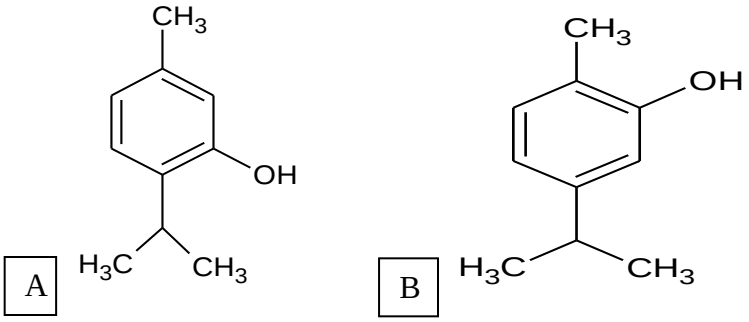


Figure 13: A) Thymol and **B) Carvacrol.**

Purpose

The purpose of this dissertation was to perform preliminary investigations on two alternative treatment avenues against *Pseudomonas aeruginosa* infections: quorum sensing inhibition and combination therapy. First, fifty folk medicinal plant extracts were screened for anti- quorum sensing properties. Next, specific attention was placed on the thyme extract, a folk-medicinal plant shown to possess anti-quorum sensing properties (Vattam et al., 2007) but also first shown by Nascimento et al., 2000 to have synergism when combined with tetracycline against resistant *Pseudomonas aeruginosa.* Furthermore, this study pursued the isolation and identification of the active component in thyme involved in the thyme-tetracycline effect, and the elucidation of its mode of action.

Rationale

There are many clues indicating that ethanolic thyme extract may act as a quorum sensing inhibitor, thus augmenting tetracycline activity. First, the thyme extract has been shown to augment activity of various antibiotics, such as ampicillin, tetracycline and chloramphenicol (Nascimento et al., 2000). Interestingly, these three antibiotics work through different modes of action (Mims et al., 1998). This indicates a more general mode of action for the plant extract. Aburjai et al. (2001) demonstrated that while the activity of tetracycline (typically active against Gram-negative organisms) is augmented by various plant extracts, activity is greatly reduced with other antibiotics such as penicillin G (typically active against Gram-positive bacteria). This suggests that the mechanism involved may be type-specific. Next, tetracycline resistance occurs by either expression of efflux pumps and/or ribosome protection expressed via the tet R protein (Rosen and Mobashery, 1998). It has been shown that quorum sensing can modulate many virulence genes including efflux pump expression (Lewis, 2001). Thus, control of quorum sensing activity by an inhibitor would present a logical explanation of these observations.

More evidence suggesting that the thyme extract may include a QSI comes to light upon examination of various green tea studies. Yam et al. (1997) studied the activity of *Camellia sinensis,* green tea, against various bacteria. They discovered that 'cup of tea' concentrations showed considerable activity against many pathogenic bacteria including 18 strains of methicillin resistant *Staphylococcus aureus* (MRSA). Tiwari et al. (2005) demonstrated that epicatechin gallate (ECGC), a compound found in *Camellia sinensis,* can produce an amplified effect when combined with the commercial antibiotic chloramphenicol against various strains of *Salmonella typhimurium.* In this study, supplementation with ECGC reduced the amount of antibiotic needed to cause growth inhibition. Tiwari suggested that this phenomenon occurred

because of "dual binding sites" which were triggered by the plant extract on the bacterial cell membrane of the pathogen. A different study demonstrated that a possible synergistic interaction between ECGC and oxacillin might be occurring during treatment of various methicillin-resistant *Staphylococcus aureus* (MRSA), that would, in effect, reverse the resistance of the strains (Hamilton-Miller and Shah, 2000). In 2003, epigallocatechin gallate (ECGC) was shown to interfere with quorum sensing activities in *E. coli* and *Pseudomonas* reporter organisms (Huber et al., 2003). Mode of action experiments can be used to determine if this is indeed the case with the thyme extract.

Specific Aims

1. Screen fifty folk-medicinal plant extracts for QSI activity.
2. Examine specifically the extract of *Thymus vulgaris* and the "thyme-tetracycline" effect:
    a) Isolate and identify the active compound in the thyme extract that augments tetracycline activity against resistant *Pseudomonas aeruginosa.*
    b) Determine the most efficient way to extract the active compound from the plant material.
    c) Determine if quorum sensing inhibition is involved in the mode of action.

MATERIALS AND METHODS

Chemicals and Reagents

The reagents used for this project—acetone, chloroform, dichloromethane, ethanol, hexane, and methanol—were histological grade and purchased from Sigma-Aldrich, which is located in St. Louis, Missouri. Media used for propagating bacterial cultures included Luria-Bertani (LB), Mueller Hinton II (MH), Tryptic soy agar (TSA), and Nutrient broth (NB) and came from Difco, located in Sparks, Maryland. Antibiotics—tetracycline free-base (purchased from MP Biomedical, Inc. in Solon, Ohio) and tobramycin sulfate salt, gentamicin sulfate salt, and kanamycin sulfate (all purchased from Sigma-Aldrich in St. Louis, Missouri). Thymol was purchased from Fisher Scientific in Fair Lawn, New Jersey. Baicalein (98%), rosmarinic acid (97%), and liquid carvacrol (98%) were purchased from Sigma-Aldrich in Steinheim, Germany. The QSIS2 assay also required the following additives: N-butanoyl-L-homoserine lactone, C_4 HSL (both from Cayman Chemical Company in Ann Arbor, Michigan), N-(3-oxododecanoyl)-L-homoserine lactone, C_{12} HSL, and 2, 3, 5-triphenyltetrazolium chloride (from Sigma-Aldrich in St. Louis, Missouri); concentrations were listed below where appropriate.

Bacterial Cultures

The QSIS2 reporter strain, *Pseudomonas aeruginosa* pLasB-SacB1, was graciously provided by Dr. Michael Givskov of BioCentrum-DTU, Technical University of Denmark, and the *Chromobacterium violaceum* ATCC 12472 used for the Chromobacterium QSI assay was obtained from the American Type Culture Collection in Manassas, Virginia. *P. aeruginosa*

ATCC 10145, obtained from the Department of Biology at Georgia State University, was used for the antibacterial studies.

Plant Samples

Plant material was either purchased from local area farmers' markets or hand-picked from surrounding fields in Atlanta, Georgia. *Thymus vulgaris* samples were purchased from a local farmers' market. Boneset, pokeweed, and yellow root were purchased from Golden Valley Herbs-Hendersonville as a 40% ethanol extract. Taxonomic identification of all plant samples was made using a minimum of two sources per specimen, including the United States Department of Agriculture Natural Resources Conservation Services website (www.plants.usda.gov) and a dichotomous key, such as Plant Families and How to Identify Them (Jacques, 1949).

Extraction

Fresh thyme (10 g) was cut into ½-inch pieces and allowed to dry in an aerated incubator at 55°C for 48 hrs. The plant material was extracted with 200 mL of ethanol in a covered flask for 24-48 hours. The resultant liquid was filtered through a cotton ball filter into a round-bottom flask and concentrated using a Buchi R-200 rotary evaporator, thus removing all the ethanol and resulting in a viscous crude plant extract. All other plant extracts were prepared as described above and tested at a concentration of 0.5 g/mL- solvent, respectively, except for ginger, sassafras, rattlesnake master, and red root, which were tested at a concentration of 0.25 g/mL. Subsequent extractions were made with slight modifications. For instance, thyme plant material

was also extracted with solvents including acetone, chloroform, ethyl acetate, hexane, methanol, methylene chloride, and petroleum ether.

Comparative studies of the extraction of the thyme plant material were also made with fresh, nonrecycled solvent and recycled solvent, or solvent collected after evaporation of a previous sample. Fresh solvent extractions were made by soaking the bulk plant material in previously unused solvent aliquots for each soak within the extraction process. For all recycled solvent extractions, minimal amounts of fresh solvent were added only if necessary to compensate for any solvent that may have been lost to evaporation during the extraction process.

Percentage Yield

The percentage yield of crude plant extracts was calculated as follows:

$$\frac{\text{Weight of crude plant extract}}{\text{Weight of starting bulk dried plant material}} \times 100 = \text{Percentage yield}$$

Disc Diffusion Assay (DDA)

Disc diffusion assay, as previously described by Mims et al. (1998) was used for all antimicrobial studies. A pure culture of *P. aeruginosa* was prepared onto a nutrient agar plates and incubated aerobically at 37˚C for 24 hours. Next, sterile saline tubes (pH 9.9) were inoculated with the fresh overnight culture of organisms producing a turbidity equivalent to 0.5 McFarland Standard (BD diagnostic systems). Turbidity was matched through visual comparison. Mueller Hinton II Agar plates, with 50 μg/mL tetracycline, were swabbed thoroughly using sterile cotton swabs saturated with the diluted bacterial suspensions. Next, 6-

millimeter test discs (BD diagnostic systems) were prepared with 20 μL of the diluted plant extract (500 mg/mL) and allowed to air dry for a minimum of three hours. The impregnated discs were placed aseptically in a radial fashion onto the inoculated plates. After 24 hours of incubation at 37˚C, active plant samples will inhibit bacterial growth, thus resulting in a "zone of inhibition" around the disc. The diameter of these zones was measured in millimeters. Positive control discs (10 μL per disc) were prepared with tobramycin sulfate/water solution (1000 μg/L). The negative control discs were treated with straight ethanol.

Thin-Layer Chromatography (TLC)

Crude plant extracts and constituents were analyzed by thin-layer chromatography (TLC). Samples were diluted in ethanol (100 mg/mL) and spotted on a 250 μm 10 x 10 mm silica gel 60 F_{254} plates (EMD Chemicals). Next, the plates were developed in 8:4:1 hexane, dichloromethane, acetone system and allowed to air-dry in a fume hood. Plates were then analyzed under an ultraviolet light for separation of compounds based on polarity.

Thin-Layer Chromatography–Bioassay

Thin-Layer Chromatography–Bioassay (TBA) **a**dapted from Slusarenko et al. (1989) was used for identification of the active antimicrobial components in the thyme extract. Plant extracts and constituents were analyzed by thin-layer chromatography (TLC). Samples were diluted in ethanol (100 mg/mL) and spotted on a 250 μm 10 x 10 mm silica gel 60 F_{254} plate (EMD Chemicals). Next, the plates were developed in 8:4:1 hexane, dichloromethane, acetone solvent system and allowed to air-dry in a fume hood. An aliquot (25 mL) of nutrient broth with 1% glycerol as added to 250 mL Erlenmeyer flasks. Next, one 10 μL loopful of *P. aeruginosa*

(grown aerobically overnight on nutrient agar at 37° C) was diluted into nutrient broth and grown at 37° C in an orbital shaker (150-200 rpm) for 24 hours. Second aliquots (25 mL) of nutrient broth were prepared with 1.5% (w/v) agar, 0.25 mL of 1% glycerol and 50 μg/mL tetracycline and referred to as TBA agar. They were later cooled to 45° C. The dye 2, 3, 5-triphenyltetrazolium chloride was added to the agar, gently agitated, and set aside. The diluted culture (1 mL) was added to the 25 mL TBA agar tubes and agitated. Finally, 50 mL of the agar (2 tubes) were carefully poured into large Petri plates containing the thin-layer chromatography plates. After 24 hours of incubation at 37°C, active compounds were identified by zones of inhibition of microbial growth. The positive control was tobramycin, and it was spotted on the TLC plate after the plate was developed; the negative control was any area of the TLC plate without extract. The experiment was run in triplicate.

Activity-Driven Fractionation

The concentrated crude extracts were separated, based on polarity, into fractional constituents, using an appropriate gradient solvent system on a solid-phase extraction (SPE) product silica 5 g/20 mL column (Fisher Scientific, Fair Lawn, New Jersey) using a twelve-port vacuum chromatography manifold.

First, the column was washed with pure hexane to prepare the silica. Next, the crude extract was dissolved into methanol (~5 mL) added to the column. Then, the column was washed with a minimal volume of hexane to allow the extract to saturate the top 5% of silica in the tube. The column was then washed with hexane, totaling approximately 90 mL of hexane, until no color was seen in the collected solvent. The first fraction was the combined hexane tubes. This process was repeated with dichloromethane, acetone, and methanol. Tubes generated from the

same solvent were recombined and roto-evaporated to remove eluting solvent from the fraction. Fractions were re-diluted as needed for used for further testing.

QSIS2 Assay

The QSIS2 assay, adapted from Rasmussen et al. (2005b), with slight modifications as described below, was used to screen plant extracts for C_4 HSL and C_{12} HLS analogues. LB Agar was made with the addition of 15.5 g sucrose (total volume 250 mL). The pH was adjusted to 5.0 with the addition of hydrochloric acid. Gentamicin (0.0247 g), kanamycin (0.0215 g), 1.1 mL of each C_4 HSL (0.00595 g/L of ethanol), and C_{12} HSL (0.00342 g/L of ethanol) plus 0.20 g of 2, 3, 5-triphenyltetrazolium chloride dye was added into 25 mL of phosphate-buffered saline (PBS). The augmented PBS (25 mL) plus 2.5 mL of overnight QSIS2 reporter strain, grown in ABT media (Rasmussen et al., 2005b), was added to the tempered LB agar and poured into Petri plates. Next, the plates were allowed to solidify for 25 minutes. Wells (7 mm) were bored into the solidified agar. Various dilutions of the plant extracts and major constituents were (50 µL per well) were added to the wells. Agar plates were allowed to sit at room temperature for one hour prior to incubation to allow the test solution ample time to diffuse into the agar. Plates were incubated at 37ºC for 12-16 hours.

When QS-mediated gene expression is activated in the QSIS2 reporter strain, sucrose mediated cell death occurs, but if QSI compounds are present in the test sample, QS-mediated cell death is blocked and the reporter strain is able to grow. When cellular growth is achieved, a zone of dark red growth is visible around the test well in only the areas where QSI molecule concentration offsets QS signal concentration. The red color is caused by the metabolism of triphenyltetrazolium chloride by the bacteria. This dye is used only to augment the bacterial

zone of growth. Antimicrobial activity was detected by a transparent clearing around the well, referred to as a "zone of inhibition." A second zone, referred to as a "zone of growth," consisted of a bright red halo around the well, which indicated QSI activity. Experiments were run in triplicate unless otherwise specified and zone diameters were measured in millimeters. All diameter measurements were recorded in millimeters.

Chromobacterial QSI Assay

The Chromobacterial QSI assay protocol was adapted from Bosgelmez-Tinaz et al. (2007) and was used for scanning samples for C_4- C_6 HSL analogues. *Chromobacterium violaceum* ATCC 12472 was propagated in 5 mL of nutrient broth and incubated in a shaker incubator for 24 hours at 30º C. LB Agar (250 mL) and LB broth with 0.3 % agar (250 mL) were used to prepare an overlay assay. First, a Petri plate was filled halfway to capacity with LB Agar and allowed to solidify for 30 minutes. Next, LB Agar (0.3%) was inoculated with 1 mL of overnight culture. The tempered agar was poured over the LB Agar base and allowed to solidify at room temperature. Wells (7 mm) were bored into the solidified agar. The test solution (50 µL per well) was added into wells. Plates were left at room temperature for another hour to allow test samples to diffuse into the agar. The plates were incubated for 16 hours at 30ºC. Violaceum color production is under QS-mediated control. If the plant extract contains a QSI activity then cellular growth will occur but pigment production will be prevented. Wells with zones of inhibited color (opaque) were considered QSI active (zone of growth), and clear zones around the well (transparent) were considered antimicrobial (zones of inhibition). Experiments were run in triplicate and zone diameters were measured in millimeters.

Garlic as the Positive Control for QSI Testing

A toluene garlic extract, adapted from Rasmussen et al. (2005b) was used as a positive control for all QSI studies. Garlic (150 g) was shredded with a commercial blender and extracted in 300 mL of toluene for 24 hours. After 24 hours, the sample was filtered through a Whatman No. 1 filter. Next, the collected material was combined with 150 mL of sterile water and mixed for 24 hours at room temperature. A separatory funnel was used to collect the toluene extract (top layer), which was subsequently used as a QSI-positive control.

Glucose and pH Testing

Glucose concentrations of the crude plant extracts were measured using True Test gold sensor strips with a True Test to-go blood/glucose meter (Home Diagnostics, Fort Lauderdale, Florida). Crude extracts were first prepared by diluting the sample to a 0.2 g/mL into deionized water. One drop of the extract was placed into a makeshift aluminum foil bowl. The meter strip was then dipped into the solution. The solution travels up the strip by capillary action. Glucose concentrations were read of the off the digital screen and recorded in dg/mL.

The pH of the samples was determined using colorpHast pH strips (EDM Chemicals). The strips were submerged in the above 0.2 g/mL extract/water samples. Results were compared to the pH scale on the package.

RESULTS: SCREENING OF FIFTY FOLK-MEDICINAL EXTRACTS FOR QSI ACTIVITY

Folklore and Scientific Data

Table 1 describes the fifty bio-ethnically relevant plant extracts that were chosen to screen for QSI activity. Bio-ethnical importance is based on either folkloric uses and/or previous scientific data.

Table 1: Folklore and Published Test Results Pertaining to the Folk-Medicinal Plants Tested.

	Common name	Scientific name	Family name	Folklore	Published test results
1	American dandelion	*Taraxacum officinale*	Asteraceae	Diuretic, tonic, urinary organs, kidneys and liver disorder (Grieve, 1995)	Active against *C. albicans*, *S. cerevisiae* (Cowan, 1999)
2	American spikenard	*Aralia racemosa*	Asteraceae	Used as a blood purifier, and as a treatment for other ailments including: asthma, cough, diarrhea, hemorrhoids, leucorrhoea and hay fever (Hutchens, 1973)	Previously investigated for its terpenoid components (Hanson and White, 1973)
3	Bamboo	*Phyllostachys aurea*	Poaceae		Rich source of hydrocyanic, benzoic acid, and tricin; isolated from bamboo is considered relatively safe compound for development as a cancer chemo-preventative

	Common name	**Scientific name**	**Family name**	**Folklore**	**Published test results**
					agent (Jiao et al., 2007)
4	Basil leaves	*Ocimum basilicum*	Lamiaceae		Rosmarinic acid produced by sweet basil has been shown to be antibacterial against planktonic *P. aeruginosa* cells (Walker et al., 2004)
5	Bay leaves	*Laurus nobilis*	Lauraceae	Brazilian uses: stomach aches, headaches, hepatic complaints (Di Stasi et al., 2002)	
6	Bitter leaf	*Vernonia amygdalina*	Asteraceae	Tonic, antibacterial, anti-tumor, anti-parasitic (Grieve, 1995)	
7	Brown seaweed	*Undaria pinnatifida*	Alariaceae		*Delisea pulchra*, a marine algae, has been shown to produce several halogenated furanones that are highly effective QSI's (Manefield et al., 2001)
8	Boneset	*Eupatorium perfoliatum*	Asteraceae	Used to treat acute bronchitis and nasopharyngeal catarrh (Habtermariam and Macpherson, 2000)	Showed to have a weak antimicrobial activity against Gram-positives such as *S. aureus* and *B. megaterium* (Habtermariam

	Common name	Scientific name	Family name	Folklore	Published test results
					and Macpherson, 2000)
9	Chaparral	*Larrea tridentata*	Zygophyllaceae	Used in a Sonoran region as a vermifuge; external uses: rheumatic arthritis, and tooth aches internal uses: inducing abortion, and menstruation and expelling afterbirth (Grant et al., 1998).	Antiviral, antifungal and anti-tutor but it also causes hepatotoxicity with prolonged use (Grant et al., 1998).
10	Chinese dandelion	*Taraxacum mongolium*	Asteraceae	Chinese folkloric uses include: inflammatory diseases and antiviral activity (Shi et al., 2008)	
11	Chinese wolfberry	*Lycium chinense P. Mill*	Solanaceae	Chinese traditional uses: as a tonic, and long life and anti-aging (Yeh et al., 2008).	
12	Chrysanthemum	*Chrysanthemum indicum*	Asteraceae	Oriental folkloric uses include fever, pneumonia, stomatitis, and colitis; the flowers were used to treat eye infections (Sassi et al., 2008)	Antimicrobial activity was found by Chrysanthemum species against *P. aeruginosa* (Sassi et al., 2008); Chlorogenic acid found in some

	Common name	Scientific name	Family name	Folklore	Published test results
					Chrysanthemum species may play a role in QSI activities (Singh et al., 2009)
13	Cinnamon	*Cinnamomum verum*	Lauraceae	Astringent, antiseptic, diarrhea, hemorrhaging of the womb (Grieve, 1995)	Cinnamaldehyde a main component in the essential oil has been shown to decrease *E. coli* biofilm formations (Niu and Gilbert, 2004), and it interferes with C_4HSL and C_6HSL QS communication (Niu et al., 2006)
14	Clove	*Syzygium aromaticum*	Myrtaceae	Antiseptic, used to treat tooth decay (Grieve, 1995)	Active against *C. albicans* biofilm formation, (Agarwal et al., 2008); Eugenol a main component in cloves also showed activity against *C. albicans* biofilms (Hu et al., 2007) but no activity against *E. coli* biofilm development (Niu and Gilbert, 2004)
15	Cranberry	*Vaccinium macrocarpon*	Ericaceae		Antibacterial (Cowan, 1999) and has shown

	Common name	Scientific name	Family name	Folklore	Published test results
					to inhibit biofilm formation on uroepithelial cells (Reid et al., 2001)
16	Dogwood	*Cornus sanguinea*	Cornaceae	Powdered bark was made into toothpaste and the root bark can be made into a scarlet dye (Wu et al., 2008)	Fruit from *Cornus officinalis* when combined with apple juice shown affect against *E. coli* O157H7; antimicrobial (Wu et al., 2008)
17	Elder berry	*Sambucus nigra*	Caprifoliaceae	Brazilian uses: muscular pain, measles, varicella, fever, cough, bad colds, hoarseness (Di Stasi et al., 2002)	Fruit contains anthocyanins, vitamins A and C, calcium, iron, and vitamin B6 (Charlesbois, 2007)
18	Fennel	*Foeniculum vulgare* Mill	Apiaceae	Used as a purgative, and to treat stomach bloating (Grieve, 1995)	
19	Fenugreek	*Trigonella foenum-graecum*	Fabaceae		Lactoferrin extracted from sprouts showed activity against *Helicobacter pylori* (Randhir et al., 2004)
20	Garlic	*Allium sativum*	Liliaceae	Brazilian uses: Hypertension, colds, topically used to treat headaches	Effective quorum-sensing inhibitor (Rasmussen et al., 2005b)

	Common name	Scientific name	Family name	Folklore	Published test results
				(Di Stasi et al., 2002)	
21	Ginger	*Zingiber officinale*	Zingiberaceae	Brazilian uses: stomach aches, bad cold, and cough (Di Stasi et al., 2002)	Showed no growth inhibition against *E. coli* ATCC 33456 (Niu and Gilbert, 2004)
22	Grapefruit rind	*Citrus paradisi*	Rutaceae	Rind and seeds used for fungal infections, loss, treatment for cellulite and weight loss (Annie's, 2005)	Ascorbic acid showed QSI activity against *C. perfringens* (Novak and Fratamico, 2004)
23	Heath aster	*Symphyotrichum ericoides*	Asteraceae	Native American uses: used in sweat baths and to revive unconscious patients (USDA, 2004)	
24	Holly berries	*Ilex opaca*	Aquifoliaceae	Berries are mildly toxic and will cause vomiting and/or diarrhea (Grieve, 1995)	
25	Holly leaves	*Ilex opaca*	Aquifoliaceae	Leaves are used to treat fevers and rheumatism (Grieve, 1995)	
26	Hymenocrater	*Hymenocrater sessilifolius*	Lamiaceae	Fever, headache wounds, heart disease (Zaidi and Crow, 2005)	Active against *C. albicans* and Gram-negative bacteria (Zaidi and Crow, 2005)

	Common name	Scientific name	Family name	Folklore	Published test results
27	Hyssop	*Hyssopus officinalis*	Lamiaceae	Tea used for rheumatism, paste used to treat bruises (Grieve, 1995)	Essential oil was shown to be bacteriostatic against *E. coli* O157H7 (Marino et al., 2001)
28	Leather leaf mahonia	*Mahonia bealei*	Berberidaceae		Active against *S. aureus*, *B. subtilis*, *B. thuringiensis* (Li et al., 2008)
29	Lemon rind	*Citrus Limon*	Rutaceae	Lemons were used in the 18th century to prevent scurvy (Baron, 2009)	
30	Lobelia	*Lobelia inflata*	Campanulaceae	Bronchitis, diaphoretic, exportant, anti-asmathic (Grieve, 1995)	A piperidine alkaloid has been shown to be active against multidrug-resistant tumor cells (Ma and Wink, 2008)
31	Lo Han Kuo	*Siraitia grosvenori*	Cucurbitaceae	Emollient used to treat dry coughs, dire thirst and constipation (Li eta l., 2006)	Two novel cucurbitane glycosides were isolated and characterized from the unripe fruit (Li et al., 2006)
32	Mint	*Mentha piperita*	Lamiaceae	Brazilian uses: topical analgesic, worms, stomach pain, bronchitis, cough (Di Stasi et al., 2002)	Species from the Lamiaceae family is antimicrobial against *P. syringae* B728a (Karamanoli and Lindow, 2006) but no QS inhibition or

	Common name	Scientific name	Family name	Folklore	Published test results
					stimulation against *A. tumefaciens* NT1 and *C. violaceum* CV0blu; active against *C. albicans* biofilms (Agarwal et al., 2008)
33	Mustard seed	*Brassica nigra*	Brassicaceae	Brazilian uses: anti-inflammatory used both internally and topically (Di Stasi et al., 2002)	
34	Neem tree	*Azadirachta indica*	Meliaceae	Used to treat measles (Lans, 2007)	Shown to be affective as a mouthwash reducing gingivitis and cariogenic bacteria (Botelho et al., 2008)
35	Olive leaves	*Olea europaea*	Oleaceae	Astringent and antiseptic, oil is used as a laxative and hair tonic (Grieve, 1995)	Inhibits bacterial and fungal growth (Pereira et al., 2007)
36	Oregano	*Origanum vulgare*	Lamiaceae	Brazilian uses: bronchitis and coughs (Di Stasi et al., 2002)	Essential oil inhibits growth against *E. coli* O157:H7 (Marino et al., 2001)
37	Pokeweed	*Phytolacca americana*	Phytolaccaceae	Extract was used to treat rheumatism and	

	Common name	Scientific name	Family name	Folklore	Published test results
				hemorrhoids (Grieve, 1995)	
38	Pomegranate	*Punica granatum*	Lythraceae	Brazilian uses: stomach aches, inflammation (Di Stasi et al., 2002)	Effectively reduced plaque when used as a mouthwash (Palombo, 2009)
39	Rattlesnake master	*Eryngium aquaticum*	Apiaceae	Dropsy, syphilis, liver problems resulting from uric acid build-up, treatment of infected wounds (Hutchens, 1973)	
40	Red ginseng	*Panax japonicus*	Araliaceae	Improves metabolism and regulates stomach and intestine functions (Xiaoguang et al., 1998)	Shown to be anti-carcinogenic (Xiaoguang et al., 1998)
41	Red root	*Ceanothus americanus*	Rhamnaceae	Astringent, mouthwash, chronic bronchitis, whooping cough, dysentery, injected as a treatment for gonorrhea (Grieve, 1995)	Ceanothic acid was found to be active against oral pathogens (Charlesbois, 2007)
42	Rosemary	*Rosmarinus officinalis*	Lamiaceae	Chinese uses: arthritis and snakebites (Lans, 2007)	
43	Sage	*Salvia officinalis*	Lamiaceae	Restores mental function and	*Salvia apiana* was found to be antimicrobially

	Common name	Scientific name	Family name	Folklore	Published test results
				improves memory (Errickson and Sedia, 2005)	active (Errickson and Sedia, 2005), essential oil was found to be bacteriostatic against 15 different organisms (Marino et al., 2001)
44	Sassafras	*Sassafras albidum*	Lauraceae	Syphilis, skin diseases, and chronic rheumatism (Grieve, 1995)	Active against helminthes (Duke, 1994)
45	Silver berry leaves	*Elaeagnus commutata*	Elaeagnaceae	Combined with sumac roots to treat syphilis (Palombo, 2009)	
46	Sweet gum fruit	*Liquidambar stryaciflua*	Hamamelidaceae	Used to treat coughs, colds, diarrhea, dysentery, and ring worm (Duke, 1994)	
47	Thyme	*Thymus vulgaris*	Lamiaceae	Whooping cough, sore throat, colic fever, cold (Grieve, 1995)	Active against viruses, bacteria, and fungi (Cowan, 1999), Thymol has been shown to be active against *C. albicans* biofilms (Braga et al., 2008)
48	Toxicanum	*Vincetoxicum stocksii*	Asclepiadaceae	Injuries, wounds, and topical skin cancers (Zaidi and Crow, 2005)	Highly active against *C. albicans* and various bacteria (Zaidi and Crow, 2005)

	Common name	Scientific name	Family name	Folklore	Published test results
49	Yellow root	*Xanthorhiza simplicissima*	Ranunculaceae	Anti-inflammatory, astringent, antimicrobial, uterotonic, immuno-stimulant (Fetler and Lloyd, 1898)	
50	Witch hazel	*Hamamelis virginiana L.*	Hamamelidaceae	Astringent, tonic, sedative, external hemorrhaging, painful swelling and tumors (Grieve, 1995)	Crude plant extract has been shown to be highly effective against periodontal bacteria (Palombo, 2009)

QSIS2 Assay Results of the Folk-Medicinal Extracts

Table 2 shows the QSIS2 assay results for both zones of inhibition, and zones of growth, recorded in millimeters. Out of fifty crude extracts tested, thirty showed a transparent clearing, or zone of inhibition, around the test well, suggesting antimicrobial activity. Thirty out of fifty crude extracts also showed a QSI zone of growth. This can be recognized by a bright red halo around the test well (Figures 14-17) at the location where the concentration of QSI molecule offsets the concentration of the signal molecule. The largest QSI values were produced by the extracts of clove (30 mm), cranberry (33 mm), chaparral (32 mm), ginger (30 mm), heath aster (37 mm), pomegranate (36 mm), oregano (31 mm) sage (37 mm), and witch hazel (33 mm).

Table 2: QSI Screening of Various Folk-Medicinal Plant Extracts using the QSIS2 Assay.

Samples		Zone of diffusion (mm)	Zone of inhibition (mm)	QSI zone of growth (mm)
1.	American dandelion	0	0	0
2.	American spikenard	0	10	18
3.	Bamboo	0	0	27
4.	Basil leaves	8	0	13, 31*
5.	Bay leaves	8	10	23
6.	Bitter leaf	0	0	29
7.	Boneset	0	0	0
8.	Brown seaweed	0	0	0
9.	Chaparral	12	21	32
10.	Chinese dandelion	8	42	0
11.	Chinese wolfberry	0	0	0
12.	Chrysanthemum	0	15	23
13.	Cinnamon	0	10	20
14.	Clove	0	19	30
15.	Cranberry	0	24	33
16.	Dogwood	0	0	0
17.	Elder berry	0	13	26
18.	Fennel	0	0	0
19.	Fenugreek	0	11	18

Samples		Zone of diffusion (mm)	Zone of inhibition (mm)	QSI zone of growth (mm)
20.	Garlic (positive control)	0	10	14
21.	Ginger	0	16	30
22.	Grapefruit Rind	0	18	0
23.	Heath aster	10	21	37
24.	Holly berry	0	0	0
25.	Holly leaves	8	0	0
26.	Hymenocrater	0	12	0
27.	Hyssop	0	15	26
28.	Leather leaf mahonia	0	10	0
29.	Lemon Rind	0	15	23
30.	Lobelia	0	0	0
31.	Lo han kuo	0	0	22
32.	Mint	0	14	21
33.	Mustard seed	0	0	0
34.	Neem tree	0	0	0
35.	Olive leaves	0	14	0
36.	Oregano	12	16	31
37.	Poke weed	0	0	0
38.	Pomegranate	12	24	36
39.	Rattlesnake master	0	15	23
40.	Red ginseng	0	0	15

Samples		Zone of diffusion (mm)	Zone of inhibition (mm)	QSI zone of growth (mm)
41.	Red root	11	14	23
42.	Rosemary	8	0	22
43.	Sage	9	12	37
44.	Sassafras	0	17	24
45.	Silver berry leaves	8	0	0
46.	Sweet gum fruit	0	15	17
47.	Thyme	0	16	25
48.	Toxicanum	0	10	0
49.	Yellow root	0	0	0
50.	Witch hazel	18	24	33

Zone of diffusion = refers to the physical diffusion of the plant extract. Typically, this appears as a color change in the agar that cannot be attributed to zone of inhibition or QSI activity.
*Sample has two QSI zones.

Images of QSIS2 Results of the Folk-Medicinal Crude Extracts

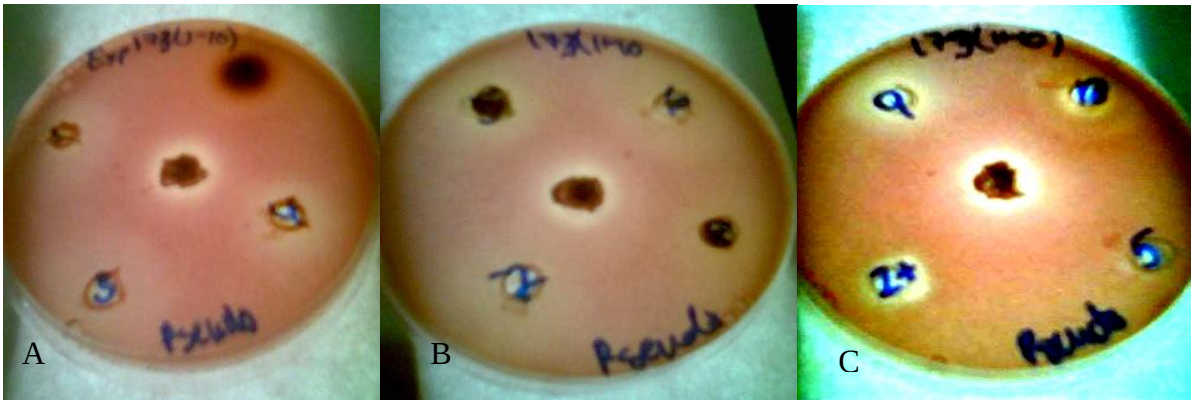


Figure 14: QSI results samples screened with the QSIS2 assay.
(A) (1) sassafras, (2) red root, (3) ginger, (4) rattlesnake master.
(B) (5) hyssop herb, (6) hymenocrator, (7) crysanthumum, (8) mint and 9) cinnamon
(C) (9) lemon rind, (10) grapefruit rind, and (11) olive leaf, (2+) garlic (positive control),

(5) internal standard.

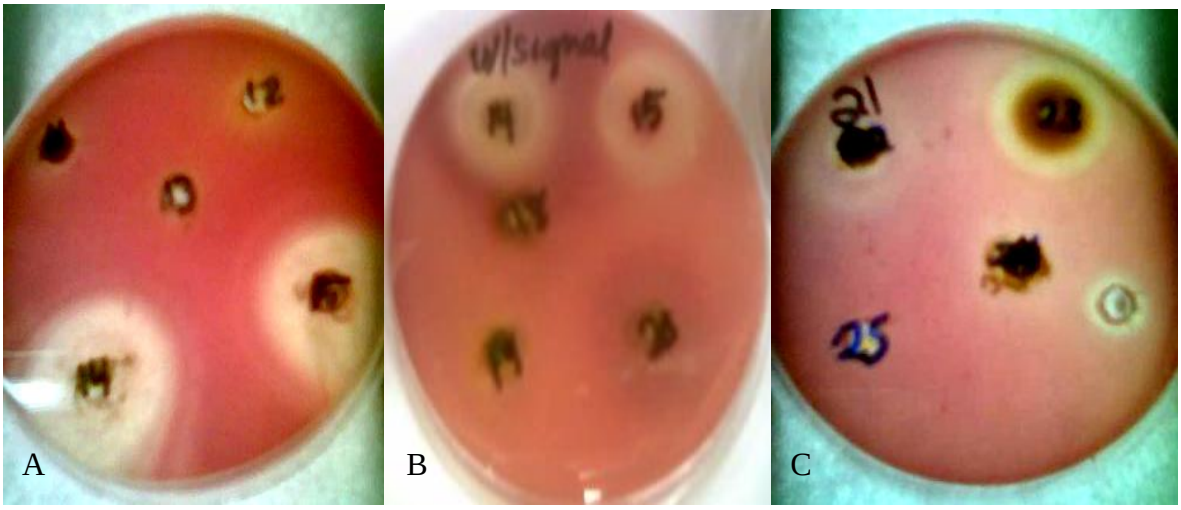


Figure 15: QSI results of crude extracts screened with the QSIS2 assay.
(A) (11) olive leaf, (12) toxicanum, (13) bamboo leaves, (14) clove, and (15) cranberry.
(B) (14) clove, (15) cranberry, (18) silverberry leaves, (19) holly leaves and (20) bay leaves.
(C) (21) rosemary, (23) pomegranate, (24) neem, and (25) mustard seed.

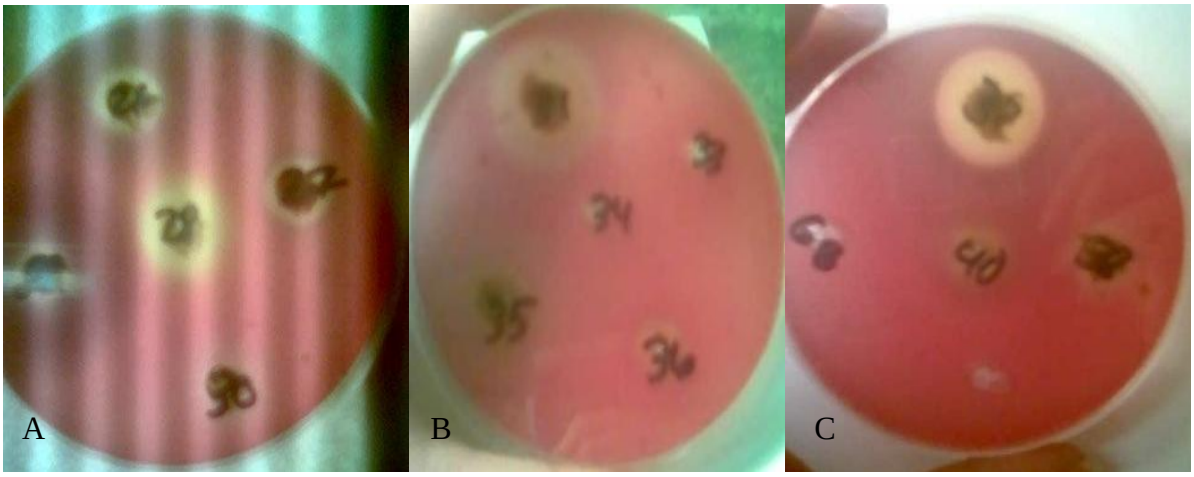


Figure 16: QSI results of crude extracts screened with the QSIS2 assay.
(A) (26) elder berry, (27) sweet gum fruit, (28) leather leaf mahonia, (29) sage, and (30) dogwood bark.
(B) (31) chaparral, (33) Chinese wolfberry, (34) boneset, (35) heath aster, and (36) fenugreek
(C) (37) oregano, (38) fennel, and (40) lobelia.

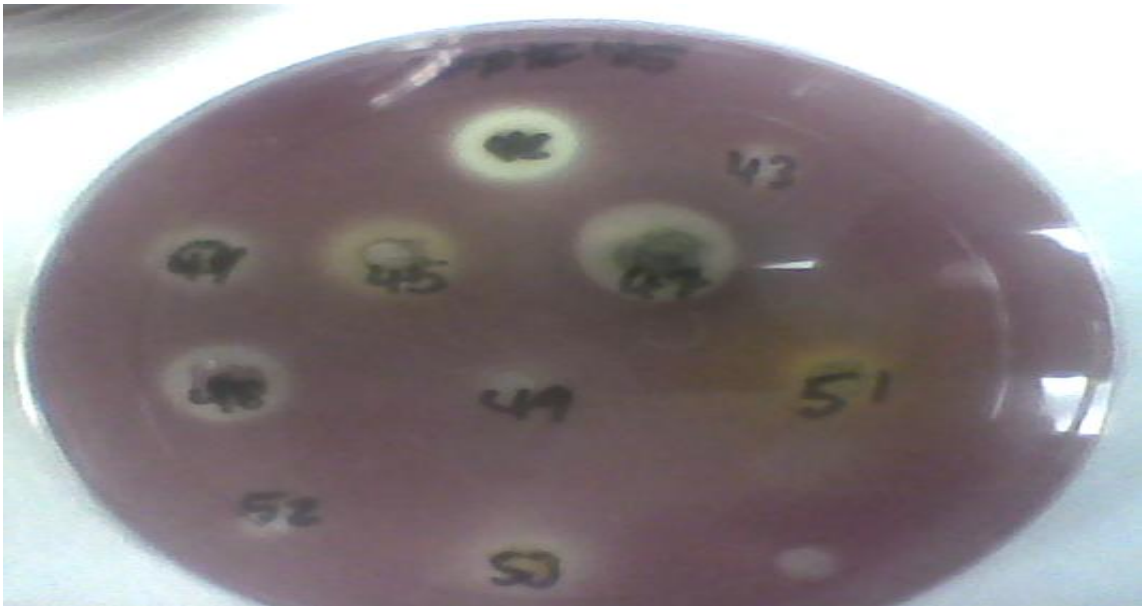


Figure 17: QSI results samples of crude extracts screened with the QSIS2 assay.
(42) witch hazel, (43) red ginseng, (44) Chinese dandelion, (45) lo han kuo, (47) thyme, (48) cinnamon, (49) holly berry, (51) yellow root, (52) American dandelion, and (53) American spikenard.

Glucose and pH levels of the Crude Extracts

Forty-two out of fifty samples were tested for pH as shown in Table 3. All samples had an acidic pH with the exception of the yellow root extract (pH 7.0). Yellow root did not show any QSI activity with the QSIS2 assay. Therefore, the pH did not seem to affect the QSIS2 assay results for this sample.

Thirty-six extracts were tested for glucose concentration, as seen below in Table 3. Preliminary sugar investigations consisting of analysis of various sugar dilutions (glucose, fructose, dextrose and sucrose) were preformed to determine which concentrations yielded false positive results with the QSIS2 assay. Glucose and dextrose did yield false positive zones of growth halos with the QSIS2 assay. Interestingly, fructose and sucrose did not affect the assay results (*data not shown*). This data suggested that samples with glucose reading of 254 mg/dL

would produce false positive halo rings of 14 mm in diameter, with the QSIS2 assay. Thirteen of the samples had a total glucose concentration of 254 mg/mL or below.

Table 3: Glucose Concentration and pH Readings of the Folk-Medicinal Extracts.

Sample Name	pH	Glucose Concentration (mg/dL)	Average glucose value (mg/dL)	QSI assay equivalent concentrations (mg/dL)
American dandelion	4.5	169, 188, 173	176.7	441.7
Bamboo	5	479, 600, 599	599	1398.3
Basil Leaves	5.0	94, 89, 87	90	225
Bay leaves	4.5	275, 276, 276	275.5	689.2
Bitter leaf	4.0	—	—	—
Boneset	4.5	—	—	—
Chaparral	4.0	59, 65, 58	60.7	151.7
Chinese dandelion	4.0	348, 350, 414	370.7	926.7
Chinese wolfberry	4.5	Hi, Hi, Hi	>660	>1650
Chrysanthemum	4	Hi, Hi, Hi	> 660	> 1650
Clove	2.5	85, 103, 88	92	230
Cranberry	—	Lo, Lo, Lo	> 20	> 50
Dogwood	4.0	Lo, Lo, Lo	>20	>50
Elder berry	4.5	—	—	—
Fennel	5	211, 279, 223	237.6	594
Ginger	5	84, 76, 81	80.3	200.8
Grapefruit rind	—	78, 70, 71	73	182.5

Sample Name	pH	Glucose Concentration (mg/dL)	Average glucose value (mg/dL)	QSI assay equivalent concentrations (mg/dL)
Heath aster	4.5	505, 533, —	519	1297.5
Holly berry	4.0	Hi, Hi, Hi	> 660	> 1650
Holly leaves	3.5	Hi, Hi, Hi	> 660	> 1650
Hymenocrater	5	Hi, Hi, Hi	> 660	> 1650
Hyssop	4.5	218, 175, Hi	—	—
Leather leaf mahonia	5.0	482, 435, 485	467.3	1168
Lemon rind	3	259, 258, 293	270	675
Lobelia	5	—	—	—
Lo han kuo	3.0	139, 128, 148	138	345.8
Mint	4	30, 30, 30	30	75
Mustard seed	6.0	—	—	—
Neem tree	4.0	—	—	—
Olive leaf	3	78, 70, 71	421	1053
Oregano	4	70,50,58	59.3	148
Pomegranate	2.0	115, 118, 117	116.7	291.7
Rattlesnake master	3	21, —,—	21	52.5
Red ginseng	4.0	Hi, Hi, Hi	> 660	> 1650
Red root	4	203, 242, 245	230	575
Rosemary	4.5	46, 48, 45	139	347.5
Sage	4.0	125, 119, 120	121.3	357.9

Sample Name	pH	Glucose Concentration (mg/dL)	Average glucose value (mg/dL)	QSI assay equivalent concentrations (mg/dL)
Sassafras	4	Lo, Lo, Lo	> 20	> 50
Silver berry leaves	6.0	38, 39, 38	39	97.5
Sweet gum fruit	3.0	—	—	—
Thyme	4.0	56, 76, 52	61.3	153.5
T toxicanum	5	366, 318, 339	341	852.5
Yellow root	7	—	—	—
Witch hazel	2.5	91, 51, 45	62.3	155.8

Crudes extracts were dissolved in water for both glucose and pH assessments at a concentration of 200 mg/mL. Lo = indicates glucose concentration below 20 mg/dl; Hi= glucose concentration content above 660 mg/dl.

Chromobacterial QSI Assay Results of the Folk-Medicinal Extracts

Table 4 lists the Chromobacterial QSI assay results of the extracts tested. Extracts were tested in triplicate and recorded as trials A, B, and C. Nineteen out of the forty-five extracts produced QSI zones of 10 mm or greater. QSI zones can be seen as opaque zones of growth with inhibited pigment production in the corresponding photos (Figures 18-20). The largest zone of activity (39 mm) was seen with the clove extract. Nine of the samples also produced a second zone which appeared to have increased pigment production.

Table 4: QSI Results of the Folk-Medicinal Plants Extracts using the Chromobacterial QSI Assay.

Samples	Zone of QSI activity (mm)			
	A	B	C	Average
American dandelion	8	8	8	8
American spikenard	14	10*	18*	14*
Bamboo	10	10	11	10
Basil leaves	14*	15*	12	14*
Bay leaves	21*	15*	18*	18*
Boneset	10	8	8	9
Brown seaweed	8	8	8	8
Chaparral	18	18*	25	20
Chinese dandelion	10	10	8	9
Chinese wolfberry	10	8	8	9
Chrysanthemum	10	9	10	10
Clove	40	43	32	39
Cranberry	19*	15*	12*	15*
Dogwood bark	8	8	8	8
Elder berry	12*	8	10	10
Fennel	12*	28*	25*	22*
Fenugreek	8	10	9	9
Garlic (positive control)	18	20	20	19
Ginger	11	10	10	10

Samples	**Zone of QSI activity (mm)**			
	A	B	C	Average
Grapefruit rind	9	9	10	9
Heath aster	12	16	10*	13
Holly berry	12	8	8	9
Holly leaves	8	8	8	8
Hymenocrater	9	9	10	9
Hyssop	12	12	11	12
Leather leaf mahonia	8, 30*	8, 30*	8, 30*	8, 30*
Lemon rind	9	9	10	9
Lo han kuo	10	8	9	9
Mint	10	9	10	10
Mustard seed	10	8	8	9
Neem tree	10	8	12	10
Olive leaves	8	8	8	8
Oregano	20	20	25	22
Pomegranate	23*	25*	27*	25*
Red ginseng	12	8	8	9
Red root	12	11	12	12
Rosemary	14*	12*	10	12*
Sage	19	20	21	20
Sassafras	20	22	20	21

Samples	Zone of QSI activity (mm)			
	A	B	C	Average
Silverberry leaves	8	8	8	8
Sweet gum fruit	8	8, 20*	8	8, 20*
Thyme	12	15	11	13
Toxicanum	10 *	10	12	11
Yellow root	12	8	11	10
Witch hazel	22	25	25	24

* Indicates a second darker purple zone was present around the well.

Images of Chromobacterial QSI Assay Results of the Folk-Medicinal Extracts

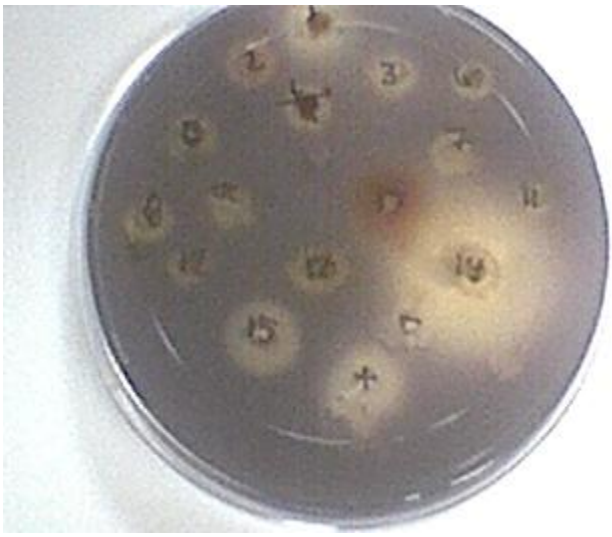


Figure 18: QSI results of crude samples screened with the Chromobacterial QSI assay. (1) Sassafras, (2) red root, (3) ginger, (5) hyssop, (6) hymenocrater, (7) chrysanthemum, (8) mint, (9) lemon rind, (10) grapefruit rind, (11) olive leaf, (12) toxicanum, (13) bamboo, (14) clove, (15) cranberry, and (+) positive control toluene garlic extract.

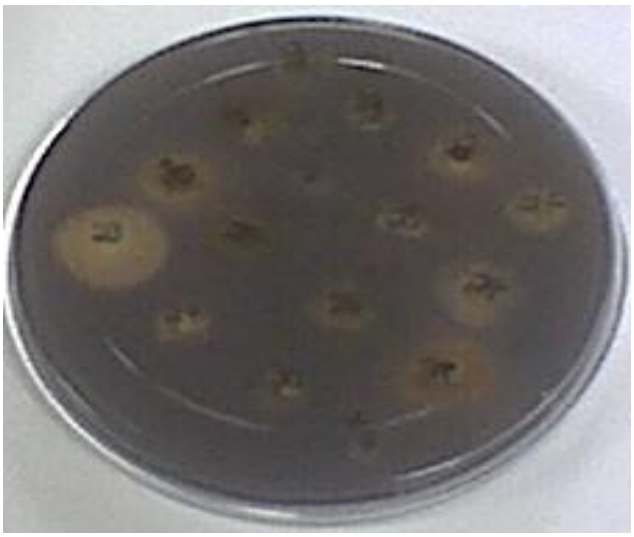


Figure 19: QSI results of the crude extracts screened with the Chromobacterial QSI assay. (17) Brown sea weed, (18) silverberry leaves, (19) holly leaves, (20) bay leaves, (21) rosemary, **(**23) pomegranate, (24) neem, (25) mustard seed, (26) elder berry, (27) sweet gum fruit, (28) leather leaf mahonia, (29) sage, (30) dogwood bark, and (31) chaparral.

Figure 20: QSI results from crude extracts screened with the Chromobacterial QSI assay. (33) Chinese wolfberry, (34) boneset, (35) heath aster, (36) fenugreek, (37) oregano, (38) fennel, (42) witch hazel, (43) red ginseng, (44) Chinese dandelion, (45) lo han kuo, (47) thyme, (49) holly berry, (51) yellow root, (52) American dandelion, and (53) American spikenard.

RESULTS: THE THYME-TETRACYCLINE EFFECT

Disc Diffusion Assay of Ethanol Extract of the Thyme Leaves

Table 5 shows the DDA results of the ethanolic extract of the thyme leaves demonstrating the lowest concentration of the extract which would exhibit inhibition activity against *P. aeruginosa* ATCC 10145. The lowest usable concentration of this extract was a dilution of 250 mg/mL (262), showing zone sizes of 8 mm and 7 mm with cultures grown on both the Mueller Hinton II (MH) and Tryptic soy agar (TSA), respectively. Activity was only seen with the addition of tetracycline in the agar at this concentration. At a higher concentration of 500 mg/mL (Figure 21) zone of inhibition diameters were increased from 8 mm to 11 mm with the addition of tetracycline. Overall observations included: (1) Tetracycline in either of the agars augments activity of the extract. (2) Larger zones of inhibition were observed in those cultures grown on MH as compared to those grown on TSA. (3) Concentrations of 500 mg/mL at 20 μL per disc produced the most consistent results on the MH agar.

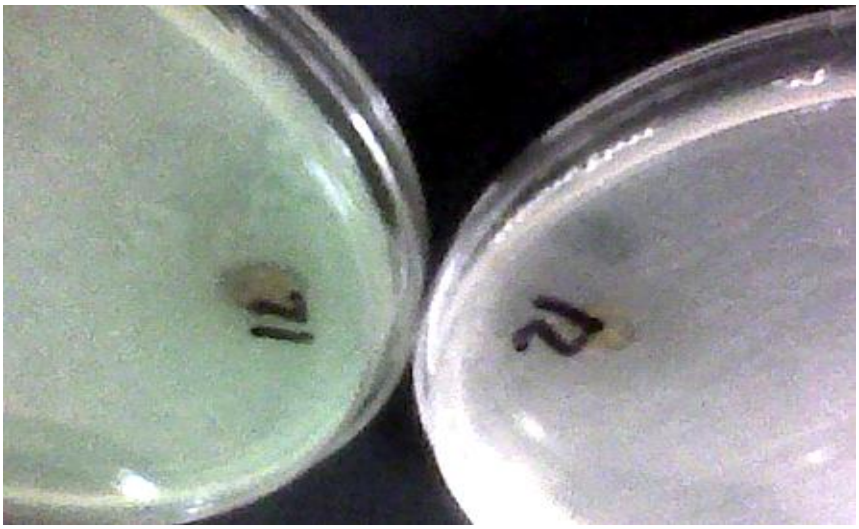


Figure 21: DDA of ethanol extract of *Thymus vulgaris* leaves at a 500 mg/mL concentration. Plate on left is Mueller Hinton II without tetracycline; plate on right is Mueller Hinton II with tetracycline.

Table 5: DDA of Ethanol Extract of *Thymus vulgaris* Leaves.

Sample description	Concentration (mg/mL)	MH (mm)	MH with tetracycline* (mm)	TSA (mm)	TSA with tetracycline* (mm)
261	500	8	11	0	7
262	250	0	8	0	7
263	125	0	0	0	0
264	62.5	0	0	0	0
Positive control (Tobramycin)	1 μg/mL	27	27	23	22
Negative control (Blank disc)	—	0	0	0	0

Inhibition values are an average of 4 replicates. Diameter of disc 6 mm, 20 μL of plant extract per disc, and 10 μL of tobramycin per disc.
*50 μg/mL of tetracycline was used.

Identification of Active Compound(s) by Thin-Layer Chromatography–Bioassay.

Figure 22 show the results of an overlay assay on a 2D thin-layer chromatography (TLC) plate which was used to separate the ethanolic leaf extract into its individual components. Activity appeared to be isolated to a single zone of inhibition located at the top third of the plate. In Figure 22, the arrow indicates the probable location of the active component(s). This compound is later referred to as compound #2. The zone of inhibition is seen regardless of the addition of tetracycline to the agar.

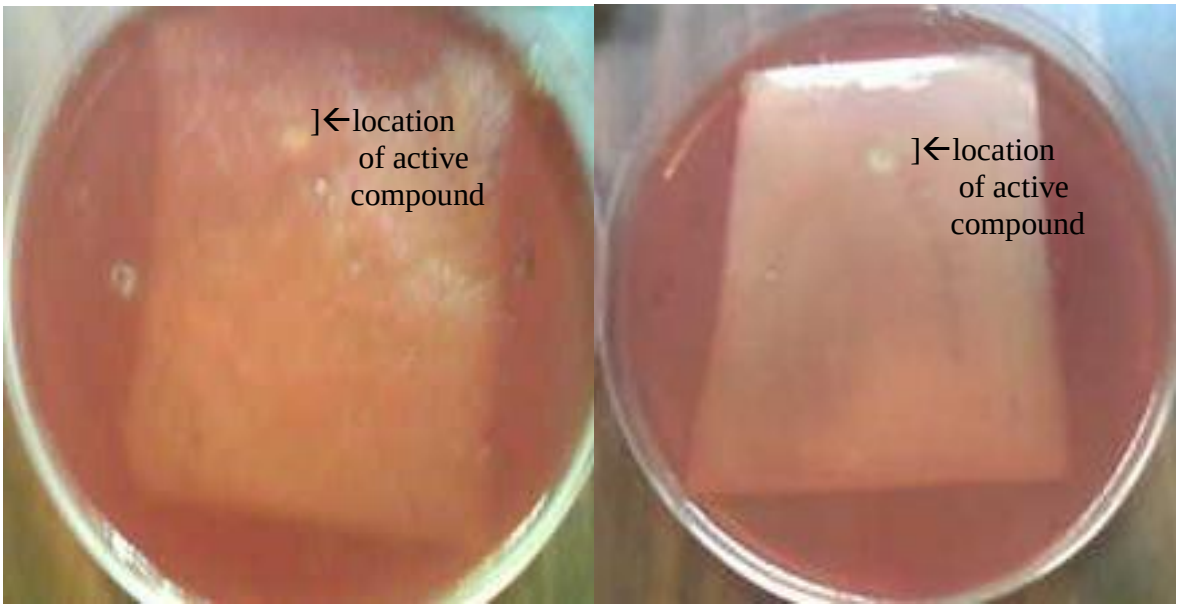


Figure 22: 2D-TBA of the thyme leaf extract.
(A) Without tetracycline in the agar.
(B) This agar contains 50 µg/mL of tetracycline; a clear zone of inhibition is seen around the active component, which is located on the top third of the plate.

Extraction of the Thyme Plant

Comparison of thyme leaves extracted in various solvents.

Figure 23 show the TLC results of thyme leaves extracted with various solvents (acetone, chloroform, ethanol, ethyl acetate, hexane, methanol, methylene chloride, and petroleum ether). Table 6 shows the corresponding percentage yield. Based on TLC results, it appears that the active component can be extracted in all the solvents tested, but the hexane extract contained the least number of inactive compounds. Hexane was also an easier solvent to work with based on its innate properties such as boiling point and hexane's ability to remove the least amount of undesirable compounds. This extract was used in several subsequent experiments.

Figure 23: TLC comparison of crude extracts of thyme leaves prepared with various solvents. Thyme leaves extracted in (by row): (1) ethanol, (2) hexane, (3) petroleum ether, (4) methylene chloride, (5) chloroform, (6) ethyl acetate, (7) acetone, (8) ethanol, (9) methanol, and (10) ethanol and (11) stems extracted in ethanol.

Table 6: Percentage Yield of Crude Extracts Produced by Extraction of Thyme Leaves in Various Solvents.

Sample description	Starting material dry weight (g)	Crude weight (g)	Yield (%)
Hexane	8.0	0.25	3.13
Petroleum ether	8.0	0.20	2.50
Methylene chloride	8.0	0.60	7.50
Chloroform	8.0	0.95	11.88
Ethyl acetate*	8.0	0.90	11.25
Acetone	8.0	1.00	12.50
Ethanol*	8.0	0.90	11.25
Methanol	8.0	0.75	9.38

Most extracts were prepared in duplicate values and are an average of two aliquots.
* Extracts not made in duplicate.

Gradient extraction of thyme leaves using recycled and non-recycled hexane.

Figure 24 shows the TLC of eight consecutive hexane filtrates. TLC results were taken of each hexane filtrate prior to combining with the previous days filtrate. Table 7 shows the

corresponding percentage yields. Based on TLC results, it appeared that the active compound(s) could be collected by three consecutive hexane filtrations 163 and 164. By the forth filtration, a second group of compounds were being extracted, those possessing a more polar nature. Graduated extraction of the thyme leaves in hexane showed that there are definite increases in the polarity of compounds removed from the bulk material as subsequent filtrates were collected. Thin-layer chromatography results (Figure 24) also revealed that by the forth extraction, the all-similar nonpolar compounds have been removed. In addition, it was interesting that there was a definite difference in the compounds when extracted with non-recycled solvent (left rows) verses recycled solvent (right rows) (Figure 24).

Figure 24: TLC results of graduated extraction of thyme leaves using recycled and non-recycled hexane.
Rows left to right are samples 152-166, ethanol leaf extract, plus thymol and carvacrol standards.

Table 7: Percent Yield from Gradient extraction of Thyme Leaves using Recycled and Non-recycled (Fresh) Hexane.

Sample	Description	Days soaked	Starting material dry weight (g)	Crude weight (g)	Yield (%)
152	Fresh hexane filtrate 1	24-hour soak	8.0	0.1	1.25
161	Fresh hexane filtrate 2	9 days	8.0	0.2*	2.5*
163	Fresh hexane filtrate 3	4 days	8.0	0.5	6.25
165	Fresh hexane filtrate 4	2 days	8.0	0.1	1.25
160	Recycled hexane filtrate 1	24 hrs	8.0	0.1	1.25
162	Recycled hexane filtrate 2	9 days	8.0	0.2*	2.5*
164	Recycled hexane filtrate 3	4 days	8.0	0.05	0.625
166	Recycled hexane filtrate 4	2 days	8.0	0.05	0.625

*Approximate values

Hexane, methanol, ethanol extractions using non-recycled solvent each of three soaks.

Figure 25 depicts TLC results of *Thymus vulgaris* leaves were consecutively extracted a total of three 24-hour soaks with non-recycled hexane, methanol, or ethanol. Figure 25 depicts the thin-layer chromatography results of these fractions. Table 8 shows the DDA results of the combined crude extracts for each solvent: ethanol aliquots (270 and 271), methanol (272 and 273), and hexane (274 and 275) using MH agar. Table 9 shows the percentage yield of all extracts. Disc diffusion assay results revealed that both methanol aliquots (272 and 273) showed an increase in inhibition activity. Zone values increased from 8 mm to 13 mm with the addition of tetracycline in the MH agar.

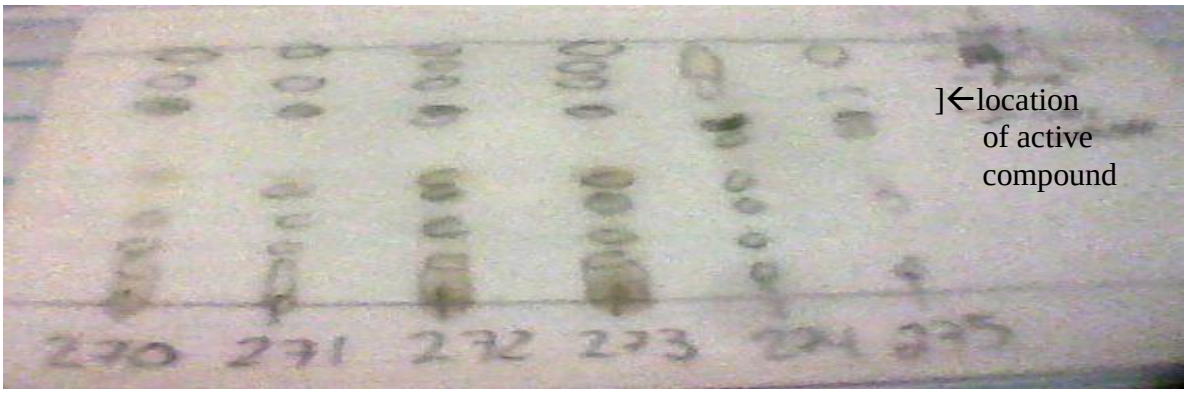


Figure 25: TLC of extracts samples 270-275 (thyme leaves) extracted in hexane, methanol, and ethanol, prepared using non-recycled solvent with each of three soaks.

Table 8: Disc Diffusion Assay Results of the Crude Extracts made with Non-recycled Solvent for each Extraction in Hexane, Methanol, and Ethanol of *Thymus vulgaris* Leaves.

Sample	Description	Mueller Hinton II (mm)	Mueller Hinton II with tetracycline (mm)
270	Ethanol crude A	7	10
271	Ethanol crude B	—	7
272	Methanol crude A	8	13
273	Methanol crude B	7	13
274	Hexane crude A	0	0
275	Hexane crude B	0	0

Experiment run in duplicate, values are an average of two runs. Two aliquots of plant material (A and B) were extracted and tested. Each extract was tested at a 333 mg/mL concentration.

Table 9: Percentage Yield of Crude Extracts made with Non-recycled Solvent.

Sample	Description	Starting material dry weight (g)	Crude weight (g)	Yield (%)
270	Ethanol crude A	10.0	0.6	6.0
271	Ethanol crude B	10.0	2.3	23.0
272	Methanol crude A	10.0	1.0	10.0
273	Methanol crude B	10.0	1.6	16.0
274	Hexane crude A	10.0	0.2	2.0
275	Hexane crude B	10.0	0.4	4.0

Two aliquots of plant material (A and B) were extracted and tested.

Hexane, methanol, and ethanol resoaks using recycled solvent each of three soaks.

Figure 26-A shows TLC results of extractions of thyme leaf samples extracted several times using recycled solvent for each of three 24-hour soak each time. Figure 26-B shows the corresponding TBA. Tables 10 and 11 (respectively) show DDA and percentage yield results for the samples tested. Disc diffusion assay results (Table 10) showed that increased inhibition activity was seen with the addition of tetracycline to the agar in all samples that were tested, while the methanol leaf extract (301) showed the greatest increase in activity with the addition of tetracycline to the MH agar. Zone values of the methanol leaf extract showed increases in activity from 9 mm to 21 mm.

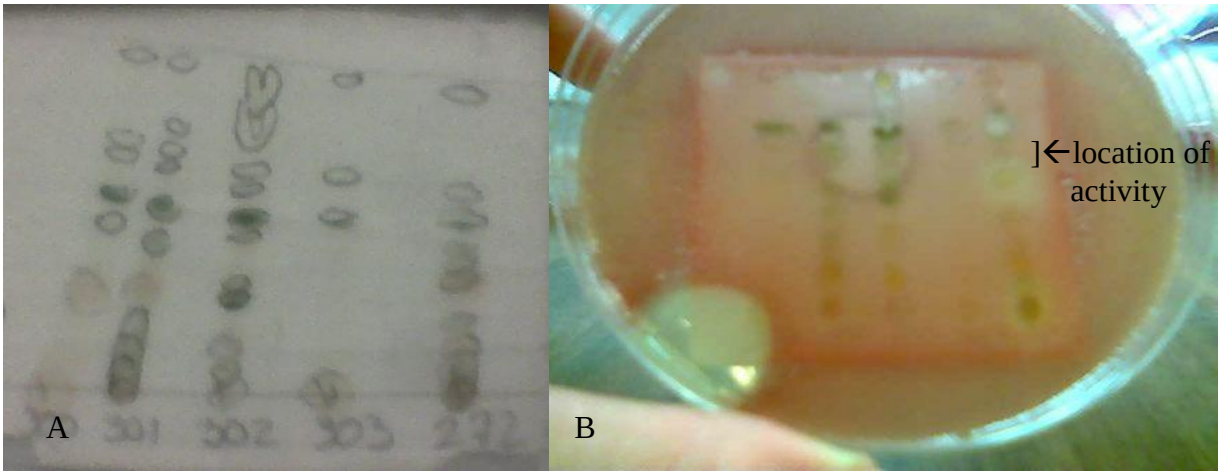


Figure 26: TLC of thyme leaf extracts (Samples 300-303, 272) extracted with hexane, methanol, and ethanol resoaks using recycled solvents with each soak. (A) TLC (B) TBA

Table 10: Disc Diffusion Assay of Hexane, Methanol, and Ethanol Resoaks using Recycled Solvent (300-303).

Sample	Description	Mueller Hinton II (mm)	Mueller Hinton II with tetracycline (mm)
300	Ethanol crude	8	17
301	Methanol crude	9	21
302	Hexane crude	0	10
303	Methanol stem crude	0	14
Positive control	Tobramycin (20 µl)	35	37
Negative control	Empty	60	0

Concentration of sample on disc (20 µl) was 333 mg/mL.
Experiment was run in duplicate; zone values are an average of two runs.

Table 11: Percentage Yield of Samples 300-303.

Sample	Description	Starting material dry weight (g)	Crude weight (g)	Yield (%)
300	Ethanol crude	10.0	1.2	12.0
301	Methanol crude	10.0	1.6	16.0
302	Hexane crude	10.0	1.7	17.0
303	Methanol stem crude	10.0	0.7	6.8

Comparison of extractions of three soaks verses one soak using non-recycled solvent each time.

Figure 27 depicts the TLC results of the thyme leaves extracted in non-recycled solvents comparing the three 24-hour soaks versus the one 24-hour soak of the bulk material. Table 12 shows the corresponding percentage yield. Disc diffusion assay results (shown in Table 13) showed that inhibition activity was seen in the E3 (305) sample and the M1, M3 (306, 307) samples for the MH without tetracycline in the agar. The results are quite different when tetracycline is in the agar. Inhibition zones greatly increase. The greatest increase in zone diameters can be seen with sample M1 (306) sample, producing zones of 18 mm. After the third soak, the inhibitive quality of the crude was reduced to 15 mm. This suggested that the most

active components are most readily removed using methanol during a single 24-hour extraction of the thyme leaves. The third soak of E3 (305) showed a higher activity than the E1 (304) single-soak sample. Zone diameters can be seen increasing from 12 mm to 15 mm. Minimal zone differences can be seen between the H1 (308) and the H3 (309) samples. Interestingly, with the methanol stem single soak (MS1) and the stem three soak (MS3). There is no activity in the MH without tetracycline but there is considerable activity with the MH with tetracycline. Also there is a considerable decrease in activity between the single soak and the three soak samples. MS1 produced zones of 16 mm while MS3 produced zones of 11 mm.

For maximum extraction of the active component(s) based on DDA results, a single 24-hour methanol soak of the leaves is sufficient to remove the active components from the plant. Therefore, while the hexane solvent is easier to handle based on innate solvent properties, the most potent crude is produced with methanol.

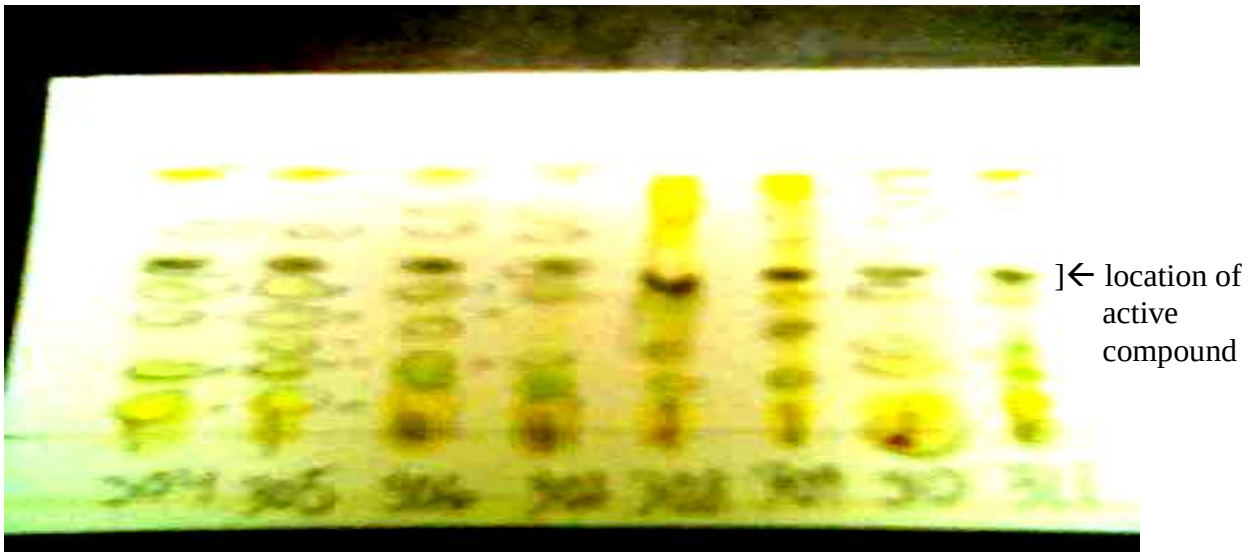


Figure 27: TLC comparison of three soaks verses one soak. Rows (Samples 304-311) are thyme samples extracted in non-recycled solvent (ethanol, methanol, hexane, methanol stem extracts).

Table 12: Percentage Yield of Crude Samples (304-311) Comparing Three Soaks Verses One Soak.

Sample	Description	Starting material dry weight (g)	Crude weight (g)	Yield (%)
304	Ethanol single soak (E1)	10.0	0.28	2.8
305	Ethanol three soaks (E3)	10.0	1.82	18.2
306	Methanol single soak (M1)	10.0	0.52	5.2
307	Methanol three soaks (M3)	10.0	1.16	11.6
308	Hexane single soak (H1)	10.0	0.14	1.4
309	Hexane three soaks (H3)	10.0	0.38	3.8
310	Methanol stem single soak (MS1)	10.0	0.21	2.1
311	Methanol stem three soaks (MS3)	10.0	0.34	3.4

Table 13: Disc Diffusion Assay of Crude Samples (304-311) Comparing Three Soaks Verses One Soak.

Sample	Description	Mueller Hinton II (mm)	Mueller Hinton II with tetracycline (mm)
304	Ethanol single soak (E1)	0	12
305	Ethanol three soaks (E3)	8	15
306	Methanol single soak (M1)	9	18
307	Methanol three soaks (M3)	8	15
308	Hexane single soak (H1)	0	11
309	Hexane three soaks (H3)	0	12
310	Methanol stem single soak (MS1)	0	16
311	Methanol stem three soaks (MS3)	0	11
Positive control	Tobramycin (20 µL per disc)	36	37
Negative control	Empty	0	0

Zone values are from an average of two experiments.

Column Chromatography of the Methanol Crude Extract

Column chromatography of the methanol single-soak crude extract (sample 307) produced four fractions. These fractions are the hexane (312), the methylene chloride (313), the acetone (314) and the methanol (315) fractions. Table 14 shows the DDA results of the methanol

crude of leaves and the four corresponding fractions. The greatest amount of activity was seen with the methylene chloride fraction (313), which showed an increase in zone sizes from 12 mm to 16 mm with the addition of tetracycline. The methanol fraction also showed an increase in zone size but only with the addition of tetracycline in the agar. Figure 28-B is a sketch of TBA results performed on the methylene chloride fraction (313), and a significant zone of inhibition was observed in the middle of the fraction lane on the TLC plate (Figure 28). This zone encircled the dark green, green, and yellow and green spots on the plate. TBA and DDA results both indicated that the highest concentration of the active component can be isolated in the center of the methylene chloride fraction of the methanol crude on the TLC plate.

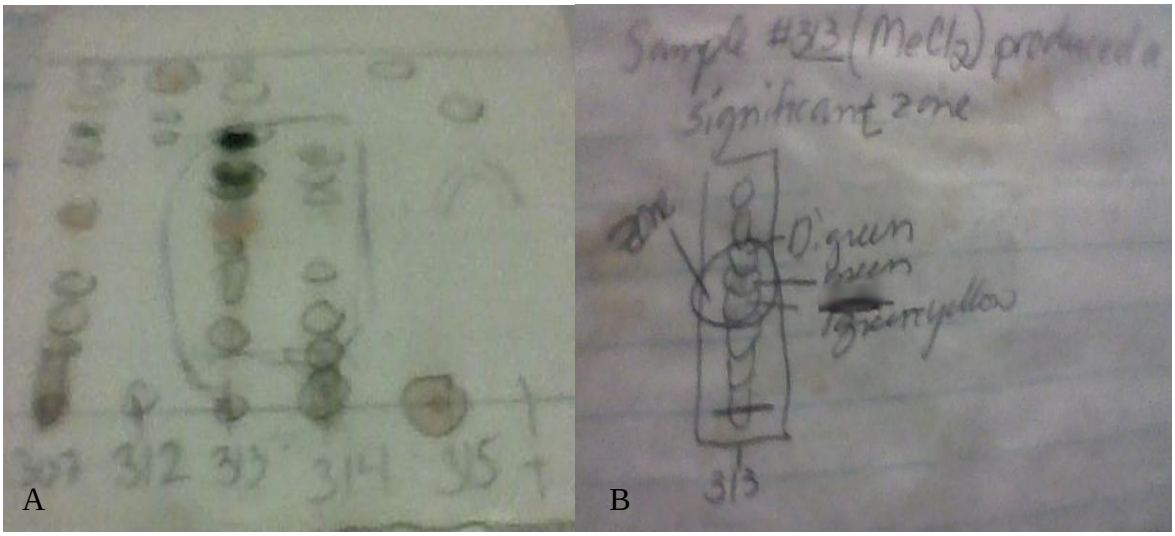


Figure 28: TLC of the methanol crude (307) and the corresponding fractions.
(A) hexane (312), methylene chloride fraction (313), acetone fraction (314), methanol fraction (315). (B) Sketch of TBA results for the Methylene chloride fraction (313)**.** The circled region in sketch B indicates the zone of inhibition seen on the TBA.

Table 14: DDA Results of the Methanol Crude Extract and its Four Corresponding Fractions.

Sample	Description	Mueller Hinton II (mm)	Mueller Hinton II with tetracycline (mm)
307	Methanol crude	14	18
312	Hexane fraction	0	0
313	Methylene chloride fraction	12	16
314	Acetone fraction	11	12
315	Methanol fraction	0	10
Positive control	Tobramycin (20 µl per disc)	33	35
Negative control	Blank disc	0	0

Fractions were re-diluted into their corresponding elution solvent at a concentration of 500 g/mL. Experiments ran in duplicate, values are an average of these experiments.

Examination of the Four Major Constituents of the Thyme Plant.

Examination of the four major constituents by thin-layer chromatography.

(Figure 29) of the four constituents revealed that rosmarinic acid and baicalein did not show any migration distance. This suggests that baicalein and rosmarinic acid samples are probably not the active component, since they did not travel up the TLC plate in the 8:4:1 hexane, methylene chloride, acetone solvent system. Thymol and carvacrol both traveled two-thirds up the plate. This was the approximate distance traveled by the active compound #2.

]←location of activity

Figure 29: TLC of four major constituents.
This is the TLC plate of the ethanol and hexane crude extracts of thyme plus the four major constituents. Rows: (1) ethanol crude of thyme leaves, (2) thymol, (3) rosmarinic acid, (4) baicalein, (5) carvacrol, (6) internal control, and (7) hexane leaf extract.

Comparisons of the Four Fractions and the Four Major Constituents

Comparisons of the fractions and the four major constituents by TLC and TBA

Figure 30-A is a sketch of the TLC plate seen in the subsequent, TBA (Figure 30-B). Zones of inhibition were seen around active samples. The three inhibition zones seen from left to right were located on the hexane crude, the thymol standard, and on the bottom-right tobramycin. The hexane crude was chosen for two reasons: (1) during a previous DDA (Table 11), this sample showed an increase in inhibition activity but only with the addition of tetracycline in the agar and (2) There are a minimum number of undesirable compounds in the sample. Figure 31 is a close-up of the hexane sample and the thymol standard. Clearly, the brightest area of the hexane inhibition zone was located in the center of the spot, which was in direct alignment with the thymol standard. These results (Figure 31) suggest that thymol may be the active component,

but that carvacrol was also in direct alignment with thymol on the TLC plate (Figure 29). This suggested that the active component was either thymol or carvacrol.

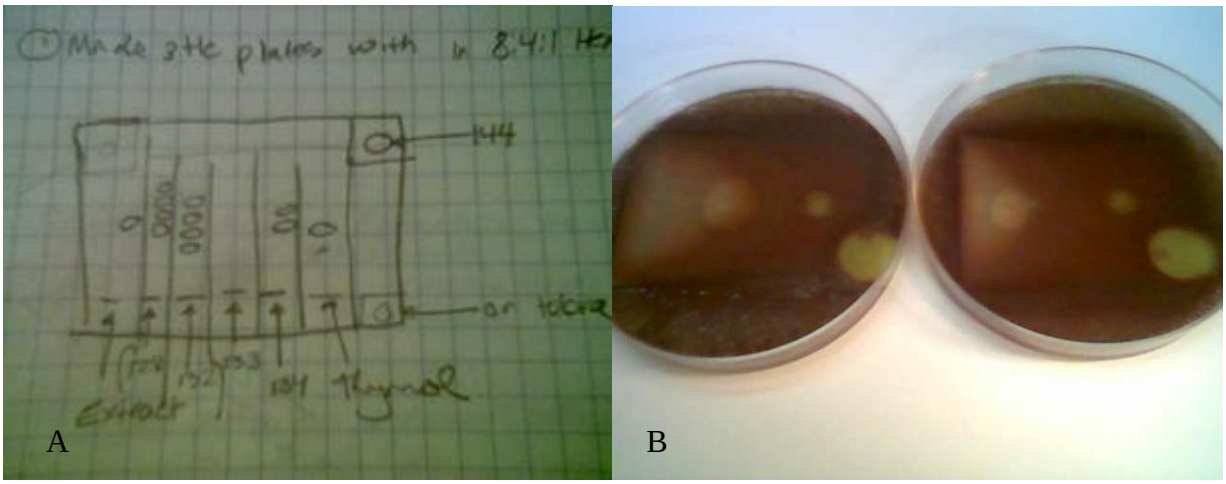


Figure 30: TLC diagram and TBA of hexane crude, thymol, and control.
(A) This is a diagram of the thin-layer chromatography plate featured in the thin-layer chromatography–bioassay (TBA).
(B) Zones of inhibition can be seen around hexane crude extract (128), the thymol standard, and the tetracycline-positive control.

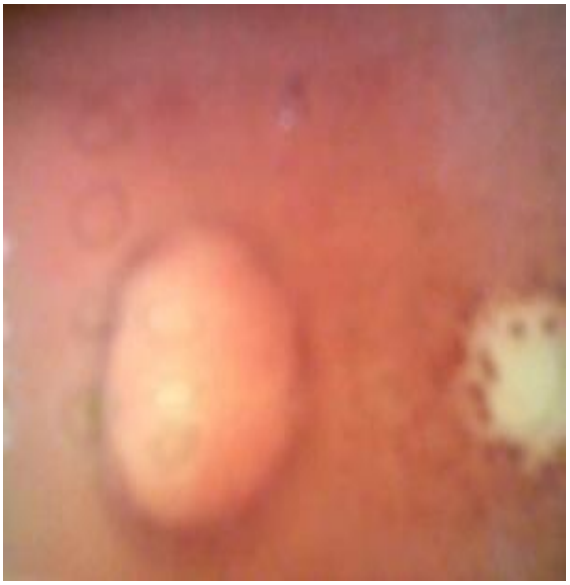


Figure 31: A close-up of rows of the above TBA.
Rows: hexane crude and thymol standard. Notice the spot on the left is brightest on the second of three spots this spot is in direct alignment with the spot on the right, the thymol standard.

Disc diffusion assay of major constituents at various concentrations

Table 15 shows the results from a DDA of the thyme plant four major constituents. Rosmarinic acid and baicalein showed no antimicrobial activity at the concentrations tested. Much higher concentrations were also tested without any activity (*data not shown*). Activity was seen with both thymol at concentrations of (4800 µg/mL) and carvacrol at concentrations of (480, 48, and 24 µg/mL) in both tetracycline and non-tetracycline samples. During a second DDA (Table 16), thymol and carvacrol standards were run at much higher concentrations. These experiments revealed that while both compounds showed antimicrobial activity, thymol showed an increase in antimicrobial activity with the addition of tetracycline to the agar. Zone size increased on average by 6 mm in diameter. Figure 32 visually depicts the increased thyme activity at 2 concentrations, 500 mg/mL and 250 mg/mL. Therefore, these results along with previous TLC (Figure 29) and TBA (Figures 30 and 31) suggested that thymol is the active component in thyme, involved in the thyme-tetracycline effect.

Table 15: DDA of Major Constituents at Low Concentrations.

Sample description	**Mueller Hinton II (mm)**	**Mueller Hinton II with tetracycline (mm)**
Baicalein (μg/mL)		
480	0	0
44	0	0
22	0	0
11	0	0
5.5	0	0
3.0	0	0
1.5	0	0
0.75	0	0
Thymol (μg/mL)		
4800 (in dark)	—	10
4800	—	10
480	0	0
48	0	0
24	0	0
12	0	0
6	—	0
3.0	0	0
1.5	0	—
0.75	0	0
Carvacrol (μl/mL)		
480	12	12
48	9	10
24	7	7
12	0	0
6	0	0
3	0	0
3.0	8	9
1.5	0	—
0.75	0	0
Rosmarinic acid (μg/mL)		
3.0	0	0
1.5	0	—
0.75	0	0
Positive control (Tobramycin)	26	25
Negative control (Blank disc)	0	0

Experiment run in triplicate; zone vales are average values of three experiments.

Table 16: Disc Diffusion Assay of Thymol and Carvacrol Constituents at Higher Concentrations.

Sample description/concentration	Mueller Hinton II	Mueller Hinton II with tetracycline
Carvacrol (mg/mL)		
1000	10*	10
500	10	10
15.6	8*	8
Thymol (mg/mL)		
500	11	15
250	9	14
125	8	8
31.25	8	9
Positive control (Ethanol leaf extract [500 mg/mL])	8	12
Negative control (Blank disc)	0	0

Experiment run in triplicate; zone vales are average values of three experiments
*Values are from an average of 2 experimental values instead of 3 replicates.

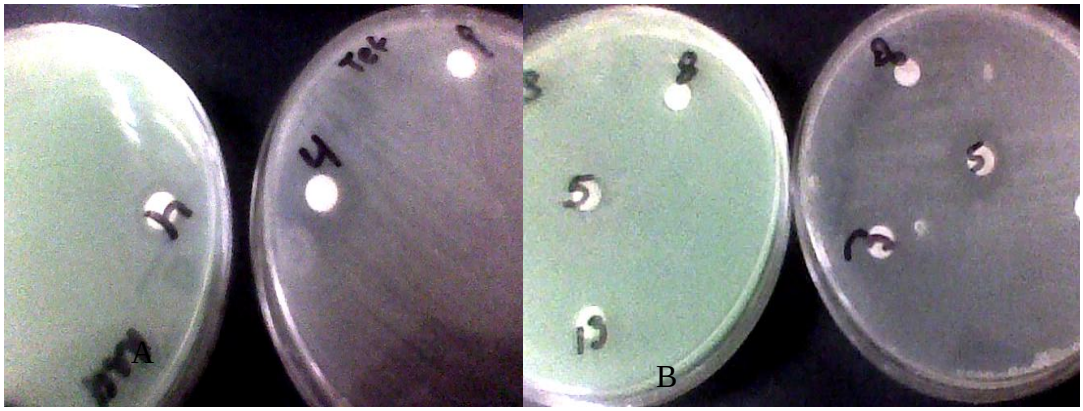


Figure 32: Disc Diffusion assay of thymol. Plate on the left is Mueller Hinton II agar without tetracycline, and plate on the right is Mueller Hinton II with tetracycline in the agar. Thymol tested at a concentration of (A) 500 mg/mL [4], (B) 250 mg/mL [5].

RESULTS: QSI ACTIVITY OF THYME AND ITS MAJOR CONSTITUENTS

QSI Activity of the Thyme Crude Extracts with the QSIS2 Assay

The results for the four crude thyme extracts chosen to assess potential anti–quorum-sensing activity with the QSIS2 assay are summarized in Table 17. The measurements are QSI zones of growth (red halos) and antimicrobial zones of inhibition (clear zones). Figure 33 is a compilation of images of representative samples demonstrating the visible activity.

The three polar extracts (ethanol signal soak crude [301], methanol single-soak crude [306], and methanol stem three-soak crude [311]), all showed considerable QSI activity. Bright red halos (seen in Figure 33 can be seen in photos B, C, and D. The most potent level of activity was seen with the methanol-stem three-soak crude extract, which produced a QSI zone of growth of 39 mm. The nonpolar hexane single-soak crude extract (308) shown in photo A of Figure 33 showed no activity at the concentration tested (0.0733 g/mL).

Table 17: QSI Activity of Crude Extracts with the QSIS2 Assay.

Sample number	Sample description (concentration)	Antimicrobial zones of inhibition (mm)			QSI zones of growth (mm)		
		A	B	C	A	B	C
301	Ethanol single-soak crude (1.000 g/mL) 301	22	17	+	27	30	+
306	Methanol single-soak crude (0.164 g/mL) 306	14	20	14	35	+	+
308	Hexane single-soak crude (0.0733 g/mL) 308	0	0	0	0	0	0
311	Methanol-stem three-soak crude (0.157 g/mL) 311	14	+	+	39	+*	+
	Positive control (Garlic)	15	—	—	25	—	—
	Negative control (Empty well)	0	—	—	0	—	—

* Indicates zones were present but very faint in color.
+ Indicates that a zone of growth was detected although a measurement was not recorded.

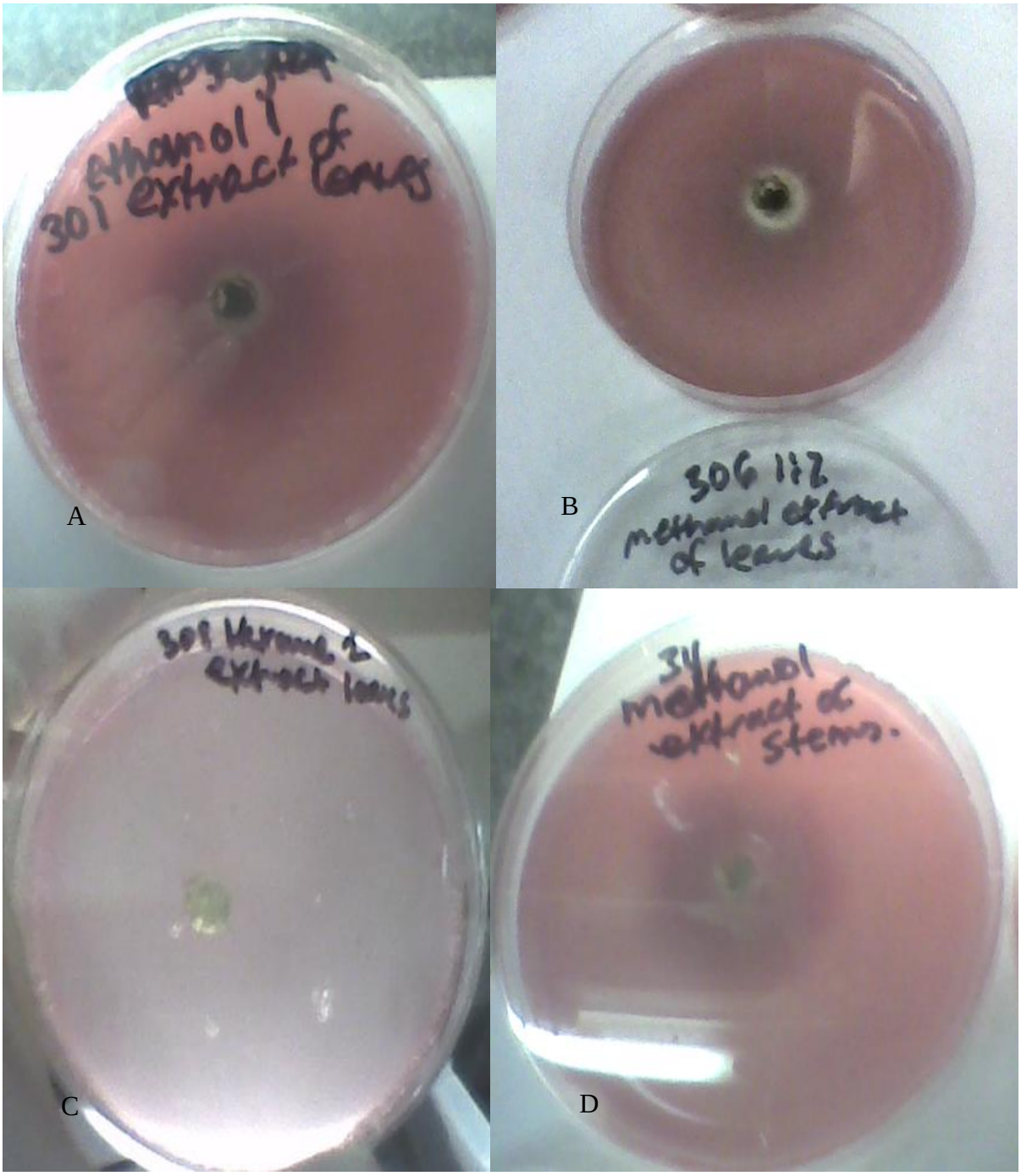


Figure 33: QSI results of various thyme extracts with the QSIS2 assay.
(A) Ethanol single-soak crude (301). (B) Methanol single-soak crude (306). (C) Hexane single-soak crude (308). (D) Methanol-stem three-soak crude (311).
* A dark red halo indicates QSI activity, and a clear zone indicates antimicrobial activity. Halos can be seen around A, B, and D (samples 301, 306, and 311).

QSI Activity of the Major Constituents Using the QSIS2 Assay

All four of the major constituents showed QSI activity with the QSIS2 reporter strain.

Baicalein -- Table 18 shows corresponding values of zones of inhibition and zones of growth. Figure 34 (A-C) depicts the results of baicalein tested at three concentrations: (A) 1µL/ mL, (B) 0.5 µL/mL, and (C) 0.125 µL/mL.

Table 18: QSI Activity of Baicalein Using the QSIS2 Assay.

Concentration (µg/mL)	Antimicrobial zone of inhibition (mm)	QSI zone of growth (mm)
4	0	20
1	0	17
0.5	0	18
0.125	0	14
Positive control (garlic)	15	25
Negative control (empty wells)	0	0

Baicalein was dissolved in methanol. This constituent was tested at various concentrations, one plate per concentration. These dilutions were not tested in triplicate.

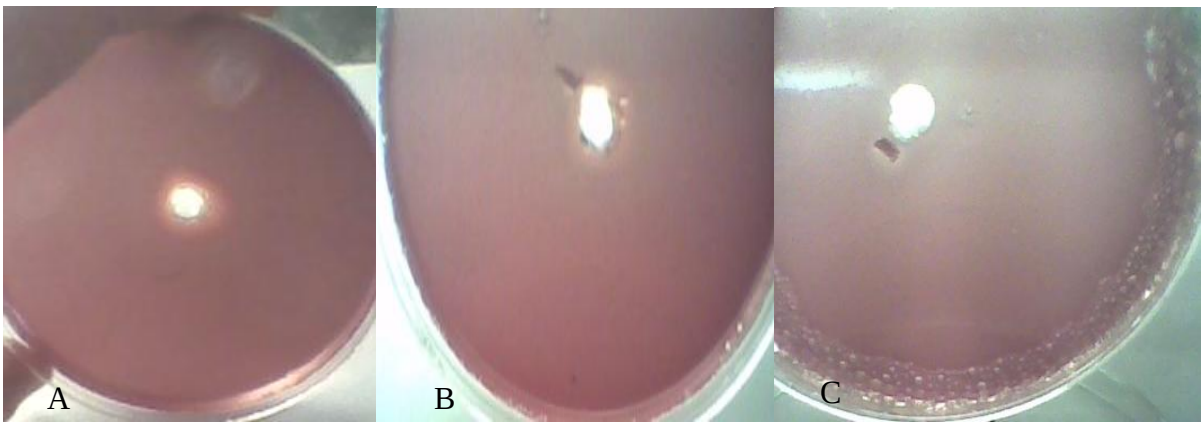


Figure 34: QSI activity of baicalein at various concentrations with the QSIS2 assay.
(A) 1 µg/mL. (B) 0.5 µg/mL. (C) 0.125 µg/mL.
Baicalein appears to produce QSI zones of growth at all three concentrations.

Thymol -- Table 19 shows the corresponding zone of inhibition and growth at concentrations of 500 mg/mL -3.9 mg/mL, constituent to solvent. Thymol showed potent antimicrobial and anti−quorum sensing activity at all the concentrations tested. Photos A and B in Figure 35 depict QSI results of thymol dissolved in methanol at concentrations 500 mg/mL and 7.8 mg/mL, respectively.

Table 19: QSI Activity of Thymol at Various Concentrations Using the QSIS2 Assay.

Concentrations (mg/mL)	Antimicrobial zone of inhibition (mm)	QSI zone of growth (mm)
500	45	+
250	46	+
125	42	+
62.5	31	+
31.3	21	+
15.6	17	26
7.8	15	22
3.9	11	12
Positive control (Garlic)	16	+
Negative control (Empty well)	0	0

+ Indicates that a zone of growth was detected although a measurement was not recorded. This constituent was tested at various concentrations, one plate per concentration. These dilutions were not tested in triplicate.

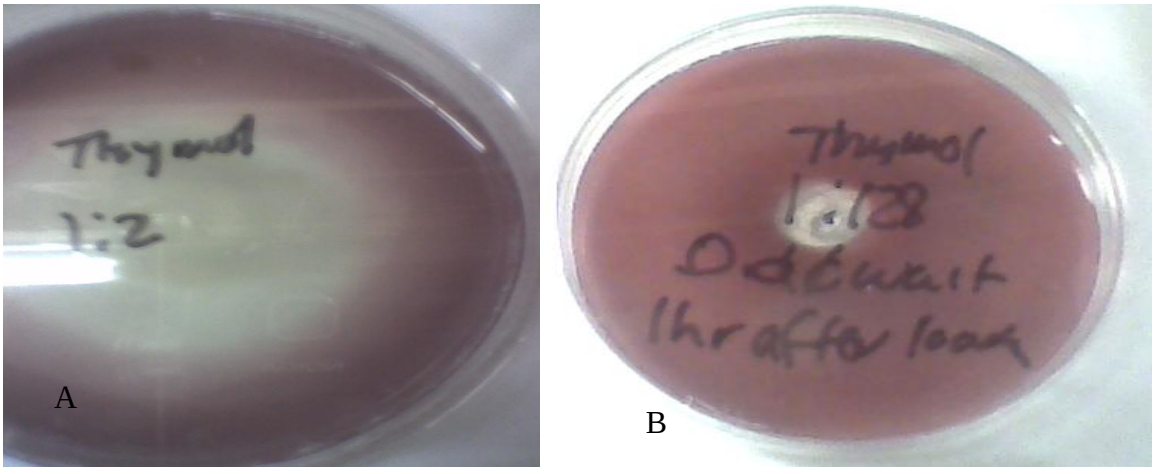


Figure 35: QSI activity of thymol at various concentrations with the QSIS2 assay.
(A) 500 mg/mL. (B) 7.8 mg/mL. Both of these concentrations showed potent zones of growth.

Rosmarinic acid -- Table 20 depicts the zones of inhibition and zones of growth at various concentrations (200-25 mg/mL). Faint QSI zones of growth were seen at the concentrations (200-50 mg/mL) of the samples tested. However, no activity was observed below the 25 mg/mL concentration. Photos A-C in Figure 36 depicts QSI results of rosmarinic acid seen with the QSIS2 assay tested at various concentrations: (A) 200 mg/mL, (B) 100 mg/mL, and (C) 50 mg/mL.

Table 20: QSI Activity of Rosmarinic Acid at Various Concentrations using the QSIS2 Assay.

Concentrations (mg/mL)	Antimicrobial zones of Inhibition (mm)	QSI zones of growth (mm)
200	20	* +
100	18	* +
50	16	* 25
25	0	0
Positive control (Garlic)	15	25
Negative control (Empty well)	0	0

Zone measurements are an average of three replicate experiments.
* Indicates zones were very faint in color.
+ Indicates that a zone of growth was detected although a measurement was not recorded.

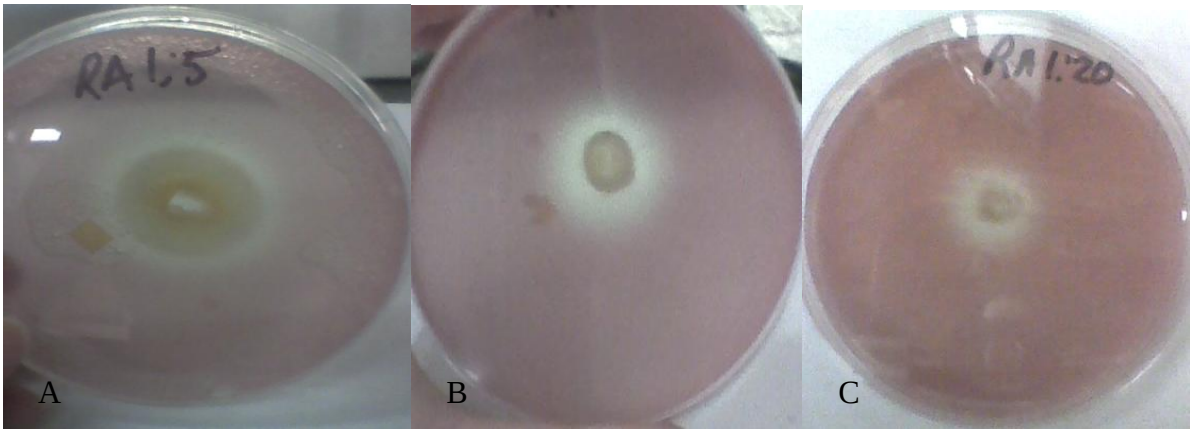


Figure 36: QSI of Rosmarinic acid at various concentrations with the QSIS2 assay.
(A) 200 mg/mL. (B) 100 mg/mL. (C) 50 mg/mL.
Faint zones of growth can be seen at all three concentrations.

Carvacrol -- Table 21 presents a more extensive analysis of liquid carvacrol samples tested at varying concentrations (1000-7.8 µL/mL). Both antimicrobial activity and anti-quorum sensing activity was seen at all concentrations, with definitive measurements recorded at 62.5-15.6 µL/mL. Photos A-C of Figure 37 depicts QSI activity of carvacrol at various concentrations: (A) 62.5 µL/mL, (B) 31.3 µL/mL, and (C) 15.6 µL/mL, respectively. Notice the red halo around the 31.3 µL/mL carvacrol well.

Table 21: QSI Activity of Carvacrol at Various Concentrations with the QSIS2 Assay.

Concentrations (µL/mL)	Antimicrobial zones of inhibition (mm)	QSI zones of growth (mm)
1000	39	+
500	40	+
250	38	+
125	30	+
62.5	27	36
31.3	18	22
15.6	16	14
7.8	11	?
Positive control (Garlic)	16	+
Negative control (Empty well)	0	0

+ Indicates a zone of growth was detected although a measurement was not recorded. This constituent was tested at various concentrations, one plate per concentration.

Figure 37: QSI activity of carvacrol at various concentrations with the QSIS2 assay. (A) 62.5 µL/mL. (B) 31.3 µL/mL. (C) 15.6 µL/mL.

Chromobacterial QSI Assay

Figure 38 (photos A and B) depicts the QSI assay results for samples of the thyme extract, baicalein, and garlic. Figure 39 (photos A and B) depicts Chromobacterial QSI assay results for rosmarinic acid, carvacrol, and thymol. Table 22 shows the corresponding antimicrobial and anti-quorum zone values. QSI zones can be seen around the thyme extract (21 mm) and the baicalein sample (18 mm). In addition, the second darker purple zone seen around the baicalein well (Figure 38-B) may suggest an increase in violacein production, thus suggesting an increase in QS activity. However, further assessment is needed.

QSI activity for three volumes (10 µL, 25 µL, and 50 µL) of the toluene garlic extract were demonstrated zone diameters 10 mm and 13 mm respectively only for volumes of 25 µL and 50 µL. This could be due to the softness of 0.3% LB agar, which can cause irregular zone sizes. Therefore, only samples with zones of greater than 10 mm were considered active. Thus, 50 µL was determined to be the optimum volume for the garlic tested.

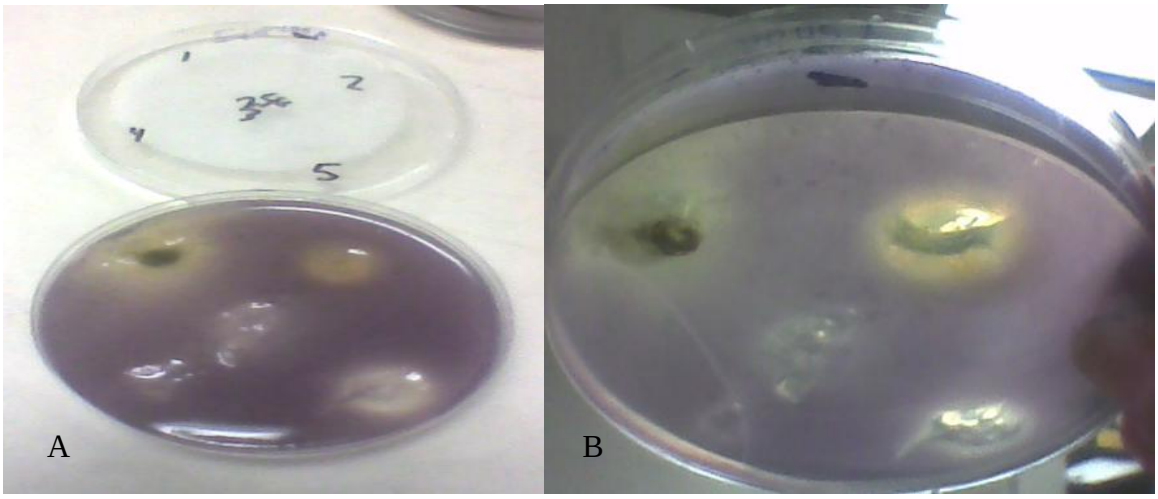
Rosmarinic acid (50 mg/mL) showed a QSI zone diameter of 10 mm, while thymol (7.8 mg/mL) and carvacrol (31.3 mg/mL) both showed significant antimicrobial and QSI activity. Figure 39-B clearly depicts the difference between the antimicrobial activity (clear zone) and

QSI activity (opaque zone). Antimicrobial zone diameters measured 15 mm for thymol and 26 mm for carvacrol. Thymol QSI zone diameters measured 22 mm, while carvacrol had zones of 34 mm.

Table 22: Chromobacterium QSI Assay of the Thyme Extract and its Main Constituents.

	Sample descriptions (concentrations)	**Antimicrobial Zone of inhibition (mm)**	**QSI Zone of inhibited color (mm)**
1	Thyme methanol leaf extract 500 mg/mL (50 µL per well)	?	21
2	Baicalein, 4µL/mL (50 µL per well)	?	18
3	Toluene garlic extract (10 µL per well)	0	0
4	Toluene garlic extract (25 µL per well)	0	10
5	Toluene garlic extract (50 µL per well)	8	13
6	Rosmarinic acid, 50 mg/mL (50 µL per well)	8	10
7	Thymol, 7.8 mg/mL (50 µL per well)	15	22
8	Carvacrol, 31.3 µl/mL (50 µL per well)	26	34
9	Internal standard	0	12
10	Negative control (empty well)	0	0

Zone measurements are an average of three replicate experiments. The toluene garlic extract was considered to be the positive control for this experiment.



Figures 38: Chromobacterial QSI assay results of the thyme extract and its various constituents. (A) The top side of the plate: (1) Thyme methanol leaf extract (500 mg/mL); (2) baicilein (4 µg/mL); (3) toluene garlic extract (50 µL per well); (4) toluene garlic extract (25 µL per well); and (5) toluene garlic extract (10 µL per well). (B) The bottom side of the plate. (From top to bottom, left to right, the samples are 1, 2, 3, 4, and 5.

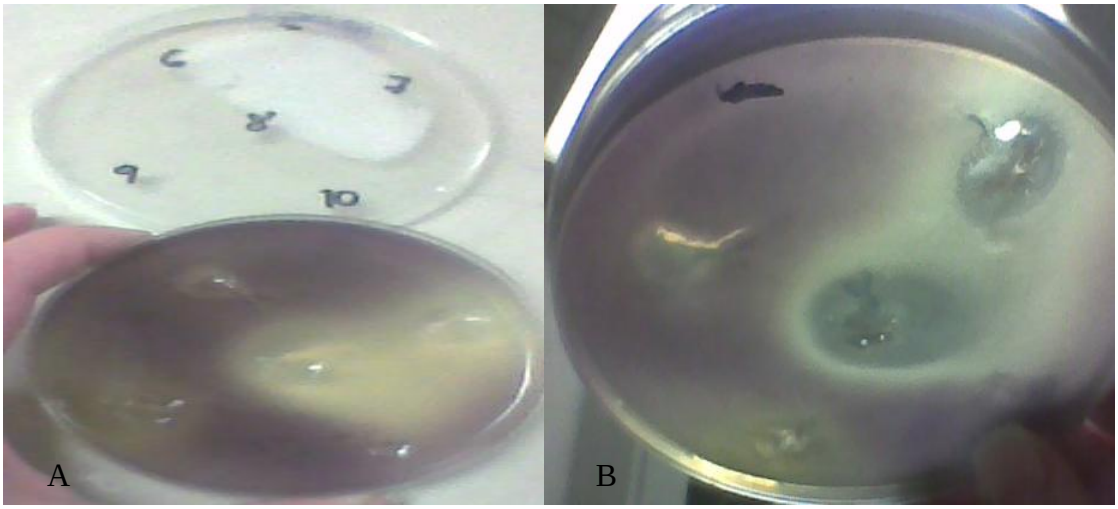


Figure 39: Chromobacterial QSI assay results of the thyme constituents. (A) The top side of the plate: (6) Rosmarinic acid (50 mg/mL); (7) thymol (7.8 mg/mL); (8) carvacrol (31.3 µl/mL); (9) internal standard; and (10) negative control (empty).
(B) The bottom side of the plate. In this view, the difference between the zones of inhibition (antimicrobial) versus the zone of inhibited color (QSI) is clear. Samples, appearing from left to right, correspond to 6, 7, 8, 9, and 10 as mentioned above. (Notice the zones of inhibition as completely clear versus the zones of growth [QSI] as opaque zone around samples 7 and 8, thymol and carvacrol, respectively.)

DISCUSSION AND CONCLUSION

The purpose of this dissertation was to perform preliminary exploration of two potential alternative modes of treatment against *Pseudomonas aeruginosa* infections: quorum sensing inhibition of folk-medicinal plant extracts and a specific example of combination drug therapy, the thyme-tetracycline effect.

Because the prevalence of multidrug-resistant *Pseudomonas* is relatively high, many researchers have begun exploring different options such as quorum sensing inhibition and combination therapy as single drug treatment options have become less and less effective. Since QSIs do not kill the pathogen, the selection pressure for development of resistance strains is greatly diminished. To date, many known QSI's such as patulin and penicillic acid, have been shown to be too toxic for human use (Rasmussen and Givskov, 2006).

Screening of Fifty Folk-Medicinal Extracts for QSI Activity

QSIS2 Assay

The first part of this study focused on screening fifty folk-medicinal plants for quorum sensing inhibitory activity. Out of 50 plants tested, thirty plant families were represented. Thirty samples showed QSI zones of growth as presented as a bright red halo. This assay detects C_4 or C_{12} HSL signaling inference. The QSIS2 assay works as such: The QSIS2 reporter stain harbors a plasmid which contains a QS promoter gene attached to the levansucrase gene encoding cell death. In the presence of sucrose and exogenous C_4 and C_{12} HSL signal molecules, QS-mediated cell death is triggered (Rasmussen et al., 2005b; Rasmussen and Givskov, 2006). When QS-mediated gene expression is activated in the QSIS2 reporter strain, sucrose mediated cell death

occurs, but if QSI compounds are present in the test sample, QS-mediated cell death is blocked and the reporter strain is able to grow. Cellular growth is achieved and a zone of dark red growth is visible around the test well in only the areas where QSI molecule concentration offsets QS signal concentration. The red color is caused by the metabolism of triphenyltetrazolium chloride by the bacteria, used only to augment the bacterial zone of growth. If the concentration of the QSI is too low or a compound in the sample has antibacterial effects then any cellular growth and or erroneous background growth will be inhibited and a "clear zone" will be seen around the test well.

Thirty samples showed a transparent or clear zone inhibited growth suggesting antimicrobial activity while twenty-four samples showed both types of activity. The plants tested which showed a transparent or clear zone around the test well these values were recorded as zones of inhibition. This lack of growth however, can be attributed to a number of factors: (1) antimicrobial activity (transparent zone) provided by a component in the plant extract, (2) a lack of a QSI compound in the extract (clear zone with the possibility of some background growth), (3) concentration of a potential QSI compound is too low in a particular region of the agar (clear zone with the possibility of some background growth). If a QSI is either not present or the concentration is too low to offset signal molecules, than QS-mediated cell death will be induced. All three scenarios produce cell death and a lack of growth around the test well.

Two variables that may lead to false positive results with the use of this QSIS2 reporter strain are test samples with high sugar content or an alkaline pH. Rasmussen et al. (2005b) stated that plant extracts with high sugar content can produce false positives with this QSIS2 reporter strain. Excess sugar content of a test sample may interfere with the sucrose mediated cell death. Krisitis and Parsek (2006) suggested that alkaline solutions of pH 8.0 or greater can cause

the acyl-homoserine lactone rings of the signal molecule to break open deeming them inactive. Both of these variables were examined for the majority of the extracts.

Preliminary investigations of various sugar concentrations (dextrose, fructose, glucose, and sucrose) and their effect on the assay were preformed to determine which concentrations would yield false positive results with the QSIS2 assay. Interestingly, fructose and sucrose did not affect the assay results. Glucose and dextrose, however, did yield false positive zones of growth halos with the QSIS2 assay. This data showed that samples with glucose concentration of 254 mg/dL produce false positive halo rings of 14 mm in diameter with the QSIS2 assay (*data not shown*). So, special care must be taken with when assessing samples with glucose concentrations of 254 mg/dL or greater. High glucose concentrations in a plant extract do not indicate lack of QSI activity just that the particular extract cannot be properly assessed with this QSIS2 assay.

Another factor that may affect QSI results is pH of the samples. The AHL molecules are very susceptible to pH changes. These molecules have been shown to have a half-life of several hours at a pH of 7.0 and several minutes at a pH of 8.0 (Krisitis and Parsek, 2006). For this reason, the pH of each extract was tested. All samples tested registered a pH of 6.0 or below with the exception of yellow root which had a pH of 7.0. Disruption of a signal molecule would prevent binding of the receptor thus allowing cellular growth and producing a false positive halo. However, pH did not affect the assay results and no QSI activity was seen with this sample.

Chromobacterial QSI assay

Nineteen of the forty-one samples showed QSI activity with the Chromobacterial QSI assay. QSI activity is observed as lack of violaceum pigment production in the assay. Samples

showing this activity most likely contain compounds which act as C_4-C_6 HSL analogues. Seven samples produced zones of increased color pigmentation. One explanation for this is that the extract might contain a second compound that augments QS-mediated pigment production.

There were a total of eleven crude extracts that had an acidic pH, low glucose content (under 360 mg/dL), and showed anti-quorum sensing activity in both of the assays. These extracts were: basil, chaparral, clove, cranberry, oregano, pomegranate, rosemary, sage, sassafras, thyme and witch hazel. The results suggest that these extracts contain compounds which interfere with C_4 HSL bacterial communication. Interestingly, five of plants were from the Lamiaceae family. Ginger and mint extracts also showed activity with the QSIS2 assay and showed little to no activity with the Chromobacterial QSI assay. These results suggest analogous interference with C_{12} HSL QS communication.

Rasmussen et al. (2005b) also screened many of these same plant extracts, and some inconsistency has been determined in comparing the two studies. Their findings showed no QSI activity for the extracts of clove, cranberry, ginseng (though this study used red ginseng) and mint. However they used a different report strain. Their QSIS1 reporter strain screens specifically for C_6 HSL analogues.

Rasmussen et al. (2005b) reported a lack of activity with their C_6 HSL analogue screening assay for ginseng, clove and mint extracts. No activity was found for the Chromobacterial QSI assay of red ginseng in this study, but we found activity with the QSIS2 assay. These results suggest that red ginseng may produce a C_{12} HSL analogue. Rasmussen et al. (2005b) also reported that the clove extract did not have QSI activity; in contrast, the assays carried out in this study showed considerable activity. One possible explanation is that the clove produces C_4 HSL analogues, compounds that may have not been identifiable with the assay used

in Rasmussen's study. The mint extract showed activity with the QSIS2 assay, which suggests C_{12} HSL communication interference as well.

Vattem et al. (2007) also screened basil, ginger, oregano, rosemary and thyme extracts with a similar Chromobacterial QSI assay type assay utilizing the *Chromobacterium violaceum* CV026 strain. This strain requires the addition of exogenous signal molecules for activity. Their findings were similar and consistent with those of this project.

Combination treatments such as antibiotic-antibiotic treatments have been discussed by many researchers (Driscoll et al., 2007). Plant extract-antibiotic combination studies have been explored by others including: Aburjai et al., 2001; Betoni et al., 2006; Nascimento et al., 2000. Several researchers (Hamilton-Miller and Shah, 2002; Rasmussen et al., 2005b) have also shown great promise using quorum sensing inhibitor-antibiotic combinations against biofilm and other types of infections.

The Thyme-Tetracycline Effect

The second part of this study focused on the medicinal folklore plant *Thymus vulgaris.* Several researchers have shown that thyme has antimicrobial (Cowen, 1999) and anti-quorum sensing activities (Vattem et al., 2007). This study demonstrates that the thyme extract can specifically interfere with *Pseudomonas aeruginosa* C_4 HSL signaling. Previous researchers (Nascimento et al., 2000) have also shown thyme has the ability to augment antimicrobial activity against resistant *Pseudomonas aeruginosa*. The combination of thyme and tetracycline is more effective at killing tetracycline resistant *P. aeruginosa* than the either of the individual components. Thus, this study set out to determine the active compound in the thyme extract that causes this effect, and to present a potential mode of action.

While thymol, a major constituent in thyme, is also well known for its antimicrobial activity, this study appears to be the first to identify thymol as the active component in the thyme-tetracycline effect.

QSI Activity of Thyme

In order to elucidate a mode of action for thyme in the thyme-tetracycline effect, various extracts of thyme and its four major constituents were assessed for QSI activity.

Thyme -- A recent report (Vatten et al., 2007) found that a water extract of thyme showed QSI activity with the *Chromobacterium violaceum* CV026. This wild type strain requires the addition of exogenous C_6 HSL signal molecules. They found that the thyme-water extract reduced QS-mediated violacein production by 60%. This study also found QSI activity with the Chromobacterium plate assay, producing QSI zones of 13 mm. This activity was substantiated by the QSIS2 assay. Four extracts of varying polarity ethanolic leaf crude, methanolic leaf crude, methanolic stem crude, and the hexane leaf crude were analyzed. Out of the four extracts, the polar extracts (methanol and ethanol leaf extracts and a methanol stem extracts) showed activity with the QSIS2 assay. However, the hexane nonpolar leaf crude extract showed no activity. Next, the four main constituents (baicalein, rosmarinic acid, thymol and carvacrol) were also examined for QSI activity using both the QSIS2 assay and the chromobacterial QSI assay.

Baicalein -- Zeng et al. (2008) reported that baicalein showed potential QSI activity against *Pseudomonas aeruginosa* using a computer-docking program. They also showed that baicalein can augment ampicillin activity against resistant *Pseudomonas sp.* Cheng et al. (2007) found synergy between baicalein and gentamicin against Gram-positive vancomycin-resistant

Enterococcus sp. The data presented herein validates their findings. Bacilien produced a strong red halo, which suggests QSI activity at all concentrations tested (Figure 3.2). The Chromobacterium plate assay also validated QSI activity. Baicalein showed QSI activity with both of the assays, but it did not show any antimicrobial activity in the DDA assays, and also it did not augment tetracycline activity.

Rosmarinic acid -- Walker et al. (2004) performed studies on the root stems of sweet basil, which produces rosmarinic acid in response to pseudomonad infections. These studies found that rosmarinic acid does effect cell growth and Rhl I/R gene expression, and can prevent biofilm formation. The QSIS2 assay showed faint zones of QSI activity at various concentrations (200-25 mg/mL). Chromobacterium studies also substantiate these results producing QSI activity at a 50 mg/mL concentration. Rosmarinic acid did show QSI activity but it did not augment tetracycline activity in the DDA assays.

Thymol and Carvacrol -- Studies have shown that thymol and carvacrol can effect biofilm formation in *Staphylococcus* (Nostro et al., 2007) and *C.albicans* (Braga et al., 2007; Braga et al., 2008). Prior to this study, thymol and carvacrol had not been tested specifically for QSI activity against *Pseudomonas aeruginosa*. This study appears to be the first to identify that thymol and carvacrol can act as potent quorum sensing inhibitory agents as demonstrated by both QSI assays. This study showed specifically that QS-mediated gene expression can be effected by both compounds. Thymol and carvacrol showed QSI activity even at minimal concentrations of 3.9 µg/mL and 7.8 µl/mL, respectively. While both compounds showed potent QSI activity, only thymol could augment tetracycline activity against *Pseudomonas aeruginosa* ATCC 10145.

These two QSI assays also indicate that the thyme extract and its four main constituents all show potential QSI activity. QSIS2 assay suggests that these compounds probably interfere

with either C_4 HSL of C_{12} HSL QS-mediated communication. The Chromobacterium QSI assay, which can be used to screen for C_4-C_6 HSL analogues, further substantiates these results by showing that the extract, along with the four main constituents, can all interfere with the C_4 HSL signaling in *C. violaceum*. This assay also shows that these compounds can repress the expression of violacein, a pigment under QS-mediated control. The significance of a compound that is able to prevent the QS-mediated gene expression of violaceum indicates that these four major constituents should also be able to prevent other QS-mediated genes from being expressed, and that other QS-mediated genes may down regulated including QS-mediated efflux pump expression. Conclusively, all of the data suggests that the thyme extract does have the ability to interfere with *Pseudomonas aeruginosa* QS-mediated gene expression. This evidence supports the idea that the thyme extract and its major constituents are all potent QSIs; hence it may be possible that the thyme extract and specifically thymol can provide tetracycline efficiency against resistant pseudomonads.

Potential Mode of Action for the Thyme-Tetracycline Effect

The collective evidence herein may be used to devise a possible mode of action that can explain the action of the thyme-tetracycline effect. Thymol was shown to be the active component in the thyme-tetracycline effect. A mode of action that may explain this effect may be that thymol is working as a C_4 HSL analogue. The Chromobacterial QSI assay shows two things. First, thymol can act as a C_4-C_6 HSL analogue at appropriate concentrations. Second, it shows that gene expression (pigmentation) can be repressed by this compound. The second assay QSIS2 reinforces the idea that thymol can act as a C_4 HSL analogue. In *Pseudomonas aeruginosa* ATCC 10145, thymol probably acts as an analogue and a competitive inhibitor for

the Rhl R transcription receptor which is under C_4 HSL control. In *Pseudomonas aeruginosa*, if Rhl-R QS-mediated gene expression is repressed then increased MexAB-OprM efflux pump gene expression is also repressed.

Sugimura et al. (2008) showed that if MexAB-OprM is suppressed by about 70 %, the cell becomes 2-4 times more susceptible to tetracycline antibiotic. MexAB-OprM expression is under QS-mediated C_4 HSL-Rhl R binding control. If C_4 HSL binding is prevented by a competitive inhibitor such as thymol, for example, tetracycline can not be expelled from the cell and concentrations increase as the molecules accumulate within the cell. Thus, efflux of tetracycline would be greatly reduced, allowing accumulation of tetracycline in the cell to levels which cause protein synthesis disruption. This would occur as tetracycline binds to the A site of the T-RNA binding site, eventually causing cell death (Poole, 2001).

The conclusion of this investigation yielded that thymol, a well known antimicrobial active constituent of *Thymus vulgaris,* and other Lamiaceae plants, can work in a synergistic fashion with ineffective doses of tetracycline to augment antimicrobial activity against tetracycline-resistant *Pseudomonas aeruginosa* ATCC 10145. A proposed mode of action for the thyme-tetracycline effect is that thymol acts as a QSI inhibitor of C_4 HSL communication, preventing MexAB-OprM efflux pump expression, thus allowing tetracycline to accumulate in the cell and cause cellular damage.

Collectively, these experiments show that quorum sensing inhibitors and maybe an important component in treating *P. aeruginosa* infections. The screening of the fifty plant extracts yielded several new QSI leads many from plant sources that have already been shown safe for human consumption. Further testing would allow researchers to explore and develop some of these leads into potential new treatments against *Pseudomonas aeruginosa* infections.

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