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The Antimycobacterial Activity of Hypericum perforatum Herb and the Effects of Surfactants

Shujie Shen
Utah State University

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THE ANTIMYCOBACTERIAL ACTIVITY OF HYPERICUM PERFORATUM HERB
AND THE EFFECTS OF SURFACTANTS

by

Shujie Shen

A thesis submitted in partial fulfillment
of the requirements for the degree
of
MASTER OF SCIENCE
in
Biological Engineering

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2012
ABSTRACT

The Antimycobacterial Activity of Hypericum perforatum Herb and the Effects of Surfactants

by

Shujie Shen, Master of Science
Utah State University, 2012

Major Professor: Dr. Charles D. Miller
Department: Biological Engineering

Due to the essential demands for novel anti-tuberculosis treatments for global tuberculosis control, this research investigated the antimycobacterial activity of Hypericum perforatum herb (commonly known as St. John’s wort, SJW), including a SJW methanol extract, purified major bioactive constituents of SJW: hypericin (Hpn), pseudohypericin (Phn) and hyperforin (Hfn). The SJW acidified methanol extract showed bactericidal activity against Mycobacterium JLS at 0.05 mg/ml culture. Purified compounds were tested at similar concentrations contained in the SJW methanol extract treatment. Among three purified bioactive compounds, only Hfn was bactericidal at 12 µg/ml. The other two compounds Phn and Hpn were not inhibitory or bactericidal at concentrations corresponding to the SJW methanol extract treatments.
The Polysorbate surfactant Tween 80, which is commonly added to the mycobacterial cultures to prevent cell clumping, was found to have inhibitory effects on the antimycobacterial activities of SJW extract and hyperforin. The addition of Tween 80 (0.05% v/v) increased the minimum bactericidal concentration (MIC) of SJW methanol extract from 0.05 to 0.33mg/ml and from 12 to 80 µg/ml for Hfn. This inhibitory effect of Tween 80 on SJW is opposite to the effect of Tween 80 on the antimycobacterial activity of rifampin and isoniazid. These observations are also in conflict with the existing permeability barrier hypothesis. A hypothesis that hyperforin molecules were sequestered in the core of Tween 80 micelles was given out to explain the repression effect of Tween 80 on hyperforin activity. The effectiveness of Tween 60, Tween 40 and Tween 20 on SJW activity was also tested. Tween 60 and Tween 40 showed the similar dose-dependent inhibitory effect on SJW extract activity with Tween 80, while the inhibitory effect of Tween 20 is much weaker.

A preliminary test was performed to detect the activity of SJW acidified MeOH extract and hyperforin on *M. tuberculosis* H37Rv strain. Results showed the MIC was 0.67mg SJW extract/ml and 200 µg Hfn/ml. In all, *M. tuberculosis* H37Rv strain is not that sensitive to SJW and hyperforin as other non-pathogenic strains tested in the present and previous studies.
PUBLIC ABSTRACT

The Antimycobacterial Activity of *Hypericum Perforatum* Herb and the Effects of Surfactants

Shujie Shen

Tuberculosis is a stubborn and lethal infectious disease. The world-wide tuberculosis surveillance reports that the new TB cases ranges from seven to eight million per year and deaths ranges from 1.3 to 1.6 million every year. The treatment of tuberculosis mainly depends on antituberculosis drugs, such as isoniazid, rifampin, pyrazinamide and ethambutol. However, drug-resistance acquisition is an inevitable process during tuberculosis treatment. Today, multi-drug resistant and the extensively drug-resistant TB cases have been widely reported around the world. Thus, the discovery of novel antituberculosis drugs is essential to effective TB treatment. *Hypericum perforatum*, also called St. John’s Wort, is a perennial herb. This research investigated St. John’s Wort’s potential use as an antituberculosis drug. In this study, we demonstrated the effectiveness of St. John’s wort methanol extract and three main bioactive constitutes against mycobacterial strains, including *Mycobacterial tuberculosis*, which is the pathogenic bacteria causing tuberculosis. We also investigated the effect of surfactants, which are commonly used in pharmaceutical and food industry, on the activity of St. John’s. The results of this study offer substantial information on the antituberculosis potential of St. John’s Wort.
ACKNOWLEDGMENTS

This project would not have been performed successfully without the support from faculty and staff of the Biological Engineering Department. I would like to express my gratitude to all of them.

Particularly, I would like to thank my advisor, Dr. Charles Miller, for the continuous support to my M.S. study and research, for his patience, motivation, enthusiasm and immense knowledge, and for his sincere encouragement. Besides my advisor, I would like to thank my thesis committee, Dr. Ronald Sims and Dr. Marie Walsh, who helped me a lot and gave me a lot of useful suggestions with this project.

I also would like to thank the previous master student, Trent W. Mortensen, whose previous research work greatly contributed to the development of this project. I would also like to thank my fellow labmates, for all the favors they did for me and for all the fun we had in the past three years.

At last, I would like to thank my family, whose support and encouragement made this possible.

Shujie Shen
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<td>Amyloid precursor protein</td>
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<td>AMK</td>
<td>Amikacin</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>BCG</td>
<td>Bacille Calmette Guerin</td>
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<tr>
<td>C$_2$</td>
<td>The carbon on the second position</td>
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<tr>
<td>C 26</td>
<td>26-carbon long</td>
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<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
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<td>CFU</td>
<td>Colony-forming units</td>
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<td>CMC</td>
<td>Critical Micelle Concentration</td>
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<td>COX</td>
<td>Cyclooxygenase</td>
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<td>DNA</td>
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<td>EMB</td>
<td>Ethambutol</td>
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<td>EtOH</td>
<td>Ethanol</td>
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<td>FAS</td>
<td>Fatty acid synthesis system</td>
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<td>FDA</td>
<td>Food and Drug Administration</td>
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<td>G/C</td>
<td>Guanine/Cytosine</td>
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<td>Hfn</td>
<td>Hyperforin</td>
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<td>Hydrophilic-lipophilic balance</td>
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<td>Hypericin</td>
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<td>HIV</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<td>H$_3$PO$_4$</td>
<td>Phosphoric acid</td>
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<td>IFN-γ</td>
<td>Interferon-gamma</td>
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<td>IGRA</td>
<td>Interferon Gamma Release Assay</td>
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<td>INH</td>
<td>Isoniazid</td>
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<td>InhA</td>
<td>Enoyl-acyl carrier protein reductase</td>
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<td>KatG</td>
<td>Catalase-peroxidase enzyme</td>
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<td>KM</td>
<td>Kanamycin</td>
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<td>LAM</td>
<td>Lipoarabinomannan</td>
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<tr>
<td>LB</td>
<td>Luria Broth media, either liquid or agar</td>
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<tr>
<td>MDR-TB</td>
<td>Multi-drug resistant tuberculosis</td>
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<td>MBC</td>
<td>Minimum Bactericidal Concentration</td>
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<td>Methanol</td>
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<td>MIC</td>
<td>Minimum Inhibitory Concentration</td>
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<td>NAA</td>
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<td>NADH</td>
<td>Nictinamide Adenine Dinucleotide</td>
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<td>NIRS</td>
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<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>OD$_{600}$</td>
<td>Optical density at a wavelength 600nm</td>
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<td>PAS</td>
<td>p-aminosalicylic acid</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PDT</td>
<td>Photodynamic therapy</td>
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Phn  Pseudohypericin
POA  Pyrazinoic acid
POE  Polyoxyethylene
PPD  Purified protein derivative
PZA  Pyazinamide
QFT-GIT  QuantiFERON®-TB Gold In-Tube test
RIF  Rifampin
RNA  Ribonucleic acid
SD  Standard deviation
SLDs  Second-line drugs
SJW  Saint John’s Wort (*hypericum perforatum* herb)
SM  Streptomycin
TB  Tuberculosis
TLC  Thin-layer chromatography
TST  Tuberculin skin test
WHO  World Health Organization
XDR-TB  Extensively drug-resistant tuberculosis
CHAPTER 1
INTRODUCTION

The Needs for Novel Anti-Tuberculosis Drug

Tuberculosis (TB), caused by the genus *Mycobacterium tuberculosis*, is a stubborn and lethal infectious disease. It spreads widely by airborne microscopic droplets from people in active TB phase when they are coughing, sneezing or talking. The main targeted organ of tuberculosis is lung, but it can also damage many other parts of the body, like bones, lymph node, joints, central nervous system and genitourinary (Smith, 2003).

The appearance of tuberculosis can be traced back to more than 5000 years ago. The battle between human beings and TB has also been around for a long time. The first true TB treatment began in 19th century with the use of fresh-air sanatoriums. In the middle of 20th century, the introduction of anti-tuberculosis drugs started the upsurge of TB treatment development. During the next decades, chemotherapy, general public health improvement, and the use of BCG vaccine greatly controlled the spread of TB (Smith, 2003). However, the emergence of drug-resistant TB and HIV co-infected TB announced the new round TB outbreaks.

Today, one-third of the world’s population is suffering from TB. In 1993, the World Health Organization (WHO) reported the estimated TB cases ranging from 7 to 8 million and the estimated deaths ranging from 1.3 to 1.6 million every year. In 2010,
those numbers were 8.8 million and 1.1 million respectively. In 2008, TB was the second leading cause of death from an infectious disease after HIV (WHO, 2011 b).

The development of chemotherapy is accompanied with the improper use of treatments. The immediate consequence of this is the drug resistance. Since the WHO launched the surveillance project to monitor the anti-tuberculosis drug resistance trends in 1994, the multi-drug resistant TB (MDR-TB) cases have been reported in every country and the extensively drug-resistant TB (XDR-TB) cases have been found in more than 50 counties (Dye, 2009). Because of the ineffectiveness of the standard six-month treatment, the treatment for drug resistant TB is an inefficient, time-consuming and expensive process (Orenstein, 2009; Mitnick, 2003). WHO recommended the minimum length of treatment for drug-resistant TB is 20 months (WHO, 2011 a) and the treatment cost for a MDR-TB patient is 10 times higher than that for a drug-susceptible TB patient (WHO, 2010).

Human immunodeficiency virus (HIV), a lentivirus, is able to cause the progressive failure to the carriers’ immune system. The rapid growth of HIV infection is accelerating the emergence and spread of drug-resistant TB (Angarano, 1998). Meanwhile MDR-TB and XDR-TB lead to a much higher mortality rates among TB/HIV coinfected patients (Campos, 2003).

Compared to the global TB situation, the western United States are not high TB occurrence areas. However, all western states still have TB surveillance system and publish TB statistic results annually. According to the surveillance TB reports, except California, Nevada and Arizona, the TB rates of other eight western states are all lower than the national rate. Utah is one of the low-incidence TB states. From 2007 to 2011, on
average 31 active TB cases were reported in Utah every year. A majority of the TB cases and TB morbidity were reported in Salt Lake Valley health district. In 2011, HIV coinfect ed TB cases and drug-resistant TB cases represented 3% and 17% of total reported TB cases, respectively. Although the overall TB situation is optimistic in the western United States, people are still keeping strong vigilance on their living area. In March 2009, a possible active TB case at Roosevelt Elementary School, Spokane, WA, caused all contacted children, staff and volunteers been isolated and tested.

While more and more countries, research institutions and health organizations are focusing on both global and their local TB situation, the progress of TB treatment is moving slowly. A breakthrough in TB treatment is always desired and essential. This research investigated the potential of the *hypericum perforatum* herb as the novel anti-tuberculosis drug.

**Hypothesis**

The *hypericum perforatum* herb has antimycobacterial activity.

**Objectives**

1. Evaluate the antimycobacterial activity of SJW methanol extract and three purified major constituents.

2. Demonstrate the effectiveness of Tween 80 on antimycobacterial activity of SJW.

3. Determine the antituberculosis activity of SJW and hyperforin on pathogenic *M. tuberculosis* strain.
CHAPTER 2
REVIEW OF LITERATURE

Mycobacteria

Genus Properties

*Mycobacterium* is a class of Gram-positive bacteria with high G+C content DNA. This genus is under the Mycobacteriaceae family, Corynebacterineae suborder and Acinomycetes order.

Mycobacteria are 0.2-0.6 μm wide and 1.0-10 μm long straight or slightly curved rods (Willey *et al.*, 2011). Most of mycobacteria are whitish or cream colored but some have carotenoid pigments that are bright yellow or orange (David, 1984). The cell wall of mycobacteria contains high levels of mycolic acids, a high-molecular-weight complex molecule. Mycolic acids are 3-hydroxy fatty acids (total 60 to 90 carbons) with an invariant C 26 aliphatic side chain at the C₂ position. The high proportion of mycolic acids causes the mycobacteria cell wall to be highly hydrophobic and acid-alcohol-fast (Ryan, 2004). The Aiehl-Neelsen and Kinyoun acid staining methods are commonly used to identify mycobacteria. In mycobacteria cultivation, the hydrophobic cell wall simulates individual cells to aggregate together or adhere tightly to the surface of culture vessels. Although for different subspecies the strength of the aggregation and adherence tendency is different, this characteristic causes difficulties for researches. To alleviate this phenomenon, a small quantity of detergent is typically added to the cultures to assist cell suspension (Ratledge, 1982).
In general, mycobacteria grow relatively slowly. However, they can be divided into two groups based on the growth rate, rapid growers showing colonies in 4-5 days and slow growers showing colonies in 7-10 days. Mycobacteria can be found in variety of habitats. Some of them are saprophytes and live in natural environments such as fresh water, salt water, soil and sand (Hartmans et al., 2006), while others are opportunistic pathogens living in the body of humans or other mammals, causing various diseases (Saviola and Bishai, 2006). Almost all pathogenic species are slow growers.

**Non-Pathogenic Mycobacteria**

Investigations of non-pathogenic mycobacteria species are increasing. More and more new species have been identified and described. Some species are used in the biocatalysis area, for instance, to modify steroid nucleus or degrade steroid side chain to produce pharmacologically active products in the steroid biotransformation process (Martin, 1984). They are also used in the conversion of alkenes to optically active epoxides (Habetscutzen, 1987; Hartmans and de Bont, 1989). Moreover, a group of mycobacterial strains which can mineralize toxic organic chemicals in the environment shows huge potential in the area of bioremediation. For example, Fathepure et al. (2005) found that a *Mycobacterium sp.* could metabolize a carcinogenic vinyl chloride. Brezna et al. (2003), Dean-Ross and Cerniglia (1996), Cheung and Kinkle (2001), Miller et al. (2004) discovered several mycobacteria species, which could utilize polycyclic aromatic hydrocarbon phenanthrene, pyrene and benzo(α)-pyrene as the sole carbon and energy source.
Pathogenic Mycobacteria

As a threat to public health, pathogenic mycobacteria species always attract more attention than nonpathogenic species. *M. tuberculosis*, the cause of human tuberculosis, is the most notorious in this family. *M. bovis, M. microti* and *M. africanum* are also the pathogenic source of tuberculosis to human, cattle or other mammalians (Adler and Rose, 1996). These four species are called the *M. tuberculosis* complex (Saviola and Bishai, 2006). Besides tuberculosis, leprosy is another serious disease and is caused by the mycobacterium species, *M. leprae*. Leprosy causes dreaded damage and lesions to skin. Other pathogenic mycobacteria species, like *M. avium* complex, *M. marinum* (Iseman, 2004), *M. ulcerans* (Johnson et al., 1999), *M. chelonae, M. fortuitum* and *M. abscessus* (Iseman, 2004), may cause infections in human, especially skin, after trauma or under immunodeficiency.

Tuberculosis

Human tuberculosis is mainly caused by *M. tuberculosis*. The process of *M. tuberculosis* infection generally has two phases, the latent and active phases. In latent TB phase, TB strains are alive but not active in the body. In this phase, individuals do not feel sick, do not have any symptoms and also cannot infect healthy individuals. Without treatment, infected people whose immune systems are weak will develop into the active TB phase. In active TB phase, tuberculosis symptom appears, such as unexplained weight loss, no appetite, night sweats, fever, weakness, persistent cough, and chest pain. This phase of infection is contagious (CDC, 2011 b).
Diagnostic Methods

How to choose a TB diagnostic method depends on the TB phase and targeted organs. The skin test and the rapid blood tests are the widely adopted diagnostic methods for latent TB. Mantoux tuberculin skin test (TST): TST is a standard primary diagnosis of *M. tuberculosis* infection. The procedure includes injecting a 0.1ml tuberculin purified protein derivative (PPD), an antigen, into the layers of skin and then visual checking for an immune response on the skin after 48 hours (CDC, 2003 a). TB Blood Tests - Interferon Gamma Release Assay (IGRA): IGRA is utilized to indicate the immune reactive strength by mixing the sample blood with TB antigen and measuring the released interferon-gamma (IFN-γ), a cytokine produced by cytotoxic T lymphocyte cells when antigen-specific immunity is triggered. Until now, the U.S. Food and Drug Administration (FDA) has approved two IGRA s, the QuantiFERON®-TB Gold In-Tube test (QFT-GIT) and T-SPOT® TB test (T-Spot), for commercial use. Compared to TST, IGRA does not have the booster effect which means previous IGRA tests can not affect subsequent TB tests (CDC, 2011 a).

For pulmonary active TB, sputum culture is the most conventional diagnostic method. In the sputum culture process, sputum samples are collected and cultured on proper medium. After several days, if the infecting organisms exist in the sputum, their growth can be visualized by microscope or detected using chemical tests. This is also employed to confirm the antibiotic sensitivity or resistance of the TB strains. The traditional sputum culture test takes 1 to 8 weeks, while a new strategy shortens the time duration to 24 hours. Nucleic acid amplification tests (NAA) are based on identifying specific DNA or RNA sequences using DNA\RNA amplification technique, like PCR,
self-sustained sequences replication, and ligase chain reaction (CDC, 2009). Otherwise, Chest X-ray and sputum cytology are routine methods combined with above-mentioned techniques in TB diagnosis.

Treatment

The tuberculosis treatment depends mainly on antituberculosis chemotherapy. All agents are classified in three ranks. The first-line antituberculosis drug indicates the most commonly used antibiotics. Currently, there are four first-line drugs: isoniazid, ethambutol, rifampin and pyrazinamide. Although the use of streptomycin in tuberculosis treatment achieved great success, the high rates of resistance rapidly reduced its efficacy and excluded it out of the first rank.

Isoniazid (isonicotinylhydrazine, INH) was discovered in 1912 and its antituberculosis activity was not determined until the 1950s. Isoniazid is an inactive prodrug that needs to be activated by the catalase-peroxidase enzyme (KatG) of M. tuberculosis. Then, the activated INH combines with nictinamide adenine dinucleotide (NADH) to form a complex. This complex binds to a key enzyme, enoyl-acyl carrier protein reductase (InhA) (Johnsson et al., 1995), and thus inhibits the type II fatty acid synthesis system (FAS), which disturbs the synthesis of cell wall mycolic acids (Takayama et al., 1975).

Rifampin (RIF) belongs to ansamycin family and used for TB treatment began in 1967. It has bactericidal effects on mycobacteria by binding the β subunit of mRNA and halting mRNA transcription (Wehrli and Staehelin, 1973).
Ethambutol (EMB), a bacteriostatic antimycobacterial agent, was adopted by TB chemo-therapy since 1966. The target of ethambutol is arabinogalactan synthesis. Arabinogalactan is considered as link between mycolic acid and peptidoglycan in the cell wall. Takayama and Kilburn (1989) proved that the presence of ethambutol promptly prevent D-arabinose connecting to arabinogalactan. Deng et al. (1995) confirmed that ethambutol inhibits arabinan core of lipoarabinomannan (LAM) synthesis. This inhibition somehow affects the incorporation of mycolic acids onto the arabinogalactan layer and leads to cell wall structure disruption and altered cell wall permeability.

Pyrazinamide (PZA) is the last drug to be added into the first-line cock-tail regimen. Although it is only a bacteriostatic agent, it has strong synergistic effect with isoniazid and rifampin (Mitchison, 1985; Heifets, 1995). Pyrazinamide is also an inactive prodrug, which needs to be activated by pyrazinamidase in bacilli to form the active pyrazinoic acid (POA). The mechanism of POA antituberculosis action is still under investigation. Some evidence implies that its action may somehow affect the fatty acid synthase I.

Due to the rapid development of resistance with using a single antibiotic, a cocktail regimen, combining four antibiotics, is recommended in tuberculosis treatment. For the common drug-susceptible pulmonary tuberculosis, a 6-month regimen with two phases is needed. The 2-month initial phase uses a combination of all four drugs and this process can eliminate a majority of the M. tuberculosis, which are rapidly multiplying and reside mainly in host cavities. The following 4-month continuation phase only uses an INH/RIF combination and focus on a subpopulation of M. tuberculosis, which
includes the slowly growing and dormant groups (CDC, 2003 b). Combinations of treatment regimens can vary with differences in drug interval and doses.

Compared to the front-line, the second-line agents have less effect, more severe side-effects, and higher production costs. They are used in therapy when the tolerance or resistance to the first-line drugs develops. Representatives of the second-line TB agents include cycloserine, ethionamide, streptomycin (SM), amikacin (AMK), kanamycin (KM), capreomycin, p-aminosalicyclic acid (PAS) and fluoroquinolones.

The third line agents include all potential antituberculosis drugs, which are not on the WHO second-line drug (SLDs) list. Amoxicillin, imipenem, clarithromycin, linezolid and clofazimine are representative of the third-line agents.

**Drug-resistant TB Strains**

Drug-resistance acquisition is almost an inevitable process during a long period chemotherapy without strict surveillance. In TB treatment, although the use of cocktail regimens substantially shortened the therapy period, the 6-month regimen is still too long to ensure patient compliance. The interruption during treatment and an inadequate treatment accelerate the conversion of TB strains from susceptible to resistant (Iseman, 1993). Based on the compensatory mutations theory and data from numerous studies, drug-resistant TB strains always have weaker competitive fitness and transmission ability (Gagneux, 2006; Andersson and Levin, 1999; Maisnier-Patin and Andersson, 2004). However, the continuously growing group of HIV carriers provides a suitable condition for the rapid spread of drug-resistant TB strains.
Although a variety of drug-resistant TB strains have been identified, the general antibiotic resistance mechanisms of these mutants are not that diverse. A majority resistance to INH is due to the catalase-peroxidase coding gene deletions or missense that result in nonfunctional enzymes which cannot activate the prodrug (Cockerill et al., 1995; Heym et al., 1995). 93% of resistance to rifampin is related to the single nucleotide mutation in the \textit{rpoB}-encoded RNA polymerase (Telenti et al., 1993). This mutation leads to the substitutions of single amino acid and prevents drug binding. Resistance to ethambutol is unclear due to the lack of understanding of ethambutol action. Several studies attribute resistance to mutant genes which code for three arabinosyltransferases, \textit{embC}, \textit{embA} and \textit{embB} (Goude et al., 2009; Lety et al., 1997; Telenti et al., 1997). For pyrazinamide resistance, 72% of resistance happens as a result of mutations in the \textit{pncA} gene (Sreevatsan et al., 1997).

Due to using antibiotics in combination, TB strains could develop resistant to several antibiotics at the same time. Multi-drug resistant TB (MDR-TB) is defined as TB strains which are at least resistant to first-line agent: INH and RIF. The extremely drug-resistant tuberculosis (XDR-TB) can be considered an extreme example of MDR-TB. It refers to TB strains which are resistant to not only the first-line agents but also the best second-line agents like fluoroquinolones and one of injectable drugs (kanamycin, capreomycin, or amikacin) (WHO, 2006).

\textit{Hypericum perforatum} (St. John’s Wort)

\textit{Hypericum} is a plant genus including about 400 species. \textit{Hypericum perforatum} L., also called commonly St. John’s wort (SJW), is the most described and investigated
subspecies because of its valuable medical properties. SJW is a 0.3-1 m tall herbaceous, perennial herb, which is native to Europe, West Asia, North Africa, and now widely distributes in most temperate areas. The leaves of SJW are stalkless, 12mm-long, and have small oil glands extended on the whole surface. The five-petalled followers of SJW are quite distinctive because of the bright golden-yellow color. Pharmacological studies show leaves, stems and flowers of SJW are the main source to obtain bioactive compounds for medicinal uses (Southwell and Bourke, 2001).

**Medicinal Uses and Bioactive Constituents**

While for thousands years SJW herb has been used for health purposes by ancient Greece, the recent increase of medicinal use of SJW was aroused by the appearance of a spate of SJW depression-alleviating activity studies in Germany during the 20th century. Thereafter, besides antidepressant activity (Gaster and Holroyd, 2000; Gharge et al., 2009), diverse medicinal properties of SJW were explored, including antitumoral (Quiney et al., 2007), anti-inflammatory (Tedeschi et al., 2003), antibacterial (Reichling et al., 2001; Saddique et al., 2010), antifungal (Saddique et al., 2010) and antiviral/antiretroviral activities (Jacobson et al., 2001; Lavie et al., 1989; Meruelo et al., 1988).

The effects of SJW herb are believed related to the active ingredients contained in the plant, some of them are metabolites and others are secondary metabolites. The concentration of each constituent ranges in individual plants because of the diversity in subspecies, ecological growth conditions and sample material processing. Until now, more than ten biochemical classes of bioactive compounds, namely naphthodianthrones derivatives, phloroglucinoles derivatives, flavonoids, procyanidines, phenylpropanes,
xanthones, proanthocyanidines amino acids, essential oil and water-soluble compounds (Greerson et al., 2001; Wurglics and Schubert-Zsilavecz, 2006; Butterweck, 2003). These bioactive compounds have been identified and investigated from different plant parts, like flowers, buds, leaves and stalk. Among them, three bioactive compounds, hypericin (Hpn) (Figure 2.1 A), pseudohypericin (Phn) (Figure 2.1 B) and hyperforin (Hfn) (Figure 2.1 C) are considered as the most primary contributors for the medicinal efficacy of SJW.

Hypericin and pseudohypericin are both naphthodianthrones derivants. Their proto-forms are located in the tiny black dots on the subaerial parts of the SJW plant (Nahrstedt and Butterweck, 1997). After isolation, proto-forms immediately convert to the comparatively more stable derivants hypericin and pseudohypericin by the influence of light (Nahrstedt and Butterweck, 1997). Hypericin is the earliest compound which was suspected to be related to antidepressant effect of SJW (Suzuki et al., 1984). However, data from studies about the antidepressant activity of hypericin are contradictory and none of the suggested action mechanisms are widely accepted. Hypericin is the most formidable natural photosensitizer, which could generate superoxide radicals or singlet oxygen molecules upon the oxygen and light stimulation. It is considered as the most promising photosensitizer candidate for antitumoral photodynamic therapy (PDT) due to its weaker toxicity and better tumor-targeting property than other candidates (Agostinis et al., 2002). In addition, experimental studies show hypericin and pseudohypericin could suppress the infection, replication and assembly of retrovirus through deactivating the reverse transcriptase of virions (Lavie et al., 1989).

Hyperforin is the major acylphloroglucinol derivative and is found in the reproductive parts (Nahrstedt and Butterweck, 1997) of SJW plant, especially the flowers.
Figure 2.1. Chemical structures of Hpn (A), Phn (B), and Hfn (C).
Hyperforin is lipophilic and only dissolves in organic solvents. It is not stable and easily degradable in light, heat and oxides. As an antidepressant compound, hyperforin has been proven to inhibit various neurotransmitters (i.e. serotonin, dopamine, noradrenaline), (Miguel et al., 2005; Muller, 2003), affect the synaptosomal ionic homeostasis (Chatterjee et al., 2001), and mediate the cytosolic pH (Muller et al., 2001; Eckert et al., 2004; Roz and Rehavi, 2003). Some evidence shows that hyperforin somehow influences the amyloid precursor protein (APP) processing which is the important target for the preventive and treatment of Alzheimer’s (Froestl et al., 2003). Hyperforin has been proven to be related to the anti-inflammatory effect of SJW. It can intervene the biosynthesis of proinflammatory eicosanoids by selectively inhibiting the activities of 5-lipoxygenase (5-LO) and cyclooxygenase (COX). Hostanska et al. (2003) reported that hyperforin has cytocidal effects in a cooperative manner with hypericin on some leukemia cells and brain glioblastoma cells. Studies by Schempp et al. (2002) suggested that hyperforin inhibits several tumour cell growth both in human and rat by inducing the mitochondria-mediated apoptosis pathway. These findings about antiproliferant and proapoptotic effects of hyperforin implied the potential pharmacological uses of hyperforin in cancer treatment.

SJW has a long history of being used as a herb remedy in wound-healing therapy. In 1959 Gainde et al. (1959) first described the antibiotic activity of SJW. After that served researchers successively reported the antibiotic activity of different SJW species (Mukherjee et al., 2002; Gibbons et al., 2002; Rabanal et al., 2002; Conforti et al., 2005) and various SJW extracts (Mazandarani et al., 2007; Conforti et al., 2005; Reichling et al., 2001; Avato et al., 2004). Their studies explored the strength variation of
antibacterial activities of SJW extracts prepared by different species materials and different solvents, and results showed the alcoholic extracts were more effective than aqueous extracts. Data indicated that SJW extracts have stronger antibacterial activity against Gram-positive strains than Gram-negative strains. Several studies clearly pointed out that hyperforin was the leading ingredient involved in SJW antibacterial efficacy (Schempp et al., 1999; Avato et al., 2004).

**Extraction and Quantification**

Plant sample diversity, light/oxygen sensitivity and poor aqueous solubility intensely impede pharmacological evaluation, quality standardization, comparison and analysis of hypericin, pseudohypericin and hyperforin (Liu et al., 2005). Harvested fresh SJW plant need to be immediately dried, sealed and stored avoiding light and oxygen to limit bioactive constitutes degradation. Common extraction procedures of SJW include Soxhlet extraction, sonication, maceration, and some modified procedures based on these. Some studies have been performed to compare and evaluate different extraction procedures of SJW, considering time, solvent and temperature (Liu et al., 2000 b; Avato and Guglielmi, 2004). High performance liquid chromatography (HPLC) is the best technique used to simultaneously identify, quantify, isolate and purify the bioactive constitutes of SJW. Thus, several HPLC assays have been developed, specifically aiming at identifying bioactive constituents in SJW extract or (Brolis et al., 1998; Ruckert et al., 2007; De los Reyes and Koda, 2001) in biological fluids (Liebes et al., 1991). Besides HPLC, mass spectrometry (MS) (Mauri and Pietta, 2000; Liu et al., 2000 a), thin-layer chromatography (TLC), nuclear magnetic resonance (NMR), near-infrared reflectance
spectroscopy (NIRS) (Rager et al., 2002), capillary electrophoresis (Jensen and Hansen, 2000) and UV-spectroscopy have also been employed individually or coupled in studies of SJW. Comparative experimental studies indicated that using a polar solvent such as MeOH or EtOH to extract SJW and performing extraction at room temperature are the key points to prepare a uniform SJW extract containing the most bioactive constitutes (Avato and Guglielmi, 2004).

**Permeability Barrier Theory**

The cell wall of mycobacteria is a composite of three layers, the innermost peptidoglycan layer, the outermost mycolic acids layer, and sandwiched arabinogalactan layers (Chhabra and Gokhale, 2009). Mycolic acids are 3-hydroxy fatty acids (total 60 to 90 carbons) which the C$_2$ position is substituted with an invariant C 26 aliphatic side chain (Willey et al., 2011). The long hydrocarbon chains of mycolic acids are supposed perpendicular to the cytoplasmic membrane and pack densely to form a tight and thick mycolate layer. This unique structure is considered as the main reason for the extremely low permeability of mycobacteria cell wall (Dmitriev et al., 2000), and a permeability barrier hypothesis was proposed to describe the process of various molecules passing cell wall. Further information implied that the permeability barrier of mycobacteria cell wall may be highly related to the intrinsic susceptibility and resistance of mycobacteria to antibiotics (Nikaido and Jarlier, 1991; Jarlier and Nikaido, 1994).

According to the permeability barrier hypothesis, different types of solutes pass the mycobacterial cell wall through different possible ways. The small hydrophilic molecules, such as isoniazid, ethambutol and pyrazinamide, cross the cell-wall through
water-filled channels (Kartmann et al., 1999; Senaratne et al., 1998). However, data showed that this permeability is much lower than for gram-negative strains. For instance, Jarlier and Nikaido (1990) detected that the permeability rate of M. chelonei cell wall for hydrophilic solutes is approximately 1000-fold lower than that of Escherichia coli. Such low permeability is probably attributed to the lower density of porins on the cell wall (Trias et al., 1992; Engelhardt et al., 2002; Kartmann et al., 1999), the extra length of porins (Engelhardt et al., 2002; Niederweis, 2003) and the crooked and narrow porin structures (Faller et al., 2004).

The hydrophobic molecules, for instance, rifamycin, macrolides, tetracyclines, and fluoroquinolones, penetrate the cell-wall via the lipid interior. This traverse is highly restricted by the low fluidity (Liu et al., 1995) of the mycobacteria cell wall. Thus raising the temperature is one of the efficient ways to enhance the penetration rate by increasing lipid fluidity. Another way to enhance the penetration rate is to increase the hydrophobicity of molecules. A serious of studies of some classes of antimycobacterial agents showed that the more hydrophobic derivatives displayed the stronger antimycobacterial activity (Yajko et al., 1990; Haemers et al., 1990). In Rastogi et al.’s (1988) study, adding a hydrophobic hydrocarbon chains onto the small hydrophilic isoniazid molecule also augmented the activity of isoniazid against M. avium. This may indicate the transfer of isoniazid penetration away from porins to lipid domains and this lipid pathway plays a more significant role than the aqueous porins in the permeability of mycobacteria cell wall (Nikaido and Jarlier, 1991). The susceptibility difference among various species to antimycobacteria agent could also relate to cell wall permeability. The proportion of α-mycolates with trans double bonds in the cell wall is the main reason
leading to low fluidity and permeability (Liu et al., 1996). Some of the more susceptible
species have a higher fluidity and permeability due to the high portion the α-mycolates
with cis double bonds and cis cyclopropane groups (Brennan and Nikaido, 1995).

Besides these two penetration mechanisms, other fairly large polycationic drugs
such as polymyxin and aminoglycosides could adsorb onto the negative charged cell-wall,
disorganize it and penetrate. This is called “self-promoted uptake” mechanism (Nikaido

**The Effect of Polysorbates**

With a high lipid content cell wall, Mycobacteria cells tend to aggregate in liquid
cultures. Tween 80 has typically been added to mycobacterial cultures to prevent cell
clumping and to provide uniform suspension of cells during antituberculosis tests (Jacobs
et al., 1991).

Tween 80 or Polysorbate 80 (polyoxyethylene (20) sorbitan monooleate) is a non-
ionic hydrophilic surfactant commonly used as an emulsifier in food industry and an
excipient in pharmaceutical industry. Tween 80 belongs to the polysorbate class, which
also includes other wildly used members: Tween 60 (Polyoxyethylene (20) sorbitan
monostearate), Tween 40 (Polyoxyethylene (20) sorbitan monopalmitate) and Tween 20
(Polyoxyethylene (20) sorbitan monolaurate). All of them have similar chemical structure:
the exactly same hydrophilic polyoxyethylene head consists of four polyoxyethylene
groups and a tetrahydrofuran group, and a slightly different lipophilic fatty acid ester tail
(Aizawa, 2011). The fatty acid tail of Tween 80 is derived from oleic acid and is an 18-
carbon chain with one double bond (Figure 2.2 A), while the tail of Tween 60 is a
saturated 18-carbon chain derived from stearic acid (Figure 2.2 B). Tween 40 has a saturated 14-carbon tail from palmitic acid (Figure 2.2 C) and Tween 20 has a saturated 12-carbon tail respectively from lauric acid (Figure 2.2 D).

There are two fundamental physicochemical parameters used to characterize surfactants.

Hydrophilic-lipophilic balance (HLB) is an index to indicate the hydrophilic or lipophilic degree of surfactants. The more hydrophobic a surfactant is, the stronger active surface it has (Moulik, 1996). A high HLB value means a high hydrophilicity and water solubility. For non-ionic surfactant, HLB value can be obtained from this equation: HLB = (mol% of hydrophilic group)/5. With different HLB values, surfactants are classified into several uses. The HLB values of polysorbate surfactants range from 15 to 17. Thus all members in polysorbate class are solubilizers, which have the function of micellar solubilization.

Another important characteristic index of surfactants is the critical micelle concentrations (CMC). In solution, the CMC is determined by the interaction between surfactant molecules themselves, solvent molecules themselves, and surfactant molecules and solvents molecules (Moulik, 1996). The CMC is a critical state that the interaction between surfactant and solvent molecules cannot compensate the self-interaction of surfactant molecules and solvent molecules. Hence, the surfactant molecules tend to form micelles with the hydrophilic head facing surrounding solvent and hydrophobic tail sequestering inside. As micelle solubilizers, these four polysorbates are able to incorporate oil species into micelle core and form a stable homogenous oil-in-water system. To test CMC, tensiometry, fluorimetry (Brito and Vaz, 1986), and
Figure 2.2. Chemical structure of polysorbates. A. Tween 80 (Polyoxyethylene-20-sorbitan monooleate), B. Tween 60 (Polyoxyethylene-20-sorbitan monostearate), C. Tween 40 (Polyoxyethylene-20-sorbitan monopalmitate), D. Tween 20 (Polyoxyethylene-20-sorbitan monolaurate).
Table 2.1. Uses of surfactant based on the hydrophilic-lipophilic balance (HLB).

<table>
<thead>
<tr>
<th>HLB value</th>
<th>Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-6</td>
<td>Water-in-oil emulsions</td>
</tr>
<tr>
<td>7-9</td>
<td>Wetting agents</td>
</tr>
<tr>
<td>8-16</td>
<td>Oil-in-water emulsions</td>
</tr>
<tr>
<td>13-15</td>
<td>Detergents</td>
</tr>
<tr>
<td>15-18</td>
<td>Solubilizers</td>
</tr>
</tbody>
</table>

spectrophotometry are the most utilized. For non-ionic surfactant, data show that the
CMC value vs. HLB value is linear, and the higher HLB corresponds to a lower CMC
(Hait and Moulik, 2001). Therefore, Tween 80 has the highest HLB, lowest CMC and the
strongest surface activity among these four polysorbate surfactants.

Table 2.2. Physicochemical parameters of polysorbates.

<table>
<thead>
<tr>
<th>Trade name</th>
<th>Chemical name</th>
<th>Molecular weight</th>
<th>HLB</th>
<th>CMC (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween 80</td>
<td>POE(20) sorbitan monooleate</td>
<td>1309.68</td>
<td>15.0</td>
<td>0.01</td>
</tr>
<tr>
<td>Tween 60</td>
<td>POE(20) sorbitan monostearate</td>
<td>1311.70</td>
<td>14.9</td>
<td>0.021</td>
</tr>
<tr>
<td>Tween 40</td>
<td>POE(20) sorbitan monopalmitate</td>
<td>1283.65</td>
<td>15.6</td>
<td>0.023</td>
</tr>
<tr>
<td>Tween 20</td>
<td>POE(20) sorbitan monolaurate</td>
<td>1227.54</td>
<td>16.7</td>
<td>0.05</td>
</tr>
</tbody>
</table>

POE: Polyoxyethylene.

**Tween 80 Influence on Mycobacteria Growth**

As a common ingredient in the mycobacteria medium, the influence of Tween 80
has been discussed for a long time and there are two major focuses. One of the subjects is
to discuss the effect of Tween 80 on the growth of mycobacteria: i) Can Tween 80 be
hydrolyzed and utilized by mycobacteria; ii) is the hydrolysis compound, oleic acid, a
nutrient or a toxin for mycobacteria? Stinson and Solotorovsky (1971) reported that *M.
avium* could hydrolyze Tween 80 in media and use oleic acid as nutrient. Dhariwal and
Venkitasubramanian (1978) determined that the lipid content of *M. phlei* had qualitative
and quantitative changes due to the presence of Tween 80 in medium. Mizuno and
Tsukamura (1978) reported that compared to glycerol and glucose, Tween 80 is a much
better carbon source to support the growth of certain slowly growing mycobacteria,
including *M. tuberculosis*. Lyon *et al.* (1963), however, observed an inhibitory effect of
Tween 80 on the growth of *M. tuberculosis* in their rotary incubation system and they
suggested that it related to the toxicity of oleic acid. Tsukamura (1990) reported that
Tween 80 dramatically inhibited the growth of *M. smegmatis* with the co-existence of
phosphate by likely interference with the synthesis nucleic acids, while no inhibitory
phenomena were observed in aqueous solution. In addition, in Saito *et al.*’s (1983)
studies, some rapidly growing mycobacteria, like *M. smegmatis*, were found that could
produce an esterase, mycobacteriocin, to hydrolyze Tween 80 and release oleic acid and
polyoxyethylenesorbitan ether, both of which have antimycobacteria activity against
some other mycobacteria species. Although a lot of published articles described their
observation during experiments, until now no direct evidence has been offered or
statement has been declared to explain how Tween 80 or oleic acid participate to support
or repress mycobacteria growth.

Another topic is the effect of Tween 80 on the antimycobacterial activity of
various antibiotic agents against mycobacteria species. Mizuguchi *et al.* (1983) reported
that the presence of Tween 80 inhibited the natural resistance of *M. intracellulare* strain
to streptomycin, kanamycin, paromomycin, viomycin, mitomycin C, rifampicin and 
isoniazid. Yamori and Tsukamura (1991) observed a paradoxical effect of Tween 80 in M.
avium complex culture on several antimycobacteria agents. In 7H10 agar medium with 
OADC enrichment the addition of Tween 80 augmented the susceptibility of M. avium 
complex to rifampicin and streptomycin, while the susceptibility to ethambutol and 
sulfadimethoxine didn’t change or was reduced. However, without OADC enrichment the 
presence of Tween 80 reduced the susceptibility of the M. avium complex to both 
rifampicin and sulfadimethoxine. Van Boxtel et al. (1990) reported that the 
susceptibilities of M. paratuberculosis to streptomycin, isoniazid, rifampin, ethambutol, 
ciprofloxacin, and penicillin G were significantly increased with the addition of Tween 
80. Masaki et al. (1990) reported that with 0.05mg/ml Tween 80 in the medium, M.
avium complex were more susceptible to Streptomycin, Kanamycin, and rifampin but not 
isoniazid and ethambutol. Hui et al. (1977) reported that the presence of Tween 80 
cauised the natural rifampin resistant M. intracellulare strain and M. smegmatis strain 
to become susceptible to rifampin. Conflicts and consistency exist among results from all 
previous studies. Some of them proposed hypothesis to explain the effect of Tween 80. In 
Hui et al.’s article (1977), he offered a possibility for the resistant–susceptible 
transformation that Tween 80 broke the permeability barrier of M. intracellulare and 
helped rifampin successfully contact with rifampin-susceptible RNA polymerase. Masaki 
et al. (1990; 1991) reported that the presence of Tween 80 caused a decrease of M. avium 
complex glycolipids. They believe this decrease was due to the interaction of Tween 80 
with cell walls, thus supporting the permeability barrier breaking hypothesis from Hui et al.
**Previous Studies**

In our previous studies, we investigated the antimycobacterial activity of SJW herb methanol extract. We utilized HPLC to quantify the pure bioactive compounds Hfn, Phn and Hpn in the SJW methanol extract. The antmycobacterial activities of Hfn, Phn and Hpn were also tested. Results showed that the minimum bactericidal concentration (MBC) of SJW methanol extract was 0.33-0.67 mg extract/ml for *M*. KMS and *M*. JLS, 0.67-1.33 mg extract/ml for *M*. MCS, and *M*. phlei and 1.33-2.66 mg extract/ml for *M*. smegmatis. Hfn, Phn, and Hpn MBC studies were performed on the most sensitive strain, *M*. JLS. Pure Phn and Hpn showed no inhibitory effect even at concentrations as high as 80 μg Phn/ml and 28 μg Hpn/ml. Although Hfn displayed inhibitory effects at 46 μg/ml and a MBC of 80 μg/ml, both concentrations are much higher than the corresponding amounts in SJW extract treatments. These results demonstrated that among the three tested bioactive compounds, Hfn was the only one contributing to SJW antmycobacterial activity.

**Summary**

In summary, a breakthrough in TB treatment for MDR-TB and XDR-TB is urgent and essential. Based on data from other articles and our previous study, SJW indeed has the antimicrobial activity against gram-positive bacteria and some mycobacteria strains. Undoubtedly, it has the potential to become a novel antituberculosis drug. However, in all previous studies, the influence of surfactants on SJW extracts and bioactive compounds were not studied.
CHAPTER 3
EFFECT OF *HYPERICUM PERFORATUM* ON MYCOBACTERIA VIABILITY

Introduction

Comparing to all the literature reviewed, our previous study designed and performed a more comprehensive SJW inhibitory/bactericidal assays. A strict experiment procedure made our results reliable and repeatable. A serious of measure was adopted to minimize the light degradation of SJW constituents as much as possible. The quantity and purity of bioactive compounds in SJW MeOH extract were detected by HPLC. Seven nonpathogenic mycobacteria strains were used in the test to make up the insufficient sample size used in all of other literature. However, our previous study didn’t consider the influence of Tween 80 that was proven should not be ignored and is hardly predicted in of all the literature reviewed. To evaluate the antimycobacterial activity of SJW more precisely and estimate the influence of Tween 80, the same assay was performed again on one of the seven mycobacteria, *M. JLS* (Mortensen *et al.*, 2012), without the presence of Tween 80. Results from presented study were compared with the previous study with the presence of Tween 80. With these results, the potential of SJW as novel antimycobacterial agent could be more distinct.
Materials and Methods

Mycobacterial Strain

*Mycobacterium* JLS was isolated by Utah State University from contaminated soil in land treatment unit at the Champion International Superfund Site, Libby, MT (Miller *et al.*, 2004). *M. JLS* is a fast-growing nonpathogenic mycobacterial subspecies (Miller *et al.*, 2007). Genomic sequencing identified 66% homology to *Mycobacterium tuberculosis* H37Rv strain (Mortensen *et al.*, 2012).

Antimycobacterial Agents

St. John’s Wort capsules were purchased from GNC Company (Glen Cove, NY); purified hypericin, hyperforin and pseudohypericin were purchased from Enzo Life Science Company (Farmingdale, NY).

SJW Acidified MeOH Extract and Purified Compounds Preparation

Contents of SJW capsules were ground into powder. SJW powder (10.8 g) was mixed with 30ml of deoxygenated acidified MeOH (containing 0.079% v/v 85% H₃PO₄) overnight at room temperature. The mixture was then centrifuged for 10 min at 4000 rpm. The supernatant was filtered using a 0.2µm sterile syringe filter and stored in a 10ml amber glass vial at -20 C. Purified Hfn, Phn and Hpn (1000 µg) were added to 250µl acidified methanol to make 4µg/µl stock solution.

LB Liquid and Agar Media

LB liquid media was prepared by dissolving 10 g Bacto Tryptone (BD, Fisher, Pittsburgh, PA), 5 g Bacto yeast extract (BD, Fisher, Pittsburgh, PA) and 10 g sodium
chloride (NaCl) (Fisher, Pittsburgh, PA) in 1 L ddH2O. This solution was sterilized by autoclave at 121 °C for 30 min and stored at room temperature covered by tin foil.

LB agar media was prepared by mixing 15g Difco agar (BD, Fisher, Pittsburgh, PA), 10g Bacto Tryptone (BD, Fisher, Pittsburgh, PA), 5g Bacto yeast extract (BD, Fisher, Pittsburgh, PA) and 10 g sodium chloride (NaCl) (Fisher, Pittsburgh, PA) in 1 L ddH2O. The solution was also autoclaved at 121°C for 30 min. After that, the hot solution were gently swirled immediately and cooled down to 55 °C. Approximately 25 ml of the solution was poured into 100x15 mm Petri dishes (Fisherbrand, Fisher, Pittsburgh, PA) to form a thin agar layer covering the bottom. Until the agar plates fully cool down to become solid, they were wrapped in plastic bag and kept at 4°C until use.

Bacterial Treatment with SJW Acidified MeOH Extract and Pure Compounds

*M. JLS* was inoculated in LB media from 15% glycerol stocks and grown at 25 °C with shaking (220 rpm). At an OD₆₀₀ of 0.02 (log-phase 10⁶ CFU/ml culture), cultures were treated with SJW crude extract and pure compounds. To minimize the bactericidal effect of the solvent MeOH itself, the amount of methanol solution added to all samples was 2% v/v of the total culture volume. Final treatment concentrations of SJW were 0.05, 0.09, 0.17 and 0.33 mg/ml culture. In our previous study (Mortensen *et al.*, 2012), amounts of Hfn, Hpn, and Phn in SJW methanol extract were quantified by HPLC. This analysis showed every 1g SJW extract contained 17.3mg Hfn, 5.8mg Hpn, and 16.2mg Phn. Based on these results, treatment concentrations of Hfn and Phn were 3, 6, 12, 23, 46, and 80µg/ml culture and treatment concentrations of Hpn were 1, 2, 4, 8, 16, and 28µg/ml culture. For each treatment, two controls were used: i) bacterial cells culture...
without any treatment and ii) bacterial cell culture treated with 2% v/v acidified MeOH. Viable bacterial cells were quantified by colony-forming unit (CFU) assays at times 0hr, 24hrs, 48hrs and 72hrs. Each sample was triplicates. The Standard deviation was calculated and marked as error bars in figures. Statistic analysis was carried on the results of SJW and hyperforin tests by SAS software, using two-way factorial ANOVA model with the significance level $\alpha=0.05$ and the multiple comparisons with the tukey adjustment.

**Results and Discussion**

**SJW Acidified MeOH Extract MBC Studies(112,538),(992,993)

Results of *M. JLS* treated with SJW in the absence of Tween 80 are shown in Figure 2. In the absence of Tween 80, SJW extract displayed a bactericidal effect even at the lowest tested concentrations of 0.05 mg SJW/ml, where 99.9% cells were dead after 24 hours. In our previous studies, bacterial inhibitory effects of SJW extract against *M. JLS* were observed at 0.33mg SJW /ml and the bactericidal effects were seen at 0.67mg SJW /ml in the presence of 0.05% Tween 80 (Mortensen et al., 2012). Unlike the effect on isoniazid and rifampin, these results suggest that Tween 80 inhibits rather than enhances the antibacterial activity of SJW extract against *M. JLS*.

**Purified Compound MBC Studies in The Absence of Tween 80**

To determine which bioactive constituent in SJW extract is responsible for the antimycobacterial effect, the antimycobacterial activity of purified Hpn, Phn and Hfn were tested with and without Tween 80. Figure 3.2-3.4 shows the effects of Hpn, Phn,
and Hfn on *M. JLS* in the presence of 0.05% Tween 80. While no antimycobacterial effects were observed with Hpn and Phn (Figure 3.2 and 3.3), concentrations of Hfn greater than 46 µg/ml showed inhibitory activity (Figure 3.4). Pure compound tests in the absence of Tween 80 are shown in Figure 3.5-3.7. No bacterial inhibitory or bactericidal activity was observed in hypericin and pseudohypericin treated cultures (Figure 3.5 and 3.6), which is consistent with what was observed in the presence of Tween 80 (Figure 3.2 and 3.3). However, in hyperforin treated samples, the bacterial inhibitory effect was observed at as low as 3 µg Hfn/ml (corresponding to 0.17 mg/ml SJW acidified MeOH extract) and bactericidal effect was displayed at 12 µg Hfn/ml (corresponding to 0.69 mg/ml SJW acidified MeOH extract) (Figure 3.7). Although the MBC of pure hyperforin is still higher than the corresponding MBC of SJW acidified MeOH extract, the antimycobacterial activity of hyperforin was much stronger in the absence of Tween 80 than in the presence of Tween 80.
Figure 3.1. Typical culturability results of *M. JLS*, in the absence of Tween 80, treated with SJW acidified MeOH extract. SJW concentrations were 0.05, 0.09, 0.17, 0.33 mg/ml culture and exposure time were 0 hrs (■), 24 hrs (■), 48 hrs (■), 72 hours (□). Controls were no methanol or SJW and methanol only treatment. Error bars are standard deviations. Two-way factorial ANOVA was used to compare the effect of time and SJW dosage, P-values <0.0001. “*” indicates that at a particular exposure time the dose treated sample is significantly different from the methanol control. The alphabetic character indicates that in a particular dosage treatment, the treatment is significantly different from other exposure times with different letters.
Figure 3.2. Typical culturability results of *M. JLS*, at the presence of 0.05% v/v Tween 80, treated with Pseudohypericin. Pseudohypericin concentrations were 3, 6, 12, 23, 46, 80 µg/ml culture and exposure time were 0 hrs (■), 24 hrs (■■), 48 hrs (■■■), 72 hours (□). Controls were no methanol or SJW and methanol only treatment. Error bars are standard deviations.

Figure 3.3. Typical culturability results of *M. JLS*, at the presence of 0.05% v/v Tween 80, treated with hypericin. Hypericin were 1, 2, 4, 8, 16, 28 µg/ml culture and exposure time were 0 hrs (■), 24 hrs (■■), 48 hrs (■■■), 72 hours (□). Controls were no methanol or SJW and methanol only treatment. Error bars are standard deviations.
**Figure 3.4.** Typical culturability results of *M. JLS*, at the presence of 0.05% v/v Tween 80, treated with Hyperforin. Hyperforin concentrations were 3, 6, 12, 23, 46, 80 µg/ml culture and exposure time were 0 hrs (■), 24 hrs (■), 48 hrs (■), 72 hours (□). Controls were no methanol or SJW and methanol only treatment. Error bars are standard deviations. Two-way factorial ANOVA was used to compare the effect of time and SJW dosage, P-values <0.0001. ”*” indicates that at a particular exposure time the dose treated sample is significantly different from the methanol control. The alphabetic character indicates that in a particular dosage treatment, the treatment is significantly different from other exposure times with different letters.

**Figure 3.5.** Typical culturability results of *M. JLS*, at the absence of Tween 80, treated with Pseudohypericin. Pseudohypericin concentrations were 3, 6, 12, 23, 46, 80 µg/ml culture and exposure time were 0 hrs (■), 24 hrs (■), 48 hrs (■), and 72 hours (□). Controls were no methanol or SJW and methanol only treatment. Error bars are standard deviations.
Figure 3.6. Typical culturability results of *M. JLS*, at the absence of Tween 80, treated with hypericin. Hypericin concentrations were 1, 2, 4, 8, 16, 28 µg/ml culture and exposure time were 0 hrs (■), 24 hrs (■), 48 hrs (■), 72 hours (■). Controls were no methanol or SJW and methanol only treatment. Error bars are standard deviations.

Figure 3.7. Typical culturability results of *M. JLS*, at the absence of Tween 80, treated with Hyperforin. Hyperforin concentrations were 3, 6, 12, 23, 46, 80 µg/ml culture and exposure time were 0 hrs (■), 24 hrs (■), 48 hrs (■), 72 hours (■). Controls were no methanol or SJW and methanol only treatment. Error bars are standard deviations. Two-way factorial ANOVA was used to compare the effect of time and SJW dosage, P-values <0.0001. “*” indicates that at a particular exposure time the dose treated sample is significantly different from the methanol control. The alphabetic character indicates that in a particular dosage treatment, the treatment is significantly different from other exposure times with different letters.
Conclusion

Excluding the interference of Tween 80, the bactericidal and inhibitory activity of SJW extract and its purified constituents have been detected and evaluated more accurately. Compared to results from our previous studies, the bactericidal and inhibitory activities of SJW acidified MeOH extract and hyperforin are both increased in the absence of Tween 80, whereas purified hypericin and pseudohypericin still did not show any bactericidal or inhibitory activities against the tested mycobacterial strains. No matter with or without Tween 80 in cultures, the bactericidal and inhibitory concentrations of Hfn were always higher than the corresponding to SJW extract treatments. Acidified methanol extract of SJW showed much stronger activity than that of the purified hyperforin. In summary, Hpn and Phn did not contribute to the anti-mycobacterial activity of SJW and Hfn only partial activity. Tween 80 partially represses activity of SJW extract and hyperforin.
CHAPTER 4
EFFECT OF SURFACTANTS ON ANTIMYCOBACTERIA ACTIVITY OF
HYPERICUM PERFORATUM

Introduction

Of all the literature reviewed, Tween 80 as an additional ingredient in the mycobacterial culture to prevent cells clumping indeed has effectiveness on antimycobacteria activity of majority agents. However, because of the inconsistence existing among their results, it is hard to summarize a rule to predict the Tween 80 action on untested antibiotic agents. In this presented studies, the action of Tween 80 on antimycobacterial activity of _hyperforin perforatum_ were detected. To further investigation, the action of three other polysorbate surfactants Tween 60, Tween 40 and Tween 20 were also tested here. The unexpected observation brings discussion about the old permeability barrier hypothesis, which was widely accepted to explain the mechanism of Tween 80 action. A new model to describe the interaction between Tween 80 micelles and antimycobacterial agent molecules is suggested out.

Material and Method

Polysorbate Surfactants and Antimycobacterial Agents

Antimycobacterial agents: isoniazid was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO); rifampin was purchased from Fisher BioReagents
Company (Fair Lawn, NJ); St. John’s Wort capsules were purchased from GNC Company (Glen Cove, NY). Tween 80, Tween 60, Tween 40 and Tween 20 were also purchased from Sigma-Aldrich Chemical Company (St. Louis, MO).

Bacterial Treatment with Isoniazid and Rifampin

Based on the MIC of isoniazid and rifampin for *Mycobacterium tuberculosis* (Zhang *et al.*, 2006), concentrations of isoniazid used to treat *M. JLS* in this study ranged from 0.02 to 20 µg/ml and concentrations of rifampin ranged from 0.5 to 50 µg/ml. Each concentration was tested on two different *M. JLS* cultures; one containing 0.05% Tween 80, and the other containing no Tween 80. Viable cell numbers were quantified by CFU assays at times 0hr and 72hrs. Each sample was duplicate.

Polysorbate Repression Test

Ten percent v/v polysorbate stock solutions were made by adding 5 ml polysorbate into 45 ml sterile deionized water. All stock solutions were filtered (0.2µm), stored at room temperature and used no more than 1 week later. In all tests, 0.33mg SJW extract/ml culture were added. For each polysorbate repression test, seven different concentrations of polysorbate were used (0%, 0.032%, 0.063%, 0.013%, 0.025%, 0.050%, 0.100% v/v) (Table 4.1). The control was *M. JLS* culture without either polysorbate or SJW MeOH crude extract. Live cell numbers were quantified by CFU method at times 0hr, 24hrs, 48hrs and 72hrs. Each sample was triplicate. The Standard deviation was calculated and marked as error bars in figures. Statistic analysis was carried on all polysorbate tests results by SAS software, using two-way factorial ANOVA model with
the significance level $\alpha=0.05$ and the multiple comparisons with the tukey adjustment.

**Table 4.1. The tested polysorbate concentrations.**

<table>
<thead>
<tr>
<th></th>
<th>0.003125%</th>
<th>0.00625%</th>
<th>0.0125%</th>
<th>0.025%</th>
<th>0.05%</th>
<th>0.1%</th>
<th>V/V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween 80 (CMC: 0.01)</td>
<td>0.026</td>
<td>0.052</td>
<td>0.104</td>
<td>0.208</td>
<td>0.415</td>
<td>0.830</td>
<td></td>
</tr>
<tr>
<td>Tween 60 (CMC: 0.021)</td>
<td>0.030</td>
<td>0.060</td>
<td>0.119</td>
<td>0.239</td>
<td>0.477</td>
<td>0.955</td>
<td></td>
</tr>
<tr>
<td>Tween 40 (CMC: 0.023)</td>
<td>0.026</td>
<td>0.051</td>
<td>0.102</td>
<td>0.204</td>
<td>0.409</td>
<td>0.818</td>
<td></td>
</tr>
<tr>
<td>Tween 20 (CMC: 0.05)</td>
<td>0.028</td>
<td>0.056</td>
<td>0.112</td>
<td>0.224</td>
<td>0.448</td>
<td>0.896</td>
<td></td>
</tr>
</tbody>
</table>

CMC: critical micelle concentrations.

**Tween 80 Micelles and SJW Binding Dye Test**

Eosin Y was got from the Walsh Lab in the Department of Nutrition, Dietetics and Food Sciences (Logan, UT). Eosin Y powder was dissolved into dd water to make 0.02mM solution. Eosin Y solution was filled into glass cuvettes, and 0.33mg/ml SJW extract was added into half of cuvettes. Different amount of Tween 80 was added into different cuvettes to make seven different concentrations (0%, 0.032%, 0.063%, 0.013%, 0.025%, 0.050%, 0.100% v/v). Thus, there were two groups of Eosin Y samples: samples in one group all had SJW and samples in another group did not. Each group contained seven samples in the difference of Tween 80 concentration. All samples were tested in the UV spectrophotometer at the wavelength of 538nm. The absorbance values were recorded. Statistic analysis was carried on the result by SAS software, using two-way factorial ANOVA model with the significance level $\alpha=0.05$ and the multiple comparisons.
with the tukey adjustment.

Results

Polysorbate Repression Tests

Polysorbate repression tests were performed to further investigate the inhibitory effect of Tween 80 on antimycobacterial activity of SJW. The same dose of SJW (0.33 mg SJW/ml), which has bactericidal effects in the absence of Tween 80, was added to all culture samples. Tween 80 was then added at various concentrations. At Tween 80 concentrations of 0.013% v/v, the bactericidal effects of SJW were efficiently diminished and only inhibitory effects were observed (Figure 4.2). Concentrations of 0.025% v/v or higher Tween 80 in media totally inhibit the antimycobacterial activity of SJW (Figure 4.2). To further investigate the action of various Tween surfactants on SJW, Tween 60 (Figure 4.3), Tween 40 (Figure 4.4) and Tween 20 (Figure 4.5), which have the similar chemical structures and properties with Tween 80, were tested. Results from each of these Tween surfactants show dose-dependent inhibitory effect on SJW antimicrobacterial activity. Tween 60 has similar inhibitory effect as Tween 80 with its inhibitory effect is weaker at 0.006% v/v and a stronger at 0.013%v/v. Tween 40 shows a little weaker inhibitory effect than Tween 80 at 0.006% v/v. Although the effect of Tween 80, Tween 60 and Tween40 are similar, the effect of Tween 20 was weaker than other three surfactants. Even at the highest concentration of 0.100% v/v, the SJW bactericidal effect was observed after 72 hours.
Effect of Tween 80 on Isoniazid and Rifampin Antimycobacterial Activity

Previous studies of Tween 80 effects on antituberculosis drugs were tested on *M. avium*, *M. intracellulare*, and *M. paratuberculosis* (Hui et al., 1977; Masaki et al., 1990; Van Boxtel et al., 1990; Yamori et al., 1991). Since *M. JLS* had not previously been tested by these two antibiotics, it was necessary to determine the activity of Tween 80 on isoniazid and rifampin (Table 4.2). After 72 hours, relative colony numbers (control colony number 100%) in cultures not containing Tween 80 and treated with isoniazid at 0.02, 0.1, and 0.2 µg/ml were 95%, 16% and 0.26%, respectively. In cultures containing Tween 80, the respective relative colony numbers were 77%, 2.4% and 0.035%. For rifampin treated samples, relative colony numbers were similar at 0.5, 10, and 50 µg/ml in the presence and absence of Tween 80 after 72 hours. However at 5 µg/ml rifampin, relative colony numbers were higher in cultures without Tween 80 (15.6% versus 0.8%). These results show the presence of Tween 80 enhances the antibacterial activities of isoniazid and rifampin against *M. JLS*.

**Tween 80 Micelles and SJW Binding Dye Test**

Dyes are commonly used for measuring critical micelle concentration of surfactants. Eosin Y is a fluorescent red dye. It shows a wavelength maximum at 518 nm in water, but the maximum wavelength will shift to 538 nm when micelles are present. The UV absorbance at 538 nm will increase with the increased micelles concentration. In this present study, such property of Eosin Y was utilized to imply the binding between Tween 80 micelles and SJW extract. Result was showed in Figure 4.6. It is obviously that
with the same amount of Tween 80 in Eosin Y solution samples containing 0.33mg/ml SJW extract always showed lower absorbance at the wavelength of 538 nm than their comparatives without SJW extract in solution.

Table 4.2. Effect of Tween 80 on antimycobacterial activity of isoniazid (A) and rifampin (B) against *Mycobacterium JLS*. The numbers in the parentheses is standard deviations.

### A

<table>
<thead>
<tr>
<th>Concen of isoniazid (µg/ml)</th>
<th>Mean of relative colony no.</th>
<th><em>M. JLS</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Tween</td>
<td>Tween 80</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.02</td>
<td>95 (3.5)</td>
<td>77 (14.7)</td>
</tr>
<tr>
<td>0.1</td>
<td>16 (3.7)</td>
<td>2.4 (2.2)</td>
</tr>
<tr>
<td>0.2</td>
<td>0.26 (0.035)</td>
<td>0.035 (0.034)</td>
</tr>
<tr>
<td>2</td>
<td>0.013 (0.0024)</td>
<td>0.017 (0.0037)</td>
</tr>
</tbody>
</table>

### B

<table>
<thead>
<tr>
<th>Concen of rifampin (µg/ml)</th>
<th>Mean of relative colony no.</th>
<th><em>M. JLS</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Tween80</td>
<td>Tween80</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.5</td>
<td>46 (3.4)</td>
<td>45 (7)</td>
</tr>
<tr>
<td>5</td>
<td>15.6 (14)</td>
<td>0.8 (0.4)</td>
</tr>
<tr>
<td>10</td>
<td>1.07 (0.07)</td>
<td>0.85 (0.5)</td>
</tr>
<tr>
<td>50</td>
<td>0.022 (0.01)</td>
<td>0.13 (0.03)</td>
</tr>
</tbody>
</table>

**Discussion**

Our results demonstrated that Tween 80 enhances the antimycobacterial activity of isoniazid and rifampin against *M. JLS* and inhibits the antimycobacterial activity of
SJW and hyperforin. Although the action of Tween 80 on isoniazid and rifampin activity against *M. JLS* is consistent with all previous reports, the inhibitory effect of Tween 80 on antimicrobacterial agents has not been reported previously. Neither the permeability barrier hypothesis (Hui *et al.*, 1977) nor the Tween 80 antimycobacterial activity hypothesis is suitable to interpret the inhibitory effect of Tween 80 on SJW and hyperforin activity. We highly suspect that the different actions of Tween 80 might be attributed to different interaction models between Tween 80 micelles and specific compounds. Isoniazid (Figure 4.1 A) is a small hydrophilic molecular. According to the structure of Tween 80 micelles, it is highly possible that isoniazid molecules are binding on the surface of Tween 80 micelles, which is consist of Tween 80 hydrophilic heads. Although rifampin molecules (Figure 4.1 B) are hydrophobic, the size of the molecule is too big to be sequestered in the core of Tween 80 micelles. Thus in the presence of Tween 80, rifampin either exists as free molecules or loosely binds on the Tween 80 surface. As a surfactant, Tween 80 may help the hydrophilic isoniazid to contact the high lipid mycobacterial cell wall more easily. On the other hand, Tween 80 would more or less damage cell wall of mycobacterial to cause the cell wall permeability increase. Thus, more free isoniazid and rifampin molecules can get into the mycobacterial cell. This may explain the antimycobacterial activity enhancement of INH and RIF in the presence of Tween 80. Hyperforin molecule (Figure 4.1 C), is smaller but more hydrophobic than rifampin. The predicted log P hydrophobicity value of hyperforin molecule is 6.32, while the log P hydrophobicity value of rifampin is only 2.36 (Wishart *et al.*, 2008). Therefore, comparing with isoniazid and rifampin, hyperforin is more likely to be sequestered in the hydrophobic core of Tween 80 micelles. Such interaction model prevent hyperforin
molecule effectively contact and traverse the mycobacterial cell wall. This can reasonably explain that the antimycobacterial activity of hyperforin decreases in the presence of Tween 80.

Based on the Tween 80 and SJW binding dye test, we preliminarily believe the binding between Tween 80 and SJW exist. In the Eosin Y solution with Tween 80 micelles, the maximum wavelength of Eosin Y shifted to 538nm is because Eosin Y molecules bind into micelles. In the SJW presence samples, hyperforin would compete with Eosin Y to bind into micelles to cause less Eosin Y binding. Thus, at the same Tween 80 concentration, the Eosin Y absorbance of sample contains SJW is lower than samples without SJW there. When the concentration of Tween 80 is high enough, both Eosin Y and hyperforin molecules have enough micelles to bind into. At this time, the Eosin Y absorbance should be the same no matter with and without SJW. Figure 4.6 displayed the competing situation at the Tween 80 concentrations from 0.032% to 0.050% v/v, and the saturated situation at the Tween 80 concentration 0.100% v/v.

In polysorbate repression results, two points are worthy of attention: i) the inhibitory effects of polysorbate on SJW are dose-dependent and ii) the inhibitory effect of Tween 20 is weaker than other tested Tween surfactants. All concentrations of polysorbate surfactants we used here are above the predicted critical micelle concentrations, suggesting micelles exist in our assay system.

Based on the studies of Aizawa (2011) and Patist et al. (2000), the size and shape of the Tween micelles are not changed throughout the tested concentration range, and only the number of micelles increased with the increased surfactant concentration. If we consider the Tween micelle interaction with hyperforin is constant, the does-depended
Figure 4.1. Chemical structure of isoniazid (A), rifampin (B) and hyperforin (C).
inhibitory effect of Tween surfactants on SJW activity could be attributed to the increase in micelle number. This is also why these four polysorbate surfactants showed dose-depended inhibitory effects. Although the molecular structure differences of polysorbate members are small, each polysorbate has a distinct critical micelle concentrations (CMC), micelle shape, micelle size, and micelle density (Hideki, 2011; Reichling et al., 2001). The CMC for Tween 80 is 0.01 mM/L (0.00144% v/v); for Tween 60 is 0.023 mM/L (0.0024% v/v); for Tween 40 is 0.027 mM/L (0.0033% v/v); and for Tween 20 is 0.05 mM/L (0.0073% v/v). Since they each have different fatty acid hydrophobic tails, the size and shape of their micelles are also different. In our studies with the four polysorbate surfactants, the inhibition ability of Tween 20 was weaker than the others, possibly because of the smaller size of the Tween 20 micelle due to the shorter hydrophobic tail (Khlebtsov et al., 2004).

**Conclusion**

Results from this chapter show that Tween 80 enhanced the antimycobacterial activity of rifampin and isoniazid, while it repressed the activity of SJW extract and hyperforin. Because of the smaller size and higher hydrophobicity of hyperforin, we highly suspect the repression action of Tween 80 on antimycobacterial activity of hyperforin is because the hyperforin molecules are sequester in the core of micelles and are prevented effectively tranversing mycobacterial cell wall. The dose-dependent repression action of Tween surfactants is related to the number and size of micelles in the solution.
Figure 4.2. Typical culturability results of *M. JLS*, in the presence of various Tween 80 concentrations, treated with 0.33 mg/ml culture SJW MeOH extract. Tween 80 concentrations range from 0.003% to 0.100% v/v of culture solution. Exposure times were 0 hrs (■), 24 hrs (■), 48 hrs (■), 72 hours (□). Controls were no SJW and no Tween 80, and SJW alone. Error bars are standard deviations. Two-way factorial ANOVA was used to compare the effect of time and various Tween 80 concentrations, P-values <0.0001. “*” indicates that at a particular exposure time the Tween 80 containing sample is significantly different from the SJW alone control. The alphabetic character indicates that in a particular Tween 80 treatment, the treatment is significantly different from other exposure times with different letters.
Figure 4.3. Typical culturability results of *M. JLS*, in the presence of various Tween 60 concentrations, treated with 0.33 mg/ml culture SJW MeOH extract. Tween 60 concentrations range from 0.003% to 0.100% v/v of culture solution. Exposure times were 0 hrs (■), 24 hrs (■), 48 hrs (■), 72 hours (□). Controls were no SJW and no Tween 60, and SJW alone. Error bars are standard deviations. Two-way factorial ANOVA was used to compare the effect of time and various Tween 60 concentrations, P-values <0.0001. “*” indicates that at a particular exposure time the Tween 60 containing sample is significantly different from the SJW alone control. The alphabetic character indicates that in a particular Tween 60 treatment, the treatment is significantly different from other exposure times with different letters.
Figure 4.4. Typical culturability results of *M. JLS*, in the presence of various Tween 40 concentrations, treated with 0.33 mg/ml culture SJW MeOH extract. Tween 40 concentrations range from 0.003% to 0.100% v/v of culture solution. Exposure times were 0 hrs (■), 24 hrs (■), 48 hrs (■), 72 hours (□). Controls were no SJW and no Tween 40, and SJW alone. Error bars are standard deviations. Two-way factorial ANOVA was used to compare the effect of time and various Tween 40 concentrations, *P*-values <0.0001. “*” indicates that at a particular exposure time the Tween 40 containing sample is significantly different from the SJW alone control. The alphabetic character indicates that in a particular Tween 40 treatment, the treatment is significantly different from other exposure times with different letters.
Figure 4.5. Typical culturability results of *M. JLS*, in the presence of various Tween 20 concentrations, treated with 0.33 mg/ml culture SJW MeOH extract. Tween 20 concentrations range from 0.003% to 0.100% v/v of culture solution. Exposure times were 0 hrs (■), 24 hrs (□), 48 hrs (■), 72 hours (□). Controls were no SJW and no Tween 20, and SJW alone. Error bars are standard deviations. Two-way factorial ANOVA was used to compare the effect of time and various Tween 20 concentrations, P-values <0.0001. "*" indicates that at a particular exposure time the Tween 20 containing sample is significantly different from the SJW alone control. The alphabetic character indicates that in a particular Tween 20 treatment, the treatment is significantly different from other exposure times with different letters.
Figure 4.6. Tween 80 micelles and SJW binding dye test. The Eosin Y absorbance results at the wavelength of 538nm: Tween 80 concentration ranging from 0 to 0.1% v/v of sample volume, (■) represents samples didn’t have SJW extract and (□) represents samples contained 0.33mg/ml SJW extract. Error bars are standard deviations. Two-way factorial ANOVA was used to show the effect of SJW existence on the absorbance of dye under each Tween 80 concentration, P-values <0.0001. “*” indicates that at a particular Tween 80 concentration the SJW containing sample is significantly different from the non-SJW containing sample.
CHAPTER 5
EFFECT OF HYPERICUM PERFORATUM ON MYCOBACTERIUM TUBERCULOSIS VIABILITY

Introduction

In Chapter 3 and our previous studies, the antimycobacterial activity of St. John’s Wort and its constituent, Hfn, against the growth of several non-pathogenic fast-grower mycobacterial strains was confirmed. Therefore, the bactericidal /inhibitory activity of SJW on pathogenic mycobacterial strains was tested. The pathogenic mycobacterial strain used in these studies was the infectious laboratory human pathogenic mycobacterial strain H37Rv, whose genome has been completely sequenced.

Materials and Methods

Bacterial Culture Preparation

*Mycobacterium tuberculosis* H37Rv was grown in Middlebrook 7H9 broth at 25°C with shaking to mid log phase. Cells were then diluted to an OD$_{600}$ of 0.010.

96-Well Plate Preparation

The most outer rows and columns of 96-well plate were filled with sterile water to maintain the humidity and prevent the evaporation of media. All wells of the column 2 and 11 were filled with 100µl media and the rest wells of the plate were filled with 50µl of media. Column 10 was considered as control filled with 50µl cell culture and 50µl media, and Column 11 was considered as control only filled with 100µl media.
Bacterial treatment with SJW acidified MeOH extract and purified hyperforin

Acidified MeOH SJW extract or purified hyperforin was added into column 2 and the concentrations were made to 5.32 mg SJW extract/ml culture or 200µl Hfn/ml culture. Pipette 50µl solution to column 3, mixed very well and then pipette 50 µl solution from column 3 to column 4. Such step was repeated until 50µl solution from column 8 was mixed into column 9, and then 50 µl solution was discarded from column 9. At last, each treatment well contained 50 µl media with SJW or hyperforin in it, and then 50µl cell culture was added into each well. The concentration of SJW and hyperforin was 2X diluted from column 2 to column 9, and each sample is triplicate. The tested drug concentrations showed in Table 5.1.

In acidified MeOH SJW extract treatment, MIC was tested both in the presence and absence of Tween 80. In the purified hyperforin treatment, MIC was only tested without Tween 80. In both SJW extract and purified hyperforin treatments, the exposure time was 6 days long. In SJW treatment data was collected only at Time 0 and Time 6-day, while in hyperforin treatment data was collected every day due to the quick degradation of hyperforin. AlamarBlue, a reagent to detect cell energy of proliferation, was used here to evaluate cell heath and then told us how many cells died under the treatment. The lower absorbance values represented less live cells, while the higher absorbance values represented more live cells in culture.
Table 5.1. The tested acidified SJW MeOH extract and hyperforin concentrations.

<table>
<thead>
<tr>
<th>Column Number</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>SJW concentrations (mg extract/ ml culture)</td>
<td>5.32</td>
<td>2.66</td>
<td>1.33</td>
<td>0.67</td>
<td>0.33</td>
<td>0.17</td>
<td>0.08</td>
<td>0.04</td>
</tr>
<tr>
<td>Hyperforin concentration (µl/ml culture)</td>
<td>200</td>
<td>100</td>
<td>50</td>
<td>25</td>
<td>12.5</td>
<td>6.25</td>
<td>3.15</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Results and Discussion

 SJW Acidified MeOH Extract Against M. tuberculosis MIC Studies

Results of M. tuberculosis H37Rv treated by SJW acidified MeOH extract are shown in Table 5.2. In the absence of Tween 80, SJW extract displayed the inhibitory effect at 0.67 mg/ culture, while in the presence of Tween 80, the same inhibitory effect was observed at 1.33mg/ cell culture. Compared with the MIC values of SJW extract against other non-pathogenic fast growers, the higher MIC values mean that the antimycobacterial activity of SJW is not as effective against growth of M. tuberculosis H37Rv. The Tween 80 in the culture still caused the repression of SJW’s action.

Table 5.2. SJW acidified MeOH extract MIC test against M. tuberculosis.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Tween 80</th>
<th>MIC (mg SJW extract/ cell culture)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SJW acidified MeOH extract</td>
<td>Yes</td>
<td>1.33</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>0.67</td>
</tr>
</tbody>
</table>

MIC: minimum inhibitory concentration.
Purified hyperforin against *M. tuberculosis* MIC studies

For the purified hyperforin treatment, results (Figure 5.1) showed that the activity was only detected at the two highest concentrations 100 µg/ml and 200 µg/ml, which corresponded to 11.56 mg SJW/ml culture. The inhibitory activity of hyperforin against *M. tuberculosis* is weaker than against *M. JLS*.

**Figure 5.1.** Typical culturability results of *M. tuberculosis* H37Rv, in the absence of Tween 80, treated with Hyperforin. Hyperforin concentrations were 3.15, 6.25, 12.5, 25, 50, 100, and 200 µg/ml culture and exposure time were day 1, day 2, day 3, day 4, day 5, day 6. Control was cell culture without hyperforin.
Conclusion

Both SJW acidified MeOH extract and hyperforin have inhibitory effect on *M. tuberculosis* H37Rv strain. The effect on *M. tuberculosis* H37Rv is not as strong as that for those non-pathogenic fast grown mycobacterial stains. Because there was only one pathogenic strain was tested, it is too early to deny the potential use of SJW or hyperforin as antimycobacterial drug. Other pathogenic mycobacterial strains need to be used in future studies.
CHAPTER 6
ENGINEERING APPLICATION AND FUTURE CONSIDERATIONS

Several potential engineering applications and related considerations of this project are listed below as well as the possible future research directions:

**Engineering Applications and Considerations**

1. To investigate the physicochemical properties of hyperforin, especially stability and solubility. This is essential to design a successful drug formulation and drug delivery system. Such studies should consider the influence of pH, buffer concentration/composition, ionic strength and drug nature hydrophobic/hydrophilic to solubility and stability.

2. To design the excipients formulation. In our previous studies, we already used HPLC to preliminarily detect the stability of hyperforin. Results showed hyperforin has poor chemical stability, especially under the exposure of light and oxygen. It is very important to find proper excipients formulation to keep hyperforin in the desired form until administration.

3. To select out the efficient delivery methods: such as oral administration, parenteral administration, transdermal administration, and aerosol administration. Such studies should consider the advantages and disadvantages of each delivery methods, the drug physicochemical properties, the duration of action, and the biological barriers.
4. Looking into the design of encapsulation materials to achieve the drug release demand, such as immediate release, nonimmediate release, delayed release, prolonged release, and controlled release. According to surfactant results in the Chapter 4, Tweens could be considered as potential constituents of such capsules.

5. About 90% of tuberculosis cases include the pulmonary infections. Aerosol delivery is a method to consider for hyperforin.

(1) To develop the micronization techniques. This can prepare hyperforin into the proper size ranges with suitable structures and therefore hyperforin can be successfully delivery to the lungs. The common technologies include spray drying and supercritical fluid manufacture.

(2) To choose the proper aerosol devices: in clinics, the widely used aerosol devices are the propellant-driven metered dose inhaler (pMDIs), dry powder inhalers (DPIs) and nebulizers. To deliver by pMDIs or nebulizers, it is critical to figure out how to homogeneously dissolve or suspend SJW or hyperforin in a selected propellant. Usually, the choices of additive, like co-solvents or surfactants are crucial. To deliver by DPIs, it is critical to figure out how to blend hyperforin into respirable particles.

(3) To detect the target tissue permeability of hyperforin by conducting the cell-bases assays. In the hyperforin case the target organ is pulmonary, so alveolar epithelium culture model is the best to try.
Future Work Recommendations

1. To investigate the strength difference of antimycobacterial activity between SJW acidified methanol extract and purified Hfn. According to results in Chapter 3, it is obvious that the anti-mycobacterial activity of Hfn is much weaker than the corresponded SJW concentrations. The investigation of the difference could be carried out based on two speculations. One possibility is that some other constituents of SJW, which have antimycobacterial activity, have additive or synergistic activities with Hfn. It is possible that the activity of purified Hfn is somehow diminished during the experiments, for example, the degradation by light or oxygen. Such studies should include using the HPLC to detect and quantify other important constituents, like adhyperforin and quercetin, in the acidified methanol extract of SJW, testing MIC and MBC of new purified candidates against non-pathogenic and pathogenic mycobacterial strains, and testing MIC and MBC of the combination of Hfn and other constitutes of SJW against mycobacterial strains for the synergistic or additive effectiveness.

2. Like tuberculosis and leprosy, an antibiotic combination regimen is commonly used in the treatment of disease caused by pathogenic mycobacterial strains. It is worth to figure out what are the drug-drug interaction between hyperforin and other clinical anti-mycobacterial drugs are additive, synergistic, or repulsive.

3. To investigate the mechanism of hyperforin activity by using DNA microarray and proteomic analysis. Such studies would include firstly using the gene arrays containing the gene of widely spread human pathogenic mycobacterial strains, for example, PanoramaTM M. tuberculosis Gene Arrays, which contains complete
genomes of both the infectious laboratory strain (H37Rv) and the recent clinical isolate (CDC-1551), to detect the changes of gene expression level induced by hyperforin, and then using the proteomic analysis to determine structures and functions of proteins induced by hyperforin.

4. To investigate the mechanism of Tween 80 on hyperforin and other anti-mycobacterial drugs. According to our discussion and results in the Chapter 4, the interaction between micelles of Tween surfactants and anti-mycobacteria drug molecules is one possibility to explain the effectiveness of Tween in the tests. It highly recommends future studies to confirm the existence of encapsulation by running NMR diffusion experiments (DOSY NMR) to show the sequestered hyperforin molecules diffuses at the same rate as the micelles formed by Tweens.

5. To demonstrate the anti-mycobacterial activity of SJW and hyperforin against other pathogenic mycobacterial strains. Although tuberculosis is the most widely spread disease caused by mycobacterial strains, it is still meaningful to test the activity of SJW and hyperforin against other pathogenic mycobacteria, such as M. leprae and M.ulcerans. It is possible that other pathogenic mycobacteria will be more sensitive to SJW and hyperforin than M. tuberculosis.
REFERENCES


APPENDICES
Appendix. Statistic Analysis
Table A.1. Statistic analysis of in the absence of Tween 80 SJW MeOH extract against M. JLS test. Experimental design information (A) and Two-way ANVOA analysis results (B).

### A

<table>
<thead>
<tr>
<th>Class</th>
<th>Levels</th>
<th>Values</th>
<th>Replicates</th>
<th>Number of Observations Read</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hfn (µg/ml culture)</td>
<td>6</td>
<td>C, M, 0.05, 0.09, 0.17, 0.33</td>
<td>2 per sample</td>
<td>48</td>
</tr>
<tr>
<td>Time (hour)</td>
<td>4</td>
<td>0, 24, 48, 72</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C, Control: no SJW MeOH extract contained.
M, MeOH Control: 0.02% v/v methanol contained; no SJW MeOH extract contained.

### B

<table>
<thead>
<tr>
<th>Source</th>
<th>Degree of Freedom</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr&gt;F</th>
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</thead>
<tbody>
<tr>
<td>Model</td>
<td>23</td>
<td>1937.78</td>
<td>84.25</td>
<td></td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Error</td>
<td>24</td>
<td>13.45</td>
<td>0.56</td>
<td>150.34</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Corrected Total</td>
<td>47</td>
<td>1951.23</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table A.2. Statistic analysis of in the presence of Tween 80 hyperforin against *M.* JLS test. Experimental design information (A) and Two-way ANVOA analysis results (B).

### A

<table>
<thead>
<tr>
<th>Class</th>
<th>Levels</th>
<th>Values</th>
<th>Replicates</th>
<th>Number of Observations Read</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hfn (µg/ml culture)</td>
<td>8</td>
<td>C, M, 3, 6, 12, 23, 46, 80</td>
<td>3</td>
<td>96</td>
</tr>
<tr>
<td>Time (hour)</td>
<td>4</td>
<td>0, 24, 48, 72</td>
<td>3 per sample</td>
<td></td>
</tr>
</tbody>
</table>

C, Control: no hyperforin contained.
M, MeOH Control: 0.02% v/v methanol contained; no hyperforin contained.

### B

<table>
<thead>
<tr>
<th>Source</th>
<th>Degree of Freedom</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr&gt;F</th>
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</thead>
<tbody>
<tr>
<td>Model</td>
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<td>978.32</td>
<td>31.56</td>
<td>35.73</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Error</td>
<td>64</td>
<td>56.54</td>
<td>0.88</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected Total</td>
<td>95</td>
<td>1034.85</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table A.3. Statistic analysis of in the absence of Tween 80 hyperforin against M. JLS test. Experimental design information (A) and Two-way ANVOA analysis results (B).

### A

<table>
<thead>
<tr>
<th>Class Level Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class</td>
</tr>
<tr>
<td>Hfn (µg/ml culture)</td>
</tr>
<tr>
<td>Time (hour)</td>
</tr>
</tbody>
</table>

C, Control: no hyperforin contained.
M, MeOH Control: 0.02% v/v methanol contained; no hyperforin contained.

### B

<table>
<thead>
<tr>
<th>Source</th>
<th>Degree of Freedom</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr&gt;F</th>
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</thead>
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<tr>
<td>Model</td>
<td>31</td>
<td>2210.65</td>
<td>71.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>64</td>
<td>83.87</td>
<td>1.31</td>
<td>54.41</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Corrected Total</td>
<td>95</td>
<td>2294.53</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table A.4. Statistic analysis of in the presence of various Tween 80 concentrations SJW MeOH extract against *M. JLS* test. Experimental design information (A) and Two-way ANOVA analysis results (B).

### A

<table>
<thead>
<tr>
<th>Class</th>
<th>Levels</th>
<th>Values</th>
<th>Replicates</th>
<th>Number of Observations Read</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween 80 (%) v/v</td>
<td>8</td>
<td>C, S, 0.003, 0.006, 0.013, 0.025, 0.05, 0.1</td>
<td>6</td>
<td>6 per sample</td>
</tr>
<tr>
<td>Time (hour)</td>
<td>4</td>
<td>0, 24, 48, 72</td>
<td></td>
<td>96</td>
</tr>
</tbody>
</table>

C, Control: no SJW MeOH extract and Tween 80 contained. 
S, SJW alone: only 0.33 mg SJW extract/ml culture contained; no Tween 80 contained.

### B

<table>
<thead>
<tr>
<th>Source</th>
<th>Degree of Freedom</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr&gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>31</td>
<td>6879.91</td>
<td>221.93</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>160</td>
<td>186.81</td>
<td>1.17</td>
<td>190.08</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Corrected Total</td>
<td>191</td>
<td>7066.73</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table A.5. Statistic analysis of the presence of various Tween 60 concentrations SJW MeOH extract against M. JLS test. Experimental design information (A) and Two-way ANVOA analysis results (B).

A

<table>
<thead>
<tr>
<th>Class</th>
<th>Levels</th>
<th>Values</th>
<th>Replicates</th>
<th>Number of Observations Read</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween 60 (% v/v)</td>
<td>8</td>
<td>C, M, 0.003, 0.006, 0.013, 0.025, 0.05, 0.1</td>
<td>6 per sample</td>
<td>96</td>
</tr>
<tr>
<td>Time (hour)</td>
<td>4</td>
<td>0, 24, 48, 72</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C, Control: no SJW MeOH extract and Tween 60 contained.
S, SJW alone: only 0.33mg SJW extract/ml culture contained; no Tween 60 contained.

B

<table>
<thead>
<tr>
<th>Source</th>
<th>Degree of Freedom</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr&gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>31</td>
<td>8692.67</td>
<td>280.41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>160</td>
<td>15.52</td>
<td>0.097</td>
<td>2891.63</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Corrected Total</td>
<td>191</td>
<td>8708.18</td>
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</table>
Table A.6. Statistic analysis of in the presence of various Tween 40 concentrations SJW MeOH extract against *M. JLS* test. Experimental design information (A) and Two-way ANVOA analysis results (B).

### A

<table>
<thead>
<tr>
<th>Class Level Information</th>
<th>Class</th>
<th>Levels</th>
<th>Values</th>
<th>Replicates</th>
<th>Number of Observations Read</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween 40 (% v/v)</td>
<td>8</td>
<td>C, M, 0.003, 0.006, 0.013, 0.025, 0.05, 0.1</td>
<td>6 per sample</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>Time (hour)</td>
<td>4</td>
<td>0, 24, 48, 72</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C, Control: no SJW MeOH extract and Tween 40 contained.
S, SJW alone: only 0.33mg SJW extract/ml culture contained; no Tween 40 contained.

### B

<table>
<thead>
<tr>
<th>Source</th>
<th>Degree of Freedom</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr&gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>31</td>
<td>8406.75</td>
<td>271.19</td>
<td></td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Error</td>
<td>160</td>
<td>108.15</td>
<td>0.68</td>
<td>401.21</td>
<td></td>
</tr>
<tr>
<td>Corrected Total</td>
<td>191</td>
<td>8514.90</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table A.7. Statistic analysis of in the presence of various Tween 20 concentrations SJW MeOH extract against *M. JLS* test. Experimental design information (A) and Two-way ANOVA analysis results (B).

### A

<table>
<thead>
<tr>
<th>Class</th>
<th>Levels</th>
<th>Values</th>
<th>Replicates</th>
<th>Number of Observations Read</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween 20 (% v/v)</td>
<td>8</td>
<td>C, M, 0.003, 0.006, 0.013, 0.025, 0.05, 0.1</td>
<td>6 per sample</td>
<td>96</td>
</tr>
<tr>
<td>Time (hour)</td>
<td>4</td>
<td>0, 24, 48, 72</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C, Control: no SJW MeOH extract and Tween 20 contained.

S, SJW alone: only 0.33mg SJW extract/ml culture contained; no Tween 20 contained.

### B

<table>
<thead>
<tr>
<th>Source</th>
<th>Degree of Freedom</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr&gt;F</th>
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</thead>
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<td>262.98</td>
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<td>&lt;.0001</td>
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<tr>
<td>Error</td>
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<td>96.37</td>
<td>0.60</td>
<td>436.62</td>
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<tr>
<td>Corrected Total</td>
<td>191</td>
<td>8248.66</td>
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</table>
Table A.8  Statistic analysis of Tween 80 micelles and SJW binding dye test. Experimental design information (A) and Two-way ANVOA analysis results (B).

A

<table>
<thead>
<tr>
<th>Class</th>
<th>Levels</th>
<th>Values</th>
<th>Replicates</th>
<th>Number of Observations Read</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween 80 (%) v/v</td>
<td>7</td>
<td>0, 0.003, 0.006, 0.013, 0.025, 0.05, 0.1</td>
<td>3 per sample</td>
<td>42</td>
</tr>
<tr>
<td>SJW</td>
<td>2</td>
<td>Yes, No</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Source</th>
<th>Degree of Freedom</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr&gt;F</th>
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</thead>
<tbody>
<tr>
<td>Model</td>
<td>13</td>
<td>6.44</td>
<td>0.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>28</td>
<td>0.00</td>
<td>0.00</td>
<td>23196.4</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Corrected Total</td>
<td>41</td>
<td>6.44</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>