5-2010

The Hypericum Perforatum Herb as an Antimycobacterial Agent and Its Implications as an Additional Tuberculosis Medication

Trent W. Mortensen
Utah State University

Follow this and additional works at: https://digitalcommons.usu.edu/etd

Part of the Biomedical Engineering and Bioengineering Commons, Medicine and Health Sciences Commons, and the Microbiology Commons

Recommended Citation
Mortensen, Trent W., "The Hypericum Perforatum Herb as an Antimycobacterial Agent and Its Implications as an Additional Tuberculosis Medication" (2010). All Graduate Theses and Dissertations. 714.
https://digitalcommons.usu.edu/etd/714

This Thesis is brought to you for free and open access by the Graduate Studies at DigitalCommons@USU. It has been accepted for inclusion in All Graduate Theses and Dissertations by an authorized administrator of DigitalCommons@USU. For more information, please contact rebecca.nelson@usu.edu.
THE HYPERICUM PERFORATUM HERB AS AN ANTIMYCOBACTERIAL AGENT AND ITS IMPLICATIONS AS AN ADDITIONAL TUBERCULOSIS MEDICATION

by

Trent W. Mortensen

A thesis submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

Biological Engineering

Approved:

Charles D. Miller
Major Professor

Ronald C. Sims
Committee Member

Marie K. Walsh
Committee Member

Soonjo Kwon
Committee Member

Byron R. Burnham
Dean of Graduate Studies

UTAH STATE UNIVERSITY
Logan, Utah

2010
ABSTRACT

Hypericum perforatum as an Antimycobacterial Agent and Its Implications as an Additional Tuberculosis Medication

by

Trent W. Mortensen, Master of Science
Utah State University, 2010

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

An immediate demand exists for new tuberculosis (TB) antibiotics due to the ever-increasing spread of drug-resistant strains. The drug-development process goes through four phases, the first (Phase 0) of which is to demonstrate and investigate drug effectiveness and toxicity. This research investigated the effectiveness of the Hypericum perforatum herb (commonly St. John’s wort (SJW)) in its growth inhibition of mycobacteria and its viability effect on human lung cells.

Organic-solvent SJW extracts were effective at inhibiting every nonpathogenic genetically sequenced Mycobacterium isolate currently available (six isolates) in preliminary studies. Quantitative studies of five Mycobacterium isolates showed an order of concentration sensitivity to the SJW methanol (MeOH) extract as (high to low) M. JLS, M. KMS, M. phlei (not sequenced), M. MCS, B. subtilis, M. smegmatis, and E. coli, with minimal bactericidal concentrations (MBCs) ranging from 0.33-2.66 mg extract/ml. The SJW compounds hyperforin (Hfn), hypericin (Hpn), and pseudohypericin (Phn) were
quantified using a novel HPLC method that utilized common HPLC equipment. A crude MeOH extract solution of 133 mg extract/ml contained 2.26 mg Hfn/ml, 0.77 mg Hpn/ml, and 2.67 mg Phn/ml. Purified Hfn had a MBC of between 6-13 µg/ml for *M. JLS* in the absence of Tween 80. Tween 80 repressed Hfn (46 µg/ml) inhibition of *M. JLS* at ≥ 0.025% (v/v). Purified Hpn and Phn showed no inhibition of *M. JLS* at all assayed concentrations, which were ≤ 27 µg/ml and ≤ 25 µg/ml, respectively. Inhibitory results from the five quantitatively assayed *Mycobacterium* samples could be extrapolated to *M. tuberculosis*, as these isolates have as high as 72% genetic homology to the pathogen.

The crude MeOH extract and Hfn were lethal to the human carcinomic alveolar epithelial lung cell line A549 at 1.3 mg extract/ml (crude extract) and ≥ 11 µg/ml (Hfn), with a Hfn LD<sub>50</sub> of 3-6 µg/ml (5.6-11.2 µmol/L). Because Hfn is antiproliferative to a list of other carcinomic cell lines in the same concentration range, the A549 cell line may be added to that list. The addition of *M. JLS* cells (5x10<sup>6</sup> cells/cm<sup>2</sup>) to penicillin-streptomycin-containing A549 culture (which killed the bacteria) did not affect A549 viability.
ACKNOWLEDGMENTS

I would like to thank the Department of Biological Engineering faculty and staff for providing excellent advice and support throughout the course of this project. I would particularly like to thank my advisor, Dr. Charles Miller, for taking a considerable amount of time to help me plan, analyze, and troubleshoot several aspects of this research. It has also been a pleasure to work with my committee members, Dr. Ronald Sims, Dr. Marie Walsh, and Dr. Soonjo Kwon, who helped me many times with this project.

I would like to thank the Huntsman Environmental Research Center for their financial support.

I would also like to acknowledge Drs. T.C. and FenAnn Shen for their many late-night and weekend hours spent with the SEM preparation and imaging.

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

Trent Mortensen
## CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>The Global Tuberculosis Problem</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Hypothesis and Objectives</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>REVIEW OF LITERATURE</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Mycobacteria</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Tuberculosis</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Drug Development and Toxicity Testing</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td><em>Hypericum perforatum</em> (St. John’s Wort)</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>HPLC Quantification of SJW Compounds</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>SJW Antimicrobial Studies</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Human Alveolar Epithelial Cells (A549)</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>Summary</td>
<td>19</td>
</tr>
<tr>
<td>3</td>
<td>HPLC-DAD QUANTIFICATION OF SJW COMPOUNDS</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Introduction</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Materials and Methods</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Results and Discussion</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>Conclusions</td>
<td>34</td>
</tr>
<tr>
<td>4</td>
<td>EFFECT OF <em>HYPERICUM PERFORATUM</em> ON MYCOBACTERIA VIABILITY</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>Introduction</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>Materials and Methods</td>
<td>41</td>
</tr>
</tbody>
</table>
Results and Discussion ................................................................. 48
Conclusions .................................................................................. 57

5. EFFECT OF HYPERICUM PERFORATUM ON
HUMAN ALVEOLAR EPITHELIAL CELLS ...................................... 84

   Introduction ............................................................................. 84
   Materials and Methods ........................................................... 84
   Results and Discussion ............................................................ 88
   Conclusions .......................................................................... 92

6. ENGINEERING APPLICATIONS AND
FUTURE CONSIDERATIONS .................................................... 102

   Engineering Applications and Considerations ....................... 102
   Future Work Recommendations ............................................. 104

REFERENCES .................................................................................. 107

APPENDIX ......................................................................................... 117
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Literature MIC values of SJW extracts and pure compounds against Gram-positive and Gram-negative bacteria</td>
<td>21</td>
</tr>
<tr>
<td>2.2</td>
<td>A comprehensive list of all organisms assayed in cited SJW inhibitory assays, showing Gram-positive strains, Gram-negative strains, and yeasts</td>
<td>22</td>
</tr>
<tr>
<td>3.1</td>
<td>Volumes, concentrations, and porosity of SJW herb and MeOH extract</td>
<td>35</td>
</tr>
<tr>
<td>3.2</td>
<td>Retention times, regression equations, and correlation coefficients ($R^2$) for the three SJW analytes from 1:50 dilutions of SJW extract</td>
<td>35</td>
</tr>
<tr>
<td>3.3</td>
<td>Assayed concentration values of SJW compounds at 1:50 dilution of the 1X crude extract</td>
<td>35</td>
</tr>
<tr>
<td>4.1</td>
<td>Inhibition demonstration of SJW extracts of various solvents and various SJW brands after one week of growth on crude-extract-containing plates</td>
<td>60</td>
</tr>
<tr>
<td>4.2</td>
<td>Inhibition demonstration of SJW extracts of various solvents and various SJW brands after two weeks of growth on crude-extract-containing plates</td>
<td>61</td>
</tr>
<tr>
<td>4.3</td>
<td>Minimum bactericidal concentrations for the seven MBC-assayed strains</td>
<td>62</td>
</tr>
<tr>
<td>5.1</td>
<td>Statistical analysis parameters for the A549 samples from the direct cell count study</td>
<td>94</td>
</tr>
<tr>
<td>5.2</td>
<td>Ryan-Einot-Gabriel-Welsh q (REGWQ) test for A549 cell treatments that received direct cell counting</td>
<td>94</td>
</tr>
<tr>
<td>5.3</td>
<td>Growth results in Ham’s media of all <em>Mycobacterium</em> isolates used in the MBC studies</td>
<td>95</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Chemical structures of Hpn, Phn, and Hfn</td>
<td>23</td>
</tr>
<tr>
<td>3.1</td>
<td>Normalized spectra of filtered-light headlamp used when working with light-sensitive compounds</td>
<td>36</td>
</tr>
<tr>
<td>3.2</td>
<td>Spectrogram of the Innova incubator-shaker photosynthetic light</td>
<td>36</td>
</tr>
<tr>
<td>3.3</td>
<td>Photon irradiance of the Innova incubator-shaker photosynthetic light as a function of distance from the light source</td>
<td>37</td>
</tr>
<tr>
<td>3.4</td>
<td>Solubility (OD&lt;sub&gt;600&lt;/sub&gt;) of 2% (v/v) SJW crude MeOH extract in various aqueous concentrations of MeOH and ACN</td>
<td>37</td>
</tr>
<tr>
<td>3.5</td>
<td>Typical chromatograms for Phn, Hfn, and Hpn standards</td>
<td>38</td>
</tr>
<tr>
<td>3.6</td>
<td>Typical chromatograms for Phn, Hfn, and Hpn in 1:50 dilution of 1X crude extract</td>
<td>38</td>
</tr>
<tr>
<td>3.7</td>
<td>Calibration curves used in the quantification of SJW compounds</td>
<td>39</td>
</tr>
<tr>
<td>3.8</td>
<td>Contour view of HPLC mAU data for SJW crude extract</td>
<td>39</td>
</tr>
<tr>
<td>3.9</td>
<td>Photoconversion/photodegradation study of SJW compounds</td>
<td>40</td>
</tr>
<tr>
<td>4.1</td>
<td>Venn diagram for genetic similarity of all currently sequenced, nonpathogenic mycobacteria to <em>M. tuberculosis</em></td>
<td>63</td>
</tr>
<tr>
<td>4.2</td>
<td>The five <em>Mycobacterium</em> strains used in the MBC studies</td>
<td>63</td>
</tr>
<tr>
<td>4.3</td>
<td>Growth plates for demonstration of inhibitory effect and comparison of extracts of different solvents and different SJW brands</td>
<td>64</td>
</tr>
<tr>
<td>4.4</td>
<td>Comparison of mycobacterial growth on LB and MB media</td>
<td>65</td>
</tr>
</tbody>
</table>
4.5 Growth comparison of five assayed Mycobacterium isolates on antibiotic-free LB plates and ampicillin-containing LB plates .......................................................... 66

4.6 Typical example of CFU quantification results using the cell dilution method ........................................................................................................... 66

4.7 Effect of MeOH on M. KMS viability .......................................................... 67

4.8 Effect of EtOH on M. KMS viability ........................................................... 67

4.9 Growth plates from dilution-in-agar MIC study of SJW EtOH extract ........................................................................................................... 68

4.10 Typical cell dilution plates used for CFU quantification in MBC studies ........................................................................................................... 69

4.11 Typical growth/death results of M. JLS from the crude extract MBC studies ........................................................................................................... 70

4.12 Typical growth/death results of M. KMS from the crude extract MBC studies ........................................................................................................... 70

4.13 Typical growth/death results of M. MCS from the crude extract MBC studies ........................................................................................................... 71

4.14 Typical growth/death results of M. smegmatis from the crude extract MBC studies ........................................................................................................... 71

4.15 Typical growth/death results of M. phlei from the crude extract MBC studies ........................................................................................................... 72

4.16 Typical growth/death results of E. coli from the crude extract MBC studies ........................................................................................................... 72

4.17 Typical growth/death results of B. subtilis from the crude extract MBC studies ........................................................................................................... 73

4.18 The time course of SJW effect on B. subtilis .............................................. 73

4.19 Growth/inhibition results for M. JLS treated with Hfn in the absence of Tween 80 ........................................................................................................... 74

4.20 Growth/inhibition results for M. JLS treated with Hfn in the presence of Tween 80 ........................................................................................................... 74
4.21 Tween 80 repression of Hfn inhibition of M. JLS with all samples except the control containing 46 µg/ml Hfn .........................................75

4.22 Growth/inhibition results for M. JLS treated with crude extract in the absence of Tween 80 .............................................................................75

4.23 Growth/inhibition results for M. JLS treated with crude extract in the presence of Tween 80 ..........................................................................................76

4.24 Growth/inhibition results for M. JLS at various concentrations of Hpn without Tween 80 added to the culture ..........................................................................................76

4.25 Growth/inhibition results for M. JLS at various concentrations of Phn without Tween 80 added to the culture ..........................................................................................77

4.26 Growth/inhibition results for the light-exposed samples (M. KMS) of the light/dark Hfn study ..........................................................................................77

4.27 Growth/inhibition curves for the foil-wrapped samples (M. KMS) of the light/dark Hfn study ..........................................................................................78

4.28 SEM images of untreated strains used in the SJW inhibitory/bactericidal assays ..........................................................................................79

4.29 SEM images of the progression of M. KMS response to SJW MeOH crude extract ..........................................................................................80

4.30 SEM images of the progression of M. smegmatis response to SJW MeOH crude extract ..........................................................................................81

4.31 SEM images of the progression of B. subtilis response to SJW MeOH crude extract ..........................................................................................82

4.32 SEM images of the response of E. coli to SJW MeOH crude extract ..........................................................................................83

5.1 The improved Neubauer Bright-Line hemacytometer setup ..........................................................................................96

5.2 Pierce BCA total protein assay for the effect of MeOH on A549 viability ..........................................................................................97

5.3 Pierce BCA total protein assay results for Hpn, Hfn, Phn and SJW MeOH crude extract at concentrations found in the 4.46 mg extract/ml crude extract ..........................................................................................97
5.4 Direct cell count results for effect of dead *M. JLS* cells, SJW MeOH crude extract, and Hfn on A549 cell viability .......................98

5.5 24-hr exposure of A549 cells to dead *M. JLS* cells, crude extract, and Hfn.................................................................99

5.6 48-hr exposure of A549 cells to dead *M. JLS* cells, crude extract, and Hfn...........................................................................100

5.7 72-hr exposure of A549 cells to dead *M. JLS* cells, crude extract, and Hfn.................................................................101
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile (also CH$_3$CN)</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>aq</td>
<td>Aqueous</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ATS</td>
<td>American Thoracic Society</td>
</tr>
<tr>
<td>B.</td>
<td>Bacillus (Genus)</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid (protein assay reagent)</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacillus Calmette Guerin TB vaccine</td>
</tr>
<tr>
<td>BD</td>
<td>Becton, Dickinson and Company</td>
</tr>
<tr>
<td>BLA</td>
<td>Biologics license application</td>
</tr>
<tr>
<td>BuOH</td>
<td>Butanol</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees centigrade (or Celsius)</td>
</tr>
<tr>
<td>C8, C12, or C18</td>
<td>Number of carbons in a hydrocarbon chain</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CH$_3$CN</td>
<td>Acetonitrile (also ACN)</td>
</tr>
<tr>
<td>(CH$_3$)$_2$CO</td>
<td>Acetone</td>
</tr>
<tr>
<td>C$<em>4$H$</em>{10}$O</td>
<td>n-butanol</td>
</tr>
<tr>
<td>CHCl$_3$</td>
<td>Chloroform</td>
</tr>
<tr>
<td>cm</td>
<td>Centimeter (10$^{-2}$ meters)</td>
</tr>
<tr>
<td>cm$^2$</td>
<td>Square centimeter</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CT</td>
<td>Computerized tomography scan</td>
</tr>
<tr>
<td>DAD</td>
<td>Diode array (or photodiode array) detector</td>
</tr>
<tr>
<td>DI</td>
<td>Deionized</td>
</tr>
<tr>
<td>DF</td>
<td>Degrees of freedom</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>E.</td>
<td>Escherichia (Genus)</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EMB</td>
<td>Ethambutol</td>
</tr>
<tr>
<td>Et$_2$O</td>
<td>Diethyl ether</td>
</tr>
<tr>
<td>et al.</td>
<td>“And others”</td>
</tr>
<tr>
<td>EtOAc</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
</tbody>
</table>
F-value  Statistical analysis parameter used to determine p-value
FBS   Fetal bovine serum
FCS   Fetal calf serum
FDA   Food and Drug Administration
g    Gram
G(-)  Gram negative
G(+)  Gram positive
GLM   General linear modeling, statistical analysis procedure
GNC   General Nutrition Centers
HCl   Hydrochloric acid
HEPES 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid
H2O   Water
H2PO4 (Ortho) phosphoric acid
Hfn   Hyperforin
HIV   Human immunodeficiency virus
HMDS  Hexamethyldisilazane
HPLC  High performance liquid chromatography
Hpn   Hypericin
hr    Hour
IDSA  Infectious Diseases Society of America
IMG   Integrated Microbial Genomes
INH   Isoniazid
JGI   Joint Genome Institute
K     1000
LB    Luria Broth media, either liquid or agar
LCA   Lung-cell toxicity assay
LD50  Half maximal lethal dose
LOD   Limit of detection
LTBI  Latent tuberculosis infection
M. (in strain names) *Mycobacterium* (Genus)
mAU  Milli \((10^{-3})\) arbitrary (or absorbance) units
MB   Middlebrook media, either liquid or agar
MBC  Minimum bactericidal concentration
MDR-TB Multidrug resistant tuberculosis
MeOH  Methanol
MIC  Minimum inhibitory concentration
min  Minute
ml   Milliliter \((10^{-3})\) liters
mm   Millimeter \((10^{-3})\) meters
mm²  Square millimeter
NAA  Nuclear acid amplification
NaHCO₃  Sodium bicarbonate
NCBI  National Center for Biotechnology Information
ND  Not determined
NDA  New drug application
nl  Nanoliter (10⁻⁹ liters)
nm  Nanometer (10⁻⁹ meters)
NMR  Nuclear magnetic resonance
NOW  A natural products company
OD  Optical density (subscript is wavelength)
p-value  Statistical analysis parameter
PAH  Polyaromatic hydrocarbon
PBL  Pacific Biolabs, company
PBS  Phosphate-buffered saline
PCR  Polymerase Chain Reaction
PDT  Photodynamic therapy
Pet.  Petroleum
Phn  Pseudohypericin
PIPES  Piperazine-N,N’-bis(2-ethanesulfonic acid)
PLGA  Poly (lactide-co-glycolide)
PPD  Purified protein derivative
PZA  Pyrazinamide
R²  Coefficient of determination
RCF  Relative centrifugal force
REGWQ  Ryan-Einot-Gabriel-Welsh q test
RF  Solute distance /solvent distance traveled
RIF  Rifampicin
RNA  Ribonucleic acid
RPM  Revolutions per minute
s  Second
SAS  Statistical Analysis System (originally), statistics software
SD  Standard deviation
Sec  A protein secretion pathway
SEM  Scanning electron microscopy
SJW  St. John’s wort
SM  Streptomycin
Std. dev.  Standard deviation
Tat  A protein secretion pathway
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>Twn</td>
<td>Tween (as in Tween 80)</td>
</tr>
<tr>
<td>U</td>
<td>Units</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram (10^{-6} grams)</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>µL</td>
<td>Microliter (10^{-6} liters)</td>
</tr>
<tr>
<td>µm</td>
<td>Micrometer (10^{-6} meters)</td>
</tr>
<tr>
<td>µmol</td>
<td>6.02 x 10^{17} (10^{-6} moles)</td>
</tr>
<tr>
<td>USU</td>
<td>Utah State University</td>
</tr>
<tr>
<td>UV-VIS</td>
<td>Ultraviolet-visible light</td>
</tr>
<tr>
<td>VT</td>
<td>Dry volume</td>
</tr>
<tr>
<td>VV</td>
<td>Void volume</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume per volume, concentration</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight (mass) per volume, concentration</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>(#)X</td>
<td>Concentration of crude extract (Table 3.1)</td>
</tr>
<tr>
<td>XDR-TB</td>
<td>Extensively drug-resistant tuberculosis</td>
</tr>
</tbody>
</table>
CHAPTER 1
INTRODUCTION

The Need for Additional Mycobacterial Antibiotics

The Global Tuberculosis Problem

Among diseases caused by the genus *Mycobacterium*, tuberculosis (TB) is the most common. This infectious disease is caused by *Mycobacterium tuberculosis* and affects about one-third of the world’s population. About 10% of those infected develop the active form of the disease in their lifetime, which usually results in pulmonary (lung) infection but can also infect other areas of the body (Smith, 2003). Those who do not develop the active disease maintain the latent disease (Jasmer et al., 2002). In 2008, there were 9.4 million new TB cases and 1.8 million TB-related deaths (WHO, 2009).

TB treatment has gone through several stages, from the fresh-air sanatoriums of the 19th century to the chemotherapeutic treatments of today (Snider, 1997). Current treatment regimens rely on strategies utilizing first- and second-line drugs, with the first-line drugs being more effective and having fewer side effects (Blumberg et al., 2003). Lack of adherence to drug regimens has led to the evolution of drug-resistant TB strains that do not respond to the best first-line medications and, in some cases, the best second-line medications (Sharma and Mohan, 2006; CDC, 2010). Therefore, novel TB medications are needed that shorten regimen duration and improve the treatment of drug-resistant and latent TB (O’Brien and Nunn, 2001). The purpose of this research was to investigate the feasibility of using the *Hypericum perforatum* herb (or more commonly St. John’s wort) for producing such medications.
**Hypothesis**

The *Hypericum perforatum* herb contains quantifiable compounds that cause cell death of *Mycobacterium* isolates but not of human lung cells.

**Objectives**

1. Establish an efficient, accurate, and precise high-performance liquid chromatography (HPLC) method for quantification of the SJW compounds pseudohypericin, hyperforin, and hypericin.

2. Demonstrate inhibitory effectiveness of SJW compounds on mycobacteria and determine the effective concentrations.

3. Determine the effect of SJW compounds on the viability of human alveolar epithelial cells.
CHAPTER 2
REVIEW OF LITERATURE

Mycobacteria

Genus Properties

*M. tuberculosis* and others of the genus *Mycobacterium* have distinct properties that set them apart from other bacteria. With regard to scientific classification, mycobacteria are their own family, Mycobacteriaceae, under the suborder Corynebacterineae, under the order Actinomycetales, under the phylum Actinobacteria in the Bacteria kingdom (NCBI, 2010).

Mycobacteria are aerobic and generally nonmotile. Since this genus does not retain a crystal-violet stain, it is classified as an acid-fast Gram-positive bacteria because it does not have an outer membrane. Mycobacteria have characteristically thick, waxy, cell walls largely composed of mycolic acids. This cell wall is composed of the following layers (from outer to inner): outer layer (capsule) lipid, mycolic acid, arabinogalactan polysaccharide, peptidoglycan, periplasm, and plasma membrane (Daffe and Reyrat, 2008). Mycobacteria are bacilli that can range in size from 0.2-0.7 µm wide and 1.0-10 µm long (Holt *et al.*, 1994). They can be classified in their pigmentation as follows: photochromagens, which produce non-pigmented colonies in the dark and pigmented colonies in the light, scotochromogens, which always produce deep yellow or orange colonies, and non-chromogens, which have a pale yellow or tan color (*M. tuberculosis* is of this type) (Jenkins, 1981). Mycobacteria can be found in water, soil, or animal tissues or secretions.
Mycobacteria are relatively slow growing, with those that form visible colonies within seven days being called rapid growers, and those taking longer being called slow growers (Wayne and Kubica, 1986). They are relatively flexible in their growth conditions, with growth temperatures (depending on strain) ranging from 25°C to over 50°C and the ability to grow on several types of media with simple nitrogen, carbon, and energy sources (Allen, 1998). Tween 80, a surfactant, is often added to mycobacterial liquid cultures to lessen clumping, which is often an issue. However, Tween 80 can also alter the outmost layer of the cell wall (Parish and Stoker, 1998).

Mycobacteria are known to have functional secretion pathways, including Sec, Tat, and mycobacterial-specific pathways, with orthologs of all known necessary secretion proteins for these pathways present in mycobacterial genomes. Secreted proteins in pathogenic mycobacteria play an important part in their virulence (McCann et al., 2009).

According to the National Center for Biotechnology Information (NCBI, 2010), there are 40 completely sequenced genome variations of 14 mycobacterium species, *M. tuberculosis* having 22 variations. There are only six nonpathogenic *Mycobacterium* isolates in that database, all of which are considered rapid growers: *M. smegmatis*, *M. gilvum* PYR-GCK, *M. vanbaalenii* PYR-1, *M. JLS*, *M. KMS*, and *M. MCS*. The latter five isolates are known for their ability to mineralize polycyclic aromatic hydrocarbons (PAHs), with the latter three of these having been isolated by researchers at Utah State University (Miller et al., 2004, 2007).
Tuberculosis

Prevalence

As of 2009, information provided by the World Health Organization (WHO) states that TB infects one-third of the world’s population, and that 10% of those infected will develop the active form of TB sometime during their lifetime. Those with active TB each infect 10-15 people, on average. TB has the highest incidence in areas of poverty and is a leading cause of death for Human Immunodeficiency Virus (HIV)-positive people, making HIV-infected persons 20 to 40 times more susceptible to active TB development (WHO, 2009).

Pathogenesis

While much about the mechanism of action for the virulence of \textit{M. tuberculosis}, the pathogen that causes TB, remains to be learned, reviews on the subject by van Crevel \textit{et al.} (2002) and Smith (2003) are informative. The severity of virulence can range from the potent, disseminating miliary tuberculosis to the self-limiting tuberculosis pleuritis (Smith, 2003). Infecting \textit{M. tuberculosis} bacilli can take localization in many parts of the body, but usually takes residence in the lungs. In a 1978 study (prior to the AIDS epidemic), it was shown that 85% of newly acquired TB cases resulted in pulmonary infection (Hopewell, 1994). This is similar to a 1993 survey of 313 TB patients, which showed 81% of the cases as pulmonary (Miller \textit{et al.}, 2000).

The events leading up to the final infection state have been studied. The chronology of TB pathogenesis has been organized into four stages (Dannenberg and Rook, 1994; Smith, 2003). (1) The first stage, lasting 3 to 8 weeks, begins with the
inhalation of aerosols containing *M. tuberculosis*. The bacilli are transferred to lung lymph nodes once they reach the alveoli. This creates the primary, or Ghon, complex. Whether or not it is possible for host macrophages to ingest and destroy the bacteria at this point depends on the bactericidal ability of the macrophages and on the TB virulence.

(2) If the mycobacteria survive this step, then monocytes in the blood and other inflammatory cells come to the infection sites in the lung. This second stage, or spreading stage, lasts about three months and consists of hematogenous spread the mycobacteria to other parts of the lungs or other organs. This stage also consists of monocyte differentiation into macrophages and subsequent ingestion (but unsuccessful destruction) of the mycobacteria. This creates a cycle of increasing numbers of both macrophages and mycobacteria.

Two to three weeks after infection, T-cell lymphocytes arrive at the infection sites, called tubercles, and activate the macrophages to destroy the mycobacteria they have ingested. This leads to the third stage (3), or inflammation stage, which begins with the stop of logarithmic mycobacterial growth and can last for 3 to 7 months. Granulomas are formed at the infection sites, with caseous centers consisting of dead macrophages that are filled with mycobacteria and an encasing of fibroblasts, lymphocytes, and blood-derived monocytes (Smith, 2003). The last stage (4), or slowing stage, can last for up to three years. In this stage, the infection may become dormant or it may spread later in life, depending on immune system strength. If the immune system weakens, mycobacteria in the granulomas may rupture their containment, leaking into the outside environment. This can lead to infection elsewhere in the lungs or body. If this happens, the outcome of this final stage event depends on the balance between (i) the outgrowth and killing of *M.*
tuberculosis and (ii) the extent of tissue necrosis, fibrosis, and regeneration (van Crevel et al., 2002).

Symptoms and Methods of Diagnosis

Symptoms. Miller et al. (2000) described the most common TB symptoms in a 1993 survey of 313 TB patients in Los Angeles County, CA, USA. With 81% of the cases pulmonary, common symptoms, in order of prevalence, were as follows: cough, fever, fatigue, weight loss, sweats, anorexia, chest pain, diarrhea, and hemoptysis (coughing up blood). Several of the symptoms were present for more than 2 weeks.

Mantoux Tuberculin Skin Test. According to the Mayo Clinic (2009) and Jasmer et al. (2002), testing for TB is done first using one or both of two tests, which are also the only current means of diagnosing latent TB. The first test is the Mantoux tuberculin skin test, which tests for a visual immune response to the purified protein derivative (PPD) of M. tuberculosis when this derivative is injected intracutaneously. The level of immune response is determined by measuring the induration (hardened skin) diameter at the injection site, not the erythema (redness).

Whole Blood Interferon-γ Assay. The second test is the whole blood interferon-γ assay. This assay tests for the presence of interferon-γ from T-lymphocytes, which have been produced in response to exposure to M. tuberculosis PPD. Another version of this test uses a different M. tuberculosis antigen, ESAT-6, to test for release of interferon-γ if the first form of the blood test has complications, such as can be caused by BCG (Bacillus Calmette Guerin TB vaccine) (Jasmer et al., 2002).
Active TB Suspected. The Mayo Clinic (2009) states that if active TB is suspected, follow-up tests could include chest X-ray, computerized tomography (CT) scan, culture tests, or nuclear acid amplification (NAA) tests. The latter test utilizes polymerase chain reaction (PCR) to amplify and detect genes unique to drug-resistant strains.

Treatment

Brief History. Scheindlin (2006) gave an overview of the history of TB treatment. TB treatment first saw some success with the use of fresh-air sanatoriums in the early-to-mid 1900s, which were places of retreat (usually in the mountains) where a healthy atmosphere aided in recovery for victims by allowing the immune system to quarantine *M. tuberculosis* bacilli into granulomas without additional infection. The discovery and isolation of *M. tuberculosis* by Robert Koch led to the development of the first TB antibiotic, streptomycin, by Waksman in 1943. Several other antibiotics were produced over the next 23 years, including isoniazid, pyrazinamide, ethambutol, and rifampicin.

First- and Second-Line TB Antibiotics. Shah *et al.* (2007) stated that first-line antibiotics for TB today are mainly isoniazid (INH), rifampicin (RIF), ethambutol (EMB), and streptomycin (SM), with the Merck Manual (2009) adding rifabutin, rifapentine, and pyrazinamide (PZA) to this list. Second-line drugs have been organized into six classes: (1) aminoglycosides other than SM (e.g., kanamycin and amikacin), (2) cyclic polypeptides (e.g., capreomycin), (3) fluoroquinolones (e.g., ofloxacin, ciprofloxacin, levofloxacin, and moxifloxacin), (4) thioamides (e.g., prothionamide and
ethionamide), (5) serine analogs (e.g., cycloserine and terizidone), and (6) salicylic acid derivatives (e.g., para-aminosalicylic acid)” (Shah et al., 2007).

Mechanisms of Action of First-Line Antibiotics. TB antibiotics cause mycobacterial inhibition through one or more of several mechanisms of action. INH receives peroxidative activation by the mycobacterial enzyme KatG, which forms species with NAD$^+$ and NADP$^+$ that inhibit enzymes that synthesize lipids and nucleic acids. It also forms nitric oxide species that inhibit metabolic enzymes (Timmins and Deretic, 2006). One of the end results of INH activity is the inhibition of mycolic acid synthesis (Mayer, 2010). RIF binds bacterial RNA polymerase, preventing RNA transcription (Wherli, 1983). EMB interferes with arabinan biosynthesis in the cell wall (Dover et al., 2008). SM interferes with aminoacyl-tRNA decoding, interfering with protein synthesis (Rattan et al., 1998). It is proposed that PZA inhibits fatty acid synthase (Dover et al., 2008).

Treatment Regimens. The American Thoracic Society (ATS), Centers for Disease Control and Prevention (CDC), and Infectious Diseases Society of America (IDSA) have produced an extensive document outlining recommended treatment regimens for TB cases of various types and giving detailed information on medications, medication combinations, and special treatment considerations for various patient subgroups (Blumberg et al., 2003). While this information is too lengthy to be reviewed in detail here, it may be generally stated that treatment for drug-susceptible TB typically begins with two or more of the first-line drugs, including INH and RIF. Initial treatment regimens depend on suspicion of severity and the presence of additional medical conditions, such as HIV. Treatment then progresses to one of various other treatment
regimens depending on test results. If drug-resistant TB is confirmed, treatment regimens employ at least three drugs to which the TB strain is susceptible in vitro.

Drug-Resistant TB Strains

Resistance Development. Drug-resistant strains of TB have emerged over time due to the lack of adherence to drug regimens (Pablos-Mendez and Lessnau, 2000). When all of the TB bacilli are not destroyed, they can multiply and in rare cases develop a genetic mutation either through DNA replication or through gene transfer from another organism, which counters the mechanism of action of an antibiotic. Such has been the case with several TB antibiotics, including INH and RIF of the first-line drugs and fluoroquinolones and the injectable drugs of the second-line medications (Rattan et al., 1998). Mutations have occurred in at least four enzyme-coding regions of *M. tuberculosis*, resulting in previously-targeted enzymes with altered binding sites to which isoniazid can no longer bind to prevent synthesis of mycolic acid. Mutations have also occurred in the DNA region coding for the RNA polymerase β subunit, which is the binding target for rifampicin. Once mutated a certain way, rifampicin can no longer bind to this subunit, and transcription goes on as usual in the cell (Sharma and Mohan, 2004).

MDR-TB and XDR-TB. In the early 1990’s, multi-drug resistant TB (MDR-TB) was discovered (Shah *et al*., 2007) and is defined as resistance to at least the first-line drugs RIF and INH (Sharma and Mohan, 2004). In 2006, a report was jointly produced by the CDC and WHO describing the emergence of extensively drug-resistant TB (XDR-TB) (CDC, 2006). XDR-TB is defined as resistance to at least RIF and INH among first-line drugs, resistance to any fluoroquinolone, and resistance to at least one second-line
injectable drug (amikacin, capreomycin, or kanamycin). By November 2009, 57
countries reported at least one case of XDR-TB (WHO, 2009). Preliminary anecdotal
evidence has also suggested some TB cases in Europe classified as extremely drug-
resistant, or XXDR (Migliori et al., 2007).

The Call for Novel TB Medications

In the 2002 American Thoracic Society (ATS) report, there is a call for novel
drugs to treat TB, both drug-susceptible and drug-resistant (Blumberg et al., 2003).
There are three reasons stated for this call: (1) the current lengthy treatment regimens,
which last for at least 6 months and which lead to non-adherence (which contributes to
unsuccessful treatment and drug resistance), (2) the increasing rate of drug-resistant TB
(Espinal et al., 2001), and (3) the need for more effective treatment of latent TB infection
(LTBI). This need is not only due to the difficulty in producing new medications, but in
large part to the lack of urgency felt by pharmaceutical companies (O’Brien and Vernon,

Drug Development and Toxicity Testing

Minimal Inhibitory Concentrations and
Minimal Bactericidal Concentrations

An important first step in developing a novel antibiotic is to know effective
dosage concentrations. Due to the variety of methods researchers have used in
determining minimal inhibitory concentrations (MICs) and minimal bactericidal
concentrations (MBCs), Andrews (2001) set forth methods to enable clear comparison of
various antibiotics. She defined MIC as the lowest concentration that will inhibit growth
after overnight incubation and MBC as the lowest concentration that will inhibit growth after subculture onto antibiotic-free media. This definition cannot be strictly applied to mycobacteria, however, due to their relatively slow growth rates. (Wayne and Kubica, 1994; Miller et al., 2004).

**Phases of Drug Development**

According to the United States Food and Drug Administration (FDA, 2010), Pacific Biolabs (PBL, 2009), and Chien et al. (2005), the process of new drug development goes through three clinical phases (after the preclinical phase) before it can be marketed. DiMasi et al. (2003) gave mean durations of and times between the clinical phases. Phase 0 is the preclinical phase. This phase includes identification of a potential pharmaceutical compound, demonstration of its (or other compounds of the same pharmacophore) effectiveness in cell or tissue cultures, gathering of toxicity data to determine dosage ranges, compound characterization, animal testing, and identification of a lead compound for clinical testing. Phase I (mean of 21.6 months), the first clinical phase, is a small-scale study on a group of healthy people to evaluate dosage and pharmacokinetic parameters and observe any toxicity indicators. Phase II (mean of 25.7 months) is a larger scale study (100-250 patients) used to demonstrate effectiveness in the intended population, gain information on side effects, and establish dosages for Phase III. Phase III (mean of 30.5 months) is a large-scale study used to gather additional data on efficacy and safety of the new drug. Once this phase has been completed, a Biologics License Application (BLA) or New Drug Application (NDA) is submitted to the FDA. If approved, the new pharmaceutical can proceed to the marketing phase.
Hypericum perforatum (St. John’s Wort)

Background

Hypericum perforatum, or St. John’s wort (SJW), is a perennial herb of the family Hypericaceae that is found in many temperate regions throughout the world. This herb is considered an invasive species in many areas and grows to a height 0.3-1 m on average. Its leaves, 1.5-4 cm long, have pellucid glands (hence the “perforatum,” Latin for “perforated”). Each plant produces seed pods and as many as 100 yellow flowers during the spring, with each flower consisting of five pedals that are usually 8-12 mm long (Kirakosyan, 2006). The highest concentrations of bioactive components are found in the aerial parts of the plant, especially the generative parts, and vary in their concentration throughout the year (Southwell and Bourke, 2001; Butterweck, 2009).

St. John’s Wort – Active Compounds

Several bioactive compounds in SJW have been identified and investigated (Greeson et al., 2001). Hypericin (Hpн) and pseudohypericin (Pнn), which are naphthodianthrones, and hyperforin (Hpfn), a phloroglucinol, have been subjects of a substantial portion of SJW research focus because of their implications in human health. Their concentrations in SJW can vary greatly depending on variables such as harvesting time, temperature, and germplasm (Couceiro et al., 2006). Chemical structures for these compounds are shown in Figure 2.1 (Bauer et al., 2001).

The Phloroglucinol Hyperforin. Hfn has been the subject of much research for its antidepressant (Gaster and Holroyd, 2000; Southwell and Bourke, 2001; Muller, 2003; Lawvere and Mahoney, 2005; Medina et al., 2006), anticancer (Agostinis et al., 2002;
Schempp et al., 2002; Hostanska et al., 2003; Quiney et al., 2007), anti-inflammatory (Tedeschi et al., 2003; Medina et al., 2006), and antibiotic activity (Gurevich et al., 1971; Laakmann et al., 1998; Ebrey, 1999; Schempp et al., 1999; Reichling et al., 2001; Avato et al., 2004; Zanoli, 2004; Beerhues, 2006; Medina et al., 2006; Milosevic and Solujic, 2006). In humans, Hfn has been found to reach blood concentrations of approximately 400 ng/ml 3-3.5 hr after a 1200 mg administration of a 5% Hfn SJW extract (Biber et al., 1998). The maximum absorbance wavelength for this compound varies depending on pH and solvent used, but 270 nm is typically used for analysis (Ganzera et al., 2002; Fourneron and Nait-Si, 2006). Hfn is soluble in organic solvents but completely insoluble in water (Holzl and Petersen, 2003).

*The Naphthodianthrones Hypericin and Pseudohypericin.* Hpn and Phn (often grouped together as “hypericins”) are photoactive compounds (Pace, 1942; Greeson et al., 2001; Southwell and Bourke, 2001; Muller, 2003) and in some instances have been reported as antibiotic compounds (Avato et al., 2004; Milosevic and Solujic, 2006). However, the antimicrobial effect of Hpn “seems to be directly related to the simultaneous effects of radiation from the sun” (Reichling et al., 2001). Hpn has also been claimed to work synergistically with Hfn in causing apoptosis in human malignant cells (Hostanska et al., 2003). Phn and Hpn are also the antiviral/antiretroviral components of SJW (Meruelo et al., 1988; Lavie et al., 1989; Jacobson et al., 2001).

In SJW extracts, Phn is typically found in concentrations two to four times higher than Hpn but can be up to ten times higher (Butterweck, 2009). These compounds are fluorescent with excitation and emission spectra dependent on solvent type used (Wynn and Cotton, 1995) and pH (Yamazaki et al., 1993) but are usually assayed at or around
(excitation/emission) 315/590 nm (Bauer et al., 2001; de los Reyes and Koda, 2001). Absorption spectra is also pH and solvent dependent but is typically assayed at or around 590 nm (Yamazaki et al., 1993, Nait-Si and Fourneron, 2004). The solubility of Hpn at 30°C is 500 µg/ml in methanol (MeOH) and 40 µg/ml (considered insoluble) in water. Aqueous solubility of Hpn can be increased to 30% that of MeOH by adding 2-hydroxypropyl-β-cyclodextrin (Holzl and Petersen, 2003). The hypericins are also soluble in an ethanol (EtOH) aqueous solution up to a dilution of 25:75 EtOH:H2O, beyond which point colloids form (Gaind and Ganjoo, 1959). Hpn and Phn are insoluble in water at ambient temperature but can be more than 40% extracted in an aqueous tea at temperatures of 60-80°C (Butterweck, 2009). Phn is slightly more soluble in polar solvents due to the extra hydroxyl unit in its chemical structure (Kirakosyan, 2006; Karioti and Bilia, 2010).

*Hypericin Photodynamic Therapy.* Hpn is one of the most potent naturally occurring photodynamic agents and is being researched for use as a photodynamic therapy (PDT) agent. Hpn can cause both apoptosis and necrosis, depending on concentration and dose of light (Agostinis et al., 2002), with apoptosis occurring at lower Hpn concentrations than necrosis (Karioti and Bilia, 2010). This compound is excited and becomes cytotoxic by wavelengths between 590-600 nm. It inhibits protein kinase C, irreversibly damages the sarco/endoplasmic reticulum and other cellular membranes, decreases cellular pH, and inhibits mitochondrial function (Sigma-Aldrich, 2009). Hpn is of great interest to the field of oncology because of the potential for direct, non-systemic cancer treatment. The mode of operation has been described for Hpn PDT, which
ultimately leads to reactive oxygen species (ROS) and subsequent oxidative tissue
damage (Agostinis et al., 2002; Kiesslich et al., 2006; Karioti and Bilia, 2010). Though
Phn is similar in chemical structure to Hpn, studies suggest that Phn is not the
photosensitizer (Vandenbogaerde et al., 1998).

*Compound Preservation and Photoconversion.* Hfn is known to be unstable and
degradate in the presence of light or oxygen (Gray et al., 2000; Orth and Shmidt, 2000). A
study by de los Reyes and Koda (2001) investigated different solvent types for
minimizing Hfn degradation. It was found that using acidified MeOH (pH 2.5 with
orthophosphoric acid) for the extraction solvent preserved Hfn better than pure MeOH,
especially at colder temperatures. Other authors performed studies in the dark to preserve
Hfn from light degradation (Avato et al., 2004). While Hfn requires protection from
light, Hpn and Phn require some exposure to light to allow the conversion of their
protoforms. It was found that storing a crude extract in an amber glass vial and exposing
to wavelengths in the range of 450-750 nm prevented Hfn degradation while allowing
conversion of the protoforms of Hpn and Phn; the optimal photoconversion range for
these compounds is 510-540 nm (Poutaraud et al., 2001b; Ruckert et al., 2007).

**HPLC Quantification of SJW Compounds**

**The Need for Quantification**

SJW is sold under various brand names and is sometimes weakly standardized,
usually according to Hpn content (de los Reyes and Koda, 2002). However, due to an
increase in research on the health benefits of other SJW bioactive compounds, there is a
push for greater regulation of these compounds as well. Several quantification
techniques for SJW compounds exist, including two-dimensional proton nuclear magnetic resonance (NMR) (which does not require compound separation for analysis) (Bilia et al., 2001). However, high-performance liquid chromatography (HPLC) has been the method of choice for quantification of these compounds (Vollmer and Rosenson, 2004), and efforts are still being made to develop improved HPLC methods.

Existing SJW HPLC Methods

Several HPLC methods have been developed for quantification of Hpn, Phn, and Hfn. Many of these methods document thorough analysis of their accuracy and reproducibility and would be effective in facilities with high-end HPLC separation and detection equipment. However, small-laboratory-scale disadvantages of many of these methods include calling for more than two mobile-phases (Mulinacci et al., 1999; Bergonzi et al., 2001; Poutaraud et al., 2001a; Avato and Guglielmi, 2004), requiring expensive or less-common analysis or detection equipment (Gray et al., 2000; de los Reyes and Koda, 2001; Tolonen et al., 2002; de Jager et al., 2004; Tatsis et al., 2007), or using relatively long run times (Bergonzi et al., 2001; Li and Fitzloff, 2001; Rager et al., 2002). A large amount of research is conducted in laboratories with only a two-pump HPLC system with diode array detection (DAD) and C18 column, and few methods match these conditions. Some of the methods struggled to obtain sharp peaks with Hpn and Phn. Ganzera et al. (2002) developed a method utilizing a less hydrophobic C12 column to sharpen the Hpn and Phn peaks. This method also used a two-pump/DAD HPLC system and was a precise method. These authors tested several columns to find a column adequate for efficient SJW compound quantification.
SJW Antimicrobial Studies

SJW Extractions

*Plant Parts Used in Extracts.* The aerial parts of the SJW plant have usually been used when authors have obtained their own plant materials for antimicrobial SJW studies (Gaind and Ganjoo, 1959; Avato et al., 2004). After harvesting (and usually drying and grounding) the plant material, various means have been taken to prepare crude SJW extracts.

*Solvents and Extraction Techniques.* Solvents used in extracts have included MeOH, EtOH, diethyl ether (Et₂O), “petrol,” chloroform (CHCl₃), acetonitrile (CH₃CN, or ACN), ethyl acetate (EtOAc), acetone ((CH₃)₂CO), n-butanol (C₄H₁₀O), dimethyl sulfoxide (DMSO), and water. Extraction techniques using these solvents have included Soxhlet extraction (Barbagallo and Chisari, 1987; Avato and Guglielmi, 2004), sonication (Avato et al., 2004; Avato and Guglielmi, 2004), maceration (Osborn, 1943; Avato and Guglielmi, 2004), digestion (Avato and Guglielmi, 2004), mixing (Yesilada et al., 1999), and boiling with water (Reichling et al., 2001). In a study comparing the first four extraction methods stated above, Avato and Guglielmi (2004) obtained the highest extraction efficiencies with sonication.

Organisms Assayed and Results

MIC comparisons for all organisms from the above-mentioned studies are seen in Table 2.1. The organisms assayed in these studies are listed in Table 2.2. All but the studies performed by Barbagallo and Chisari (1987) and Reichling et al. (2001) reported a much greater inhibition of Gram-positive strains than Gram-negative strains by SJW
extracts and compounds. Some of these studies found that the essential oil, tanning agents, and Hpn were not responsible for inhibition (Neuwald and Hagenstrom, 1954; Gaind and Ganjoo, 1959), conflicting with the study of Avato et al. (2004), which showed Hpn as having antimicrobial activity. No combination of pure compounds was seen in any of these studies and merits investigation.

**Human Alveolar Epithelial Cells (A549)**

The A549 human alveolar epithelial cell line is a carcinomic, immortal cell line isolated from a 58-year-old man with lung cancer (Giard et al., 1973). These cells contain keratin, which is useful for staining purposes (ATCC, 2009). Due to their carcinomic heritage, they can be perpetually subcultured, which makes them useful for ongoing study. It is also possible to measure various inflammatory markers in these cells in response to a treatment (Bhowmik et al., 2000). These cells have been found to be relatively good models for a type of alveolar epithelial cells that are potential targets of macromolecule drug delivery (Foster et al., 1998).

A study performed by Lin et al. (1998) attempted to mimic a *M. tuberculosis* infection. This study investigated chemokine production in response to A549 cell contact with *M. tuberculosis* cells. It was found that only treatment with living bacteria that were able to achieve intracellular growth induced an inflammatory response.

**Summary**

In summary, MDR-TB and XDR-TB have reached a dangerous point. Mutations in the genome of *M. tuberculosis* are making this bacterium increasingly resistant to chemotherapy; therefore, new TB medications are needed. The SJW herb has shown
inhibitory ability for several Gram-positive bacterial strains, but previous studies have only included two mycobacterial isolates in SJW inhibitory assays (Table 2.2). As the mycobacterial cell wall composition is different than that of other Gram-positive strains (it contains more mycolic acid), SJW MIC and MBC values for Gram-positive strains cannot be directly translated to mycobacteria. Therefore, direct studies of the effect of SJW extracts and compounds on mycobacteria were performed in this research (Chapter 4). In order to determine inhibitory parameters, it is necessary to have accessible means whereby SJW compounds can be quantified. While several SJW HPLC assays exist in literature, there has been a lack of an assay that utilizes a two-pump HPLC system with DAD detection, C18 column, and which does not take a large amount of time to complete; such a method was developed during the course of this study (Chapter 3).

Finally, an effective antibiotic must not only be able to kill target bacteria, but must also not harm the host. Blood Hfn levels after SJW ingestion do not reach concentrations above literature MIC/MBC values of assayed Gram-positive bacteria. Therefore, other methods for Hfn administration, such as inhalation, merit investigation. No study seen thus far has assayed for effect of SJW extracts or compounds on human alveolar epithelial cells (A549). Hence, this research also investigated the effect of SJW crude extract and compounds on A549 viability (Chapter 5).
Table 2.1 Literature MIC values of SJW (except Osborn’s study, which used *Hypericum elodes* and *Hypericum olympicum*) extracts and pure compounds against Gram-positive and Gram-negative bacteria. Gram-positive inhibition was dominant in most studies.

<table>
<thead>
<tr>
<th>Author(s) (Year)</th>
<th>Extract or Pure Compound</th>
<th>Gram(+) MIC</th>
<th>Gram(-) MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osborn (1943)</td>
<td>Ground herb pulp (in H₂O) (other <em>Hypericum</em>)</td>
<td>Not given, but inhibits</td>
<td>No inhibition</td>
</tr>
<tr>
<td>Neuwald &amp; Hagenstrom (1954)</td>
<td>(CH₃)₂CO, Pet. Ether</td>
<td>Not given, but inhibits</td>
<td>No inhibition</td>
</tr>
<tr>
<td>Gaind &amp; Ganjoo (1959)</td>
<td>CHCl₃, (CH₃)₂CO, EtOH</td>
<td>1:10⁴-1:10² dilution</td>
<td>No inhibition</td>
</tr>
<tr>
<td>Gurevich, et al. (1971)</td>
<td>Hyperforin</td>
<td>0.1-1.0 µg/ml</td>
<td>400 µg/ml</td>
</tr>
<tr>
<td>Barbagallo &amp; Chisari (1987)</td>
<td>CHCl₃, H₂O, MeOH, Petrol</td>
<td>0.31-1.25 mg extract/ml</td>
<td>1.25-2.5 mg extract/ml</td>
</tr>
<tr>
<td>Schempp et al. (1999)</td>
<td>Hyperforin</td>
<td>0.1-1.0 µg/ml</td>
<td>No inhibition</td>
</tr>
<tr>
<td>Yesilada et al. (1999)</td>
<td>BuOH, CHCl₃</td>
<td>7.8-250 µg extract/ml</td>
<td>Not assayed</td>
</tr>
<tr>
<td>Reichling et al. (2001)</td>
<td>Boiling H₂O extract</td>
<td>1.3-30 mg herb/ml</td>
<td>15-60 or &gt;60 mg herb/ml</td>
</tr>
<tr>
<td>Avato et al. (2004)</td>
<td>CHCl₃, EtOAc, MeOH, Pet. Ether</td>
<td>25-300 or &gt;1000 µg extract/ml</td>
<td>No inhibition</td>
</tr>
<tr>
<td></td>
<td>Hyperforin, Hyperforin salt</td>
<td>25-100 µg/ml</td>
<td>No inhibition</td>
</tr>
<tr>
<td></td>
<td>Hypericin</td>
<td>12.5-25 or &gt;1000µg/ml</td>
<td>No inhibition</td>
</tr>
<tr>
<td></td>
<td>Flavonoids</td>
<td>&gt;1000 µg/ml</td>
<td>No inhibition</td>
</tr>
<tr>
<td>Milosevic &amp; Solujic (2006)</td>
<td>EtOH</td>
<td>3.5 mg extract/ml</td>
<td>1.25-3.5 mg extract/ml</td>
</tr>
</tbody>
</table>
Table 2.2  A comprehensive list of all organisms assayed in cited SJW inhibitory assays, showing Gram-positive strains (left column), Gram-negative strains (center column), and yeasts (right column). Corresponding authors of studies are shown in bottom right of table.

<table>
<thead>
<tr>
<th>Gram-Positives</th>
<th>Gram-Negatives</th>
<th>Yeasts</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacillus cereus</strong> i</td>
<td><strong>Agrobacterium tumefaciens</strong> j</td>
<td><strong>Aspergillus niger</strong> i</td>
</tr>
<tr>
<td><strong>Bacillus mycoides</strong> d,j</td>
<td><strong>Azotobacter chroococcum</strong> i</td>
<td><strong>Candida albicans</strong> d,f,h,i</td>
</tr>
<tr>
<td><strong>Bacillus subtilis</strong> c,d,i,j</td>
<td><strong>Bacillus pyocyaneus</strong> c</td>
<td><strong>Candida tropicalis</strong> i</td>
</tr>
<tr>
<td><strong>Corynebacterium diphtheriae</strong> c,f</td>
<td><strong>Citrobacter freundii</strong> h</td>
<td><strong>Cryptococcus laurentii</strong> i</td>
</tr>
<tr>
<td><strong>Corynebacterium michiganense</strong> d</td>
<td><strong>Enterobacter aerogenes</strong> h</td>
<td><strong>Fusarium avenaceum</strong> d</td>
</tr>
<tr>
<td><strong>Enterococcus faecalis</strong> d,f,h,i</td>
<td><strong>Enterobacter cloacae</strong> j</td>
<td><strong>Fusarium oxysporum</strong> j</td>
</tr>
<tr>
<td><strong>Listeria monocytogenes</strong> h</td>
<td><strong>Erwinia carotovora</strong> i</td>
<td><strong>Mucor plumbeus</strong> d</td>
</tr>
<tr>
<td><strong>MRSA</strong> h</td>
<td><strong>Escherichia coli</strong> a,b,c,e,f,h</td>
<td><strong>Penicillium chrysogenum</strong> d</td>
</tr>
<tr>
<td><strong>Mycobacterium phlei</strong> d</td>
<td><strong>Helicobacter pylori</strong> g</td>
<td><strong>Penicillium canescens</strong> j</td>
</tr>
<tr>
<td><strong>Mycobacterium tuberculosis</strong> c</td>
<td><strong>Klebsiella pneumoniae</strong> h,j</td>
<td><strong>Saccharomyces cerevisiae</strong> i</td>
</tr>
<tr>
<td><strong>Sarcina lutea</strong> d</td>
<td><strong>Proteus mirabilis</strong> h</td>
<td></td>
</tr>
<tr>
<td><strong>Staphylococcus aureus</strong> a,b,c,d,e,f,h,i</td>
<td><strong>Proteus vulgaris</strong> e</td>
<td></td>
</tr>
<tr>
<td><strong>Staphylococcus epidermidis</strong> h</td>
<td><strong>Pseudomonas fluorescens</strong> j</td>
<td></td>
</tr>
<tr>
<td><strong>Staphylococcus oxford</strong> e</td>
<td><strong>Pseudomonas glycinea</strong> j</td>
<td></td>
</tr>
<tr>
<td><strong>Staphylococcus saprophyticus</strong> h</td>
<td><strong>Pseudomonas phaseolicola</strong> j</td>
<td></td>
</tr>
<tr>
<td><strong>Streptococcus agalactiae</strong> f</td>
<td><strong>Pseudomonas aeruginosa</strong> c,f,h</td>
<td></td>
</tr>
<tr>
<td><strong>Streptococcus haemolyticus</strong> c</td>
<td><strong>Salmonella choleraesuis</strong> h</td>
<td></td>
</tr>
<tr>
<td><strong>Streptococcus mutans</strong> e</td>
<td><strong>Salmonella paratyphi A</strong> c</td>
<td></td>
</tr>
<tr>
<td><strong>Streptococcus pyogenes</strong> f,h</td>
<td><strong>Salmonella paratyphi B</strong> c</td>
<td></td>
</tr>
<tr>
<td><strong>Streptococcus sanguis</strong> e</td>
<td><strong>Salmonella typhi</strong> b,c</td>
<td></td>
</tr>
<tr>
<td><strong>Streptococcus viridans</strong> c</td>
<td><strong>Shigella dysenteriae</strong> c</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Shigella flexneri</strong> h</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Vibrio cholerae</strong> c</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Author(s) (Year)</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>a. Osborn (1943)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>b. Neuwald &amp; Hagenstrom (1954)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>c. Gaind &amp; Ganjoo (1959)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>d. Gurevich et al. (1971)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>e. Barbagallo &amp; Chisari (1987)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>f. Schempp et al. (1999)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>g. Yesilada et al. (1999)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>h. Reichling et al. (2001)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>i. Avato et al. (2004)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.1 Chemical structures of Hpn (a), Phn (b), and Hfn (c) (Bauer *et al*., 2001).
CHAPTER 3
HPLC-DAD QUANTIFICATION OF SJW COMPOUNDS

Introduction

Compounds in the SJW herb are of interest to the medical community for their antidepressant, antibiotic, anticancer, antiviral, and anti-inflammatory activity. A simple high-performance liquid chromatography (HPLC) method was developed during the course of these studies that uses common HPLC separation and analysis equipment for the quantification of pseudohypericin (Phn), hyperforin (Hfn), and hypericin (Hpn). This 30-min method requires only two mobile phases, a multi-wavelength UV/VIS detector (such as photodiode array), and a C18 column. The concentrations of the three compounds in the methanol (MeOH) SJW extract were determined. This method is particularly useful for laboratories that do not have the more expensive equipment called for by many SJW HPLC analytical protocols.

Materials and Methods

Acidified MeOH Preparation

Acidified MeOH, pH 2.5, was prepared using 85% phosphoric acid (H₃PO₄, Mallinckrodt Chemicals, Phillipsburg, NJ) (de los Reyes and Koda, 2001). The pH was lowered dropwise with a 9” glass Pasteur pipette (Fisherbrand, Fisher, Pittsburgh, PA). Previously, volume dispensation of this pipette was measured using a laboratory balance and dividing the average mass of 30 drops of acid by its density (1.685 g/ml for 85% H₃PO₄) to obtain an average of 17.98 µL of 85% H₃PO₄ per drop. A glass pH probe
calibrated using aqueous buffers was used to determine that 11 drops per 250 ml MeOH (Fisher Chemicals, Pittsburgh, PA), or a solution of 0.079% (v/v) H₃PO₄, brought the measured pH to 2.5. Oxygen was then removed by sparging with nitrogen for approximately 10 min. Evaporation was minimized by capping the bottle with Parafilm that had a small puncture for gas escape.

**SJW Crude Extract and Pure Compound Preparation**

SJW crude extract preparations were performed in low-intensity green filtered-light using a headlamp (Quad Tactical LED, Princeton Tec, Trenton, NJ) with wavelength emissions above the degradation ranges (400 nm) (Figure 3.1). Hfn, Hpn, and Phn standards (Enzo Life Sciences, Plymouth Meeting, PA) were brought to concentrations of 4000 µg/ml by adding acidified MeOH with syringes. These were stored in 1-ml amber glass crimp vials (Fisherbrand, Fisher, Pittsburgh, PA) at -20°C.

SJW powder used for making the crude extract was prepared by grinding the contents of SJW capsules (GNC, Pittsburgh, PA) to a fine powder using a mortar and pestle and storing at 4°C. A “1X” MeOH extract was prepared by adding 30 ml acidified MeOH to 10.8 g SJW powder in a 50-ml centrifuge tube (Corning, Corning, NY). A “2X” extract was similarly prepared by adding 15 ml acidified MeOH to 10.8 g SJW powder. The contents were mixed by vortexing and shaking and stored at room temperature overnight. After vortexing and shaking again the next day, the mixtures were centrifuged for 10 min at 4000 RPM (3452 RCF). The supernatant was 0.2-µm filtered and stored in a 10-ml amber glass vial at -20°C.
Determination of Herb Volume, Porosity, and Extract Concentrations

Specific herb volume was determined by adding 30 ml acidified MeOH to 10.8 g of SJW powder, mixing thoroughly, and dividing the volume increase by herb mass. Porosity was determined using equation 1. The void volume ($V_v$) was the difference of the dry herb volume ($V_T$) and the true (wet) volume.

$$Porosity = \frac{V_v}{V_T}$$ (1)

Extract masses of the 2X and 1X SJW solutions were determined by measuring the dry weight of 1-ml (in triplicate) of these extracts. This was done by subtracting the premeasured mass of the weigh boats from the oven-dried (60°C) extracts after two days and averaging the triplicate for each extract. Using a scale, it was determined that a 1-ml syringe (BD, Franklin Lakes, NJ) was more accurate for dispensing this organic solution than a pipette with plastic tip, even after wetting the tip.

SJW Solubility Testing

Preliminary HPLC work revealed problems with precipitation of organic SJW extract in the mobile phase, causing clogging of the pre-column. Therefore, a solubility test was devised to determine the solubility ranges of SJW MeOH extract in the common HPLC solvents MeOH and acetonitrile (ACN) (Honeywell Burdick & Jackson, West Chester, PA). Aqueous concentrations of these solvents were prepared from 0-100% (v/v) in 10% increments. 1X SJW MeOH extract was then added at a 2% (v/v) concentration. Optical density measurements at 600 nm ($OD_{600}$) were taken to determine maximum solubility ranges of SJW MeOH extract in these solvents.
HPLC Analysis

*Mobile Phase Preparation.* Mobile phase A was 80:20 acetonitrile:water and 0.035% (v/v) 85% H₃PO₄. Mobile phase B was 99:1 acetonitrile:water and 0.035% (v/v) 85% H₃PO₄. Degassing was performed using a Fisher Scientific sonicator (FS60, Fisher, Pittsburgh, PA) for 45 min, with bottles wrapped in Parafilm punctured with small holes to allow gas escape.

*Line and Pump Priming.* Line and pump priming was achieved by pulling 10 ml through the tubing, discarding, then pulling another 10 ml through the tubing and using this to prime each respective pump. The detector and pumps were run for 10 min (with the pumps at 50:50 A:B at 0.5 ml/min), after which a blank run was performed to clean the column and run the pumps at higher flow rates.

*HPLC Method.* The HPLC instrument used was a Beckman System Gold model 125S equipped with a 168 photodiode array (or simply “diode array”) detector (DAD). Injection volume was 60 µl into a 20 µl loop to compensate for laminar flow issues and done at a rate of approximately 5 µl/s. To prevent column overloading when analyzing the crude extract, a 1:50 dilution (2% v/v) was used (this volume was also used most commonly in the SJW inhibitory studies). The HPLC method was as follows: at 0 min 100:0 A:B at 1.5 ml/min; at 8 min a 0.5-min flow rate increase to 2.5 ml/min; at 11 min a 0.5-min flow rate decrease to 1.5 ml/min and a 1-min A-B gradient to 0:100 A:B; at 16 min a 0.5-min flow rate increase to 2.5 ml/min; and at 28 min a 0.5-min flow rate decrease to 1.5 ml/min and a 1-min B-A gradient to100:0 A:B to equilibrate the column in preparation for the next run. The method ended at 30 min.
**Loop and Injector Cleaning.** Loop and injector cleanings between runs were done by flushing 1 ml of mobile phase A using a 1-ml blunt-end syringe with a Parafilm wrapping around the needle to allow for sealing against the injection port. Some cleaning solution was allowed to flow backwards out from the port to clean the port entrance. Sample injection syringe cleaning was done using several (usually three) EtOH rinses followed by several acidified MeOH rinses.

**Peak Analysis and Preparation of Calibration Curves.** Analysis of peak area was performed using the Gold Noveau integration software with absorbance readings taken at the following wavelengths: Phn: 590 nm; Hfn: 273 nm; Hpn: 578 nm. Threshold and peak width settings had to be changed from time to time, especially for the hypericins, to cause the peak integration baseline to span the bottom of the peak.

Calibration curves were prepared through selection of the most accurate and precise of three calibration curves prepared for each of the three active compounds. The calibration curves for Phn and Hpn consisted of five points (as these were more difficult to quantify) in singlet, while the calibration curve for Hfn consisted of four points in duplicate. Final Phn and Hpn calibration curves spanned concentrations from 12.5-100 µg/ml, and the final Hfn calibration curve spanned concentrations from 5-100 µg/ml. The three compounds were usually mixed together and analyzed simultaneously, allowing for more efficient calibration curve preparation. Acidified MeOH was the diluent used to prepare the solutions.
Repeatability Testing

Three sets of calibration curves allowed for the investigation of the repeatability of this method. Repeatability was determined by investigating the variation between sets of regression equations for each compound, given a certain area.

Determination of Active Compound
Photoconversion or Photodegradation

Photoconversion/photodegradation of SJW compounds were investigated by preparing a fresh 10-ml amber glass vial of 1X SJW extract and placing it in a New Brunswick Scientific Innova 42 photosynthetic incubator-shaker, shaking approximately 5 cm beneath the photosynthetic light at a photon irradiance of 223 µmole/m²/s/nm. The spectrum and light intensity of this incubator are shown in Figures 3.2 and 3.3, respectively, with the spectrum almost completely above 400 nm. Samples (250 µl each) were taken from the vial at times 0, 15 min, 30 min, 1 hr, and 2 hr, and active compound concentrations were analyzed using the regression equations from the calibration curves.

Results and Discussion

Herb Volume, Porosity, and Extract Concentrations

Wet (true) and dry volumes and porosity of the ground SJW herb, as well as concentrations of the extract, are given in Table 3.1. It can be seen from these values that the MeOH dissolved a substantial part of the herbal mass (46.1% for 1X and 46.5% for 2X). Hence, about 46% of the herb mass is soluble in MeOH using the extract preparation methods outlined above. Though the MeOH volume added for the 1X
solution was twice the volume as the MeOH added for the 2X solution, to the same mass of SJW herb, this did not double the 1X solution volume (as the herb itself occupies considerable volume at these concentrations). The 1X solution had a 67% higher volume than the 2X solution. The 2X extract had approximately the same percentage greater measured mass (68%) than the 1X extract. If the 2X extract had been saturated, its percentage mass increase (compared to 1X) would have been less than the percentage volume increase of 1X. Therefore, these results show that the 2X extract had not reached the solubility limit of SJW in MeOH. The extracts were observed qualitatively to have a high viscosity and a dark, tar-like appearance, even after filtering through a 0.22 µm filter.

**SJW Solubility**

The OD$_{600}$ data for SJW MeOH extract solubility in MeOH and ACN is shown in Figure 3.4. Greatest solubility was found to be at values greater than 70% MeOH or between 60-80% ACN. It was found that if the beginning mobile phase was outside of the SJW solubility range, it caused analyte precipitation and clogging in the pre-column. Therefore, the “A” mobile phase of 80% ACN$_{aq}$ was found to be effective at quickly eluting the non-targeted polar compounds. By using this mobile phase isocratically for the first 10 min of the run, it also provided clear resolution of compounds through the elution of Phn. Afterward, there was no precipitation as the gradient moved to 99% ACN.
Chromatograms, HPLC Parameters, and Standard Curves

Typical chromatograms for the Phn, Hfn, and Hpn standards are shown in Figure 3.5, and their corresponding crude extract chromatograms are given in Figure 3.6. Calibration curves for these compounds are shown in Figure 3.7. Retention time ranges, regression equations from the calibration curves, and correlation coefficients are shown in Table 3.2. Compound concentrations in a 1:50 dilution of the 1X extract are shown in Table 3.3. It should be noted that as the concentrations of the hypericins increased, the retention times decreased; therefore, there were small time ranges for the retention times of these compounds. Also, though formal limit of detection (LOD) tests were not performed, the hypericins were generally difficult to detect at concentrations lower than 25 µg/ml. Hfn was easily detectable at all concentrations attempted. The lowest attempted concentration of Hfn was 1 µg/ml, which produced a sharp, easily integratable peak, so lower concentrations of this analyte should also be integratable. Figure 3.8 shows a contour view of the DAD data for a typical crude extract HPLC run, with X-axis time, Y-axis wavelength, and Z-axis (coming toward the viewer) milli-arbitrary (or absorbance) units (mAU), which has magnitude marked by color. Magnitude color indicators (from highest to lowest) are red, yellow, green, light blue, dark blue, and purple. Analysis was performed at 590 nm for Phn, 273 nm for Hfn, and 578 nm for Hpn. This view shows the numerous compounds in SJW that are able to be effectively resolved using this HPLC method.
Repeatability

This method was found to be highly repeatable for Hfn and less repeatable for Phn and Hpn, as shown in the compound concentration values from each calibration curve, along with their means and standard deviations, in Table 3.3. As the standards used in the creation of both the method and the first set of calibration curves were somewhat old (six weeks) and returned relatively imprecise values for the hypericins, it was decided to not make the first set the official calibration curves for this method.

The second and third calibration curve regression equations for Phn and Hfn returned concentration values within 2% of each other. It was decided to use the regression equation from third calibration curve for Hfn, as the four points in this calibration curve came from duplicate analysis using fresh stock solution. The regression equations from the second set of standard curves were used for the hypericins; even though these calibration curves were performed in singlet, they each consisted of five points (as opposed to three points in the third set due to difficulty in integrating concentrations below 25 µg/ml), produced higher correlation coefficients, and were prepared with fresh stock solution. The Hpn calibration curve used successfully integrated peaks at 5 µg/ml and 10 µg/ml, which was another reason it was used.

Photoconversion/Photodegradation of Active Compounds

Normalized concentration data for the three SJW analytes after exposure to the Innova photosynthetic light are shown in Figure 3.9. There was a 20% increase in Phn, a 4% decrease in Hpn, and a 41% decrease in Hfn. The loss in Hfn was considered to not be worth the relatively small photoconversion-gain in Phn, so photoconversion was not
carried out in any additional experiments. These novel results show degradation of Hfn at wavelengths above 400 nm, contrary to reported values on the subject (Ruckert et al., 2007).

Discussion

As the use of MeOH in HPLC analysis is often preferred due to its relatively low cost, several attempts were made to use this solvent in our analysis. However, this solvent did not have the hydrophobicity necessary to elute the highly nonpolar Hpn within the target 30-min timeframe. Also, the primary purpose of this HPLC method is for efficient analysis, not preparative purposes, and does not use an exorbitant amount of solvent. Therefore, the benefits outweigh the drawbacks in the use of ACN along with MeOH.

The development of a 30-min SJW HPLC quantification method with a C18 column for Phn, Hfn, and Hpn proved to be a difficult task. This was primarily due to the chemical properties of the hypericins, which caused relatively wide peak bases, tailing, and in the case of Hpn, a long retention time. To narrow the Phn and Hpn peaks in this method, flow rates were increased from 1.5 ml/min to 2.5 ml/min during the elution of both of these peaks. Though less hydrophobic columns, such as a C8 or C12 column, are able to provide a sharper peak shape for the hypericins, the C18 column is more common, and that is why this method was developed. However, the less hydrophobic column is recommended if available, in which case a different method would have to be used (such as that outlined by Ganzera et al., 2002).
It was also determined that injecting three times the sample loop volume helped to overcome laminar flow issues and purge the mobile phase from the loop, thereby injecting a pure 20 µl of sample into the column. It was found that this allowed for greater consistency in peak area, as shown by the relatively high correlation coefficients. It was also found that if there was not enough analyte volume to inject three times the loop volume, a very slow injection of a smaller volume could also achieve consistency.

The last part of the HPLC method goes through an equilibration phase to prepare the column for the next run, which can be started immediately should the need exist. After preparing this method initially, the HPLC run time was allowed to go far beyond 30 min to ensure that Hpn was the last SJW compound to exit the column, which it was. Therefore, this method allows for the user to immediately begin another run.

Conclusions

A SJW HPLC method for the quantification of Phn, Hfn, and Hpn within 30 min has been developed and shown to be effective. This method requires only two mobile phases, a UV/VIS detector (such as photodiode array), and a C18 column. It also avoids clogging of the extract in the precolumn. While this method may be used for the quantification of Phn and Hpn, it is most accurate and repeatable for the quantification of Hfn. It has been found that wide Phn and Hpn peaks is a common problem in C18 column analysis of SJW, and for increased accuracy in their analysis, a method utilizing a less hydrophobic column is recommended. The biggest advantage of our method is for researchers who are interested in investigating SJW who only have access to the most common HPLC separation and detection equipment.
Table 3.1 Volumes, concentrations, and porosity of SJW herb and MeOH extract. Only the 2X and 1X extracts were assayed using HPLC. The other dilutions are dilutions of the 1X extract and are listed for reference for other chapters (usually added to cultures at 1:50 dilutions). SJW extract concentration is mg solids, not solution, per ml.

<table>
<thead>
<tr>
<th>SJW Herb</th>
<th>Wet volume (ml/g)</th>
<th>Dry volume (ml/g)</th>
<th>Porosity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ground Herb</td>
<td>0.694</td>
<td>1.39</td>
<td>0.501</td>
</tr>
<tr>
<td>SJW Extract</td>
<td>mg herb/ml solvent</td>
<td>mg herb/ml solution</td>
<td>mg extract/ml solution</td>
</tr>
<tr>
<td>2X</td>
<td>720</td>
<td>480</td>
<td>223</td>
</tr>
<tr>
<td>1X</td>
<td>360</td>
<td>288</td>
<td>133</td>
</tr>
<tr>
<td>1/2X</td>
<td>180</td>
<td>144</td>
<td>67</td>
</tr>
<tr>
<td>1/4X</td>
<td>90</td>
<td>72</td>
<td>33</td>
</tr>
<tr>
<td>1/8X</td>
<td>45</td>
<td>36</td>
<td>17</td>
</tr>
<tr>
<td>1/16X</td>
<td>23</td>
<td>18</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 3.2 Retention times, regression equations, and correlation coefficients ($R^2$) for the three SJW analytes from 1:50 dilutions of SJW extract. The regression equations came from the second set of calibration curves for Phn and Hfn and from the third set for Hfn.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pseudohypericin</th>
<th>Hyperforin</th>
<th>Hypericin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention time</td>
<td>10-10.5 min</td>
<td>15 min</td>
<td>22.5-23.5 min</td>
</tr>
<tr>
<td>Regression equation</td>
<td>$Y=1419.9x-3739$</td>
<td>$Y=10553x-1063.2$</td>
<td>$Y=5100.1X-6667.2$</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.996</td>
<td>0.999</td>
<td>0.995</td>
</tr>
</tbody>
</table>

Table 3.3 Assayed concentration values of SJW compounds at 1:50 dilution of the 1X crude extract. Three sets of calibration curves were prepared for Phn and Hfn, and two were prepared for Hpn to test repeatability. The second sets of calibration curves were used for Phn and Hpn, and the third set was used for Hfn. Compound concentrations are listed at this dilution for ease in comparison with the inhibitory studies in Chapters 4 and 5, as this is the most common concentration (v/v) that the crude extract was added to cultures.

<table>
<thead>
<tr>
<th>Cal. Curve Set</th>
<th>Pseudohypericin (µg/ml)</th>
<th>Hyperforin (µg/ml)</th>
<th>Hypericin (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>34.4</td>
<td>43.3</td>
<td>26.4</td>
</tr>
<tr>
<td>2</td>
<td><strong>53.4</strong></td>
<td>46.2</td>
<td><strong>15.4</strong></td>
</tr>
<tr>
<td>3</td>
<td>54.4</td>
<td><strong>45.5</strong></td>
<td>NA</td>
</tr>
<tr>
<td>Mean</td>
<td>47.4</td>
<td>45</td>
<td>20.9</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>11.3</td>
<td>1.5</td>
<td>7.8</td>
</tr>
</tbody>
</table>
Figure 3.1 Normalized spectra of filtered-light headlamp used when working with light-sensitive compounds. The green filter was mostly used, as its emission corresponded with the photoconversion (from their protoforms) wavelengths of the hypericins (510-540 nm) and prevented Hfn degradation, which occurs below 400 nm (Poutaraud et al., 2001b).

Figure 3.2 Spectrogram of the Innova incubator-shaker photosynthetic light. Very little emission is seen below 400 nm (Hfn-damaging wavelengths).
Figure 3.3  Photon irradiance of the Innova incubator-shaker photosynthetic light as a function of distance from the light source. The photoconversion/photodegradation experiment was carried out at a distance of 5 cm with a photon irradiance of 223 µmol/m²/s/nm.

Figure 3.4  Solubility (OD₆₀₀) of 2% (v/v) SJW crude MeOH extract in various aqueous concentrations of MeOH and ACN. Lower OD equals higher solubility. The crude extract had highest solubility at concentrations of ≥ 70% MeOH or 60-80% ACN.
Figure 3.5  Typical chromatograms for Phn, Hfn, and Hpn standards, with retention times of approximately 10, 15, and 22.5 min, respectively. The 273 nm chromatogram for Hfn is below (blue), and the 590 nm chromatogram for the hypericins is above (red).

Figure 3.6  Typical chromatograms for Phn, Hfn, and Hpn in 1:50 dilution of 1X (2.66 mg extract/ml) crude extract, with retention times of approximately 10.5, 15, and 23.5 min, respectively. The 273 nm chromatogram for Hfn is below (blue), and the 590 nm chromatogram for the hypericins is above (red).
Figure 3.7 Calibration curves used in the quantification of SJW compounds. The Hfn curve had the highest correlation coefficient, followed by Phn, then by Hpn. The Hfn calibration curve shown was the third curve prepared for this compound, and the hypericins were each their second calibration curves prepared.

Figure 3.8 Contour view of HPLC mAU data for SJW crude extract. X-axis is time (min), Y-axis is wavelength (nm), and Z-axis (coming toward the viewer) is absorbance (mAU). Red represents highest absorbance, followed by yellow, green, light blue, dark blue, and purple.
Figure 3.9 Photoconversion/photodegradation study of SJW compounds. A 20% concentration increase was observed in Phn, a negligible concentration change was observed in Hpn, and a 40% concentration decrease was observed in Hfn. While Ruckert et al. (2007) state that Hfn degradation occurs at wavelengths below 400 nm, these findings show significant Hfn degradation above 400 nm.
CHAPTER 4

EFFECT OF *HYPERICUM PERFORATUM* ON MYCOBACTERIA VIABILITY

**Introduction**

Of all the literature reviewed, only two *Mycobacterium* strains, one time each, have been included in SJW inhibitory/bactericidal assays. This is an insufficient sample size if one is to understand the response of this genus to SJW extracts and compounds. To create a larger data set on the response of mycobacteria to SJW, seven nonpathogenic mycobacteria (six of which are the only genetically sequenced nonpathogenic *Mycobacterium* strains currently available) were used in a series of inhibitory and bactericidal studies presented here. These strains contain as much as 72% homology to the *M. tuberculosis* genome (see Figure 4.1 for a Venn diagram comparison of these genomes). With these results, investigators should be able to better predict how pathogenic mycobacteria might respond to the SJW herb.

**Materials and Methods**

**Comparison of Mycobacterial Genomes**

Mycobacterial genomes were compared using the Integrated Microbial Genomes (IMG) section of the Department of Energy (DOE) Joint Genome Institute (JGI) website (www.jgi.doe.gov). Under the menus “Find Genes,” “Phylogenetic Profilers,” “Single Genes,” the “Find Genes In” option was selected for *M. gilvum* PYR-GCK, and the “With Homologs In” option was selected for all nonpathogenic *Mycobacterium* isolates and for *M. tuberculosis* CDC1551. Similarity cutoffs were as follows: Max. E-value: 1e-
SJW Extract and Pure Compound Preparation

SJW extracts and pure compound solutions were prepared in the manner outlined in Chapter 3. The solvent used for preparing SJW extracts in most of the inhibitory studies was MeOH. However, acetone, ethanol, ethyl acetate, chloroform, and water were also used for a study comparing the inhibition of SJW extracts of different solvents. SJW from GNC (Pittsburgh, PA) was used for most studies. However, the Nature’s Way (Springville, UT) and NOW (Bloomingdale, IL) brands of SJW were also used in a study comparing SJW brands. Extracts used in the earliest studies were not 0.2-μm filtered, but nearly all studies used filtered extract. Dilutions of the crude extract were made by diluting the 1X, not 2X, stock solution (Table 3.1).

Media Preparation

Luria broth (LB) and agar, as well as Middlebrook broth (MB) and agar, were prepared according to protocols in the Appendix. Ampicillin was added to the media for one study comparing growth with and without it and was added from stock solution to a working concentration of 50 μg/ml. Tween 80 (Sigma, St. Louis, MO) was often added at a 0.5% (v/v) concentration of a 10% (w/v) stock solution, making a working concentration of 0.05%.
Culture Preparation

Cultures and Growth Conditions Used. The *Mycobacterium* cultures used for the inhibitory/bactericidal studies were *M. JLS*, *M. KMS*, and *M. MCS* (isolates from Utah State University), *M. smegmatis* (#14468, ATCC, Manassas, VA), *M. phlei* (ATCC #BAA-486), and *M. vanbaalenii* PYR-1 and *M. gilvum* PYR-GCK (isolates obtained through a combined Joint Genome Institute sequencing effort). Gram-negative controls were *Pseudomonas putida* (obtained on USU campus) and *Escherichia coli* (ATCC #25922). The Gram-positive control was *Bacillus subtilis* (ATCC #6051). A qualitative study was performed that compared the growth of mycobacteria on LB and MB media, and LB media was used for all other studies. All cultures were incubated at 37°C except for *B. subtilis*, which was incubated at 30°C. Broth cultures were incubated in shaking conditions at 220 RPM for 12-ml culture tubes (BD Falcon, Franklin Lakes, NJ) or 150 RPM for 48-well plates (BD Falcon, Franklin Lakes, NJ).

Freezer Stock Preparation. Freezer stocks of the bacterial isolates were prepared by adding 500 µl of turbid culture to 500 µl of 30% (v/v) sterile glycerol, bringing the final cell solution to 15% (v/v) glycerol, in a 1.5 ml microcentrifuge tube (Fisher, Pittsburgh, PA). This was kept at -80°C.

Broth Culture Preparation. Broth cultures were inoculated by adding a volume of bacterial freezer stock (typically between 50-200 µl, with more volume for shorter time to turbidity) to liquid media. Inoculations were typically done in 5 ml media in 12-ml culture tubes. At the beginning of experiments, cultures were diluted with LB media in a 50-ml centrifuge tube (Corning, Corning, NY) (typically about a 1:100 dilution) to an optical density (OD$_{600}$) of between 0.01-0.05 using a spectrophotometer (Bio-Spec Mini
00834, Shimadzu, Columbia, MD). If Tween 80 was to be added, it was also done at this time prior to dispensing the inoculated media into the assay containers, which were either 12-ml culture tubes or 48-well plates.

Agar Plate Culture Preparation. Agar plate cultures were prepared either for working culture storage or for SJW inhibitory assays. For streak plates, a small amount of bacteria was streaked in small portion of the plate along the edge (about 1/5 of the plate). This first section required either a small volume (5-10 µl) of liquid culture or a small amount (barely visible on a pipette tip) of bacteria from another plate. Using a pipette tip (usually a 1.5-ml tip), this section of the plate was then streaked over to the next 1/5 of the plate using a new tip. This process was repeated, each time using a new tip, until all sections of the plate had been streaked. If several strains were used on one plate, then the plate was divided into sections, and each strain was streaked only in its section.

Culture Treatment

Inhibitory Assays. Inhibitory assays (not bactericidal assays) were performed on SJW-containing agar plates. These studies used plates that had SJW crude extract or solvent (for controls) added to the plates at their coolest point before solidification. The plates were then swirled to mix and allowed to solidify before treating with cultures. Crude extract was added at equal concentrations for a study comparing inhibition of three different SJW brands and extracts of six different solvents, and it was added at different various concentrations for a study investigating MIC values. Plates with no growth were above the minimal inhibitory concentration (MIC), and plates with growth were below it.
When pipetting SJW crude extract solution or organic solvents, greater accuracy was achieved by “wetting” the tip, pipetting up and down once or twice before the final aspiration and dispensing. Pipetting accuracy was measured using a laboratory scale to verify accurate weight using the solvent density.

*Bactericidal Assays.* Bactericidal assays were performed in SJW-containing broth media. After culture preparation, SJW crude extract or pure compounds were added to the cultures at various concentrations in low-intensity green-filtered-light conditions. Crude extract studies were carried out in culture tubes (except when used for comparison in the pure compounds MBC studies, in which case a 48-well plate was used), while pure compound studies were carried out in 48-well plates (except for one study comparing the effect of light exposure on Hfn inhibition of *M*. KMS, in which case 12-ml culture tubes were used). For accurate dispensing of volumes, syringes were used to dispense the SJW treatments. For volumes between 250-1000 µl, a 1-ml graduated syringe (BD, Franklin Lakes, NJ) was used. For volumes less than 250 µl, graduated glass syringes were used (Hamilton, Reno, NV). After EtOH/MeOH toxicity studies, SJW crude extract and pure compounds were added at concentrations at or below 2% (v/v). Cultures were then kept at the appropriate temperature (stated above), and cell samples were taken at various time points for cell counting (method outlined below).

*Effect of Tween 80 on Hfn MBC Study.* A study investigating the effect of various concentrations of Tween 80 on Hfn MBC was performed using *M*. JLS as a mycobacterial representative. Cultures containing six concentrations of Tween 80 (2-fold dilutions) were added to a 48-well plate in triplicate and all treated with the same Hfn concentration of 46 µg/ml. A no-Tween 80 control, as well as a no-Tween 80/Hfn
control, were included. CFU counts were taken each day for 3 days, with the first CFU count taken before Hfn addition.

**Colony Forming Unit (CFU) Quantification-Cell Dilution Method**

The cell dilution method was by performed by doing the following: in a 96-well Costar plate (Fisher, Pittsburgh, PA), 90 µl of LB media was placed in columns 2-8 using an Eppendorf eight-channel pipette (Fisher, Pittsburgh, PA) with Eppendorf ep tips (Fisher, Pittsburgh, PA). After thoroughly resuspending a cell culture using a larger pipette, 30 µl were taken and placed into a well in column 1. After column 1 had been filled with samples from eight cultures, the multichannel pipette was used to perform serial dilutions, changing tips between each column. Minor but important techniques included the following: (1) ensuring a tight fit of the pipette tips on the pipette; (2) thoroughly mixing the contents of the wells in each column before transferring to the next column (via stirring and pipetting up and down several times with the pipette tips); (3) keeping the tips off the bottom of the wells to avoid sudden aspiration (which leads to excessive volumes); and (4) visually inspecting the tips for precision and to ensure the presence of solution in the tips before transferring to the next column.

Each cell dilution was spotted on an antibiotic-free LB agar plate. Blowing out with the pipette when applying spots to a plate was avoided due to spraying when the bubble in the spot burst, which would cause rogue colonies and less-accurate CFU counts. Instead, pipette volume was adjusted to slightly higher (0.5 µl higher) than desired dispensing volume (10 µl) to compensate for the volume not dispensed from not blowing out. After securing four pipette tips on the pipette, the four most dilute wells in
a row of the 96-well plate were thoroughly mixed, and four spots were placed on the plate. Uniform spots were obtained by slowly dispensing the liquid (forming droplets on the tips) and slowly lowering the drops to the surface (touching the media with the tips was avoided, as spraying could occur with pressure build-up if the tip was touching the agar). Without changing tips, the four most concentrated wells in the same row were then thoroughly mixed and placed on the agar plate in the same manner. The plates were allowed to sit in the laminar flow hood until the spots absorbed into the media (about 30-45 min), after which they were wrapped in Parafilm and placed in an incubator (37°C for all strains except for *B. subtilis* and *E. coli*, which were incubated at room temperature due to their higher growth rates).

After visual colony growth on the plates (about four to five days for mycobacteria and one day for *E. coli* and *B. subtilis*), colonies were counted. Two counts were taken from the two spots that had the highest number of countable colonies. These values were then back-calculated to yield CFU/10 µl, then multiplied by 100 to yield CFU/ml. The two CFU counts from each plate were averaged as the final CFU count, with the error being the standard deviation. A “time 0” count was always taken, usually before the addition of SJW crude extract or compounds, followed by additional counts at various time points.

**Scanning Electron Microscopy (SEM) Cell Fixing**

Scanning electron microscopy (SEM) imaging was performed for all the strains without SJW treatment, and imaging was performed for *M. KMS, M. smegmatis, B. subtilis*, and *E. coli* after treatment with the crude extract at various concentrations and
time points. Cell fixation and imaging procedures were performed by Dr. TC Shen and Dr. FenAnn Shen of the USU Physics Department. Cell fixation protocols are in the Appendix.

**Results and Discussion**

**Comparison of Mycobacterial Genomes**

Homology comparisons of all nonpathogenic *Mycobacterium* genomes with *M. tuberculosis* are shown as a Venn diagram in Figure 4.1. The outer, non-percentage numbers indicate number of genes. The outer percentages indicate percent genes unique to each isolate. Middle percentages indicate percent genes shared with *M. tuberculosis*. Inner percentages indicate percent genes of *M. tuberculosis* that are incorporated into each isolate, which is of greatest interest for this study. Percent *M. tuberculosis* genome incorporation is as follows (from greatest to least): *M. MCS* (72%), *M. smegmatis* (69%), *M. KMS* (69%), *M. vanbaalenii* (67%), *M. JLS* (66%), and *M. gilvum* (65%). This comparison sheds greater light on the selection of organism for a *M. tuberculosis* surrogate. *M. smegmatis* is commonly used as a pathogenic surrogate, but for tuberculosis studies, perhaps *M. MCS* would be a more appropriate choice, with three percentage points greater similarity. *M. MCS* was found to have the greatest morphology similarity to *M. smegmatis* out of all assayed isolates from these studies; colonies from these isolates were found to grow more laterally and have a more “dry” appearance on an agar plate than other isolates, and there was a higher degree of cell clumping in liquid culture than other isolates. The high degree of clumping of these two isolates did make CFU quantitation difficult, however. Therefore, isolates that exhibited less clumping,
such as \textit{M. JLS} or \textit{M. KMS}, were often chosen to act as mycobacterial representatives in studies requiring many samples that could only accommodate one or two strains. The isolates assayed the most through these studies are shown in Figure 4.2: \textit{M. JLS}, \textit{M. KMS}, \textit{M. MCS}, \textit{M. smegmatis}, and \textit{M. phlei}.

**Demonstration of Inhibitory Effect of Extracts of Different Solvents and Different SJW Brands**

The inhibition/growth results of \textit{M. JLS}, \textit{M. KMS}, \textit{M. MCS}, \textit{M. vanbaalenii}, \textit{M. gilvum}, and Gram-negative-control \textit{Pseudomonas putida} control after one week and two weeks are shown in Tables 4.1 and 4.2, respectively. Plates where fungal contamination occurred (as this study did not use filtered crude extract) are denoted as “not determined” (ND). Growth inhibition occurred in all organic extracts of SJW, with the exception of the Nature’s Way chloroform extract; plates with this SJW brand showed growth of \textit{M. gilvum} PYR-GCK in the ethyl acetate plate and growth of all strains except \textit{M. vanbaalenii} PYR-1 in the chloroform plate. The cause for this is uncertain, though settling of the relatively high-density chloroform (specific gravity 1.48) may have had an effect. No inhibition occurred in any plates containing water SJW extract except the GNC \textit{M. MCS} plate. The results show very little differences between the different brands of SJW and between different types of organic extracts. As GNC SJW was easy to obtain and showed the least amount of fungal contamination, and since MeOH was the most commonly used solvent in other SJW studies in literature, GNC SJW and MeOH were used for the remaining studies. Images of the GNC plates for each type of solvent, along with the controls, are shown in Figure 4.3.
It is interesting to note the pigmentation differences between the extracts of the various solvents. The dark pigmentation caused problems in colorimetric assays throughout the study (i.e., spectrophotometric growth assessment or protein assay). The ethyl acetate extract was much less pigmented in media than the others yet still inhibited mycobacterial growth. Hence, future studies may warrant the use of the ethyl acetate extract and LB media to reduce pigmentation and make colorimetric/spectrophotometric assays a more viable option.

Comparison of Mycobacterial Growth on Different Media Types

While no significant difference was seen in cell growth on LB or MB media, there was significant difference seen in cell pigmentation, particularly in the USU isolates *M. JLS*, *M. KMS*, and *M. MCS* (Figure 4.4). The LB plates produced cells with a deeper yellow pigmentation, making them easier to see. Due to the easier visibility of the cells on LB, the relative ease of making this media, and its lower cost, it was decided to use LB media for the remainder of this study.

Ampicillin Resistance of Mycobacteria

The effect of ampicillin (50 µg/ml) on the growth of the five mycobacterial isolates is shown in Figure 4.5. While there was not a dramatic visual difference in cell growth, the ampicillin did have an effect on cell pigmentation; the cause for this is unknown. As cell visibility was a necessity for this study (for CFU counting), antibiotics were not used in other studies.
CFU Quantification

Typical results for the cell-dilution CFU quantification method are shown in a *M.* JLS example in Figure 4.6. The CFU concentration in this figure would be $1.1 \times 10^8$ CFU/ml according to the most dilute spot and would be $6.1 \times 10^7$ CFU/ml according to the next most dilute spot, yielding an average of $8.55 \times 10^7$ CFU/ml and a standard deviation of $3 \times 10^7$. Spectrophotometric CFU quantification was attempted in preliminary studies. However, mycobacterial clumping decreased accuracy, and the results of such attempts are not shown here. While the cell dilution CFU quantification method was more laborious, it proved to be more precise.

EtOH/MeOH Toxicity Testing

The cell growth/death results for *M.* KMS in MeOH and EtOH over a six-day period are shown in Figures 4.7 and 4.8, respectively. Of both solvents, only the 4% EtOH concentration caused a decrease in *M.* KMS CFU population (10-fold decrease in the first four days), which rebounded somewhat in the following two days. There were no significant differences seen between any of the other solvent concentrations and their no-solvent controls. It was necessary to evaluate the toxicity of EtOH and MeOH to ensure any inhibitory/bactericidal effect was not due to the solvents. MeOH and EtOH were the solvents chosen for this study since they were the most commonly used SJW solvents in literature. The lower toxicity of MeOH reinforced the decision to use it as the SJW solvent for the duration of these studies. In the MBC studies, the 2% (v/v) concentration was found to be the minimum organic solvent concentration that would still allow a high enough SJW crude extract concentration to consistently inhibit all
mycobacteria at more than one assayed concentration. Hence, SJW extracts of various concentrations were most commonly added to cultures at 2% (v/v) concentration for the inhibitory/bactericidal studies.

**Crude Extract MIC Studies**

Agar plates from the inhibitory studies are shown in Figure 4.9. A trend of no growth at the higher SJW concentrations and growth at the lower SJW concentrations was seen, but a distinct threshold was not seen. No growth was seen from the 8% - 1% plates (except one of the 1% plates), small growth was seen on the 0.5% - 0.25% plates and growth was seen on all other plates (except one of the 0.03% plates). Therefore, a tentative MIC value from these plates would be between the 0.5% and 1% concentrations (of 1X stock solution). However, with the understanding of the inhibitory effects of EtOH, which was shown to adversely affect cell growth at a 4% concentration in the solvent toxicity studies, inhibitory effect cannot be exclusively credited to the SJW extract. Since any EtOH inhibition was uniform throughout all the plates (all plates had the same volume added, regardless of SJW concentration of the additive) and would only serve to reduce CFU population, the 0.5% - 1.0% 1X MIC value should be fairly accurate.

**Crude Extract MBC Studies**

A typical set of CFU dilution growth plates for each strain, along with zoomed-in views of colonies from these strains, are shown in Figure 4.10. All plates shown are from the 48-hr time point except *B. subtilis*, which is from the 24-hr time point (shown for clarity). The absence of growth of cells that had been exposed to higher SJW
concentrations can be seen. The MeOH control and no-MeOH/SJW control can be seen at the bottom two sections of the bottom plate in each image.

Typical growth/death results are shown in Figures 4.11 through 4.17. MBC values are shown in Table 4.3. Since the definition of MBC is the minimum concentration that kills $\geq 99.9\%$ (three orders of magnitude) of the culture over a given period of time (which is considered 72 hr for this study), the 0.67 mg extract/ml culture would be considered a bactericidal concentration for M. MCS and M. phlei. From most sensitive to least sensitive, the results show concentration sensitivity to the SJW MeOH extract in the following order: M. JLS and M. KMS, M. phlei, M. MCS, B. subtilis, M. smegmatis, and E. coli, which showed no sensitivity. It should be noted that earlier studies showed that even though these differences exist in sensitivity over time, it was found that B. subtilis reacted the most quickly. This conclusion was derived from earlier MBC studies, which considered the “time 0” time point to be immediately after adding the SJW extract. Within the 0.5-hr to 1-hr timeframe it took to perform the first CFU quantification after SJW extract addition, the B. subtilis cultures had already been killed at the higher concentrations. This effect is seen in Figure 4.18. The 2.66 mg extract/ml concentration killed the cells within this timeframe, and the 0.67 and 1.33 mg extract/ml concentrations saw a rebound in cell growth over two days. The rebound effect may be attributable to the fact that this cell dilution method does not detect CFU concentrations under 100 cells/ml and to the relatively fast doubling time of 30-45 min (depending on medium) for B. subtilis (Leitch and Collier, 1996). This growth may also be attributable to resistant mutants and merits further study. This immediate effect was not seen in any other strain assayed.
It should be noted that Tween 80 (0.05% final concentration) was used in these crude extract MBC studies. In the light of the purified compound MBC results shown below, the MBC values would likely be lower in the absence of Tween 80.

**Purified Compound MBC Studies**

*Hfn Inhibition and the Effect of Tween 80.* In the absence of Tween 80, Hfn caused bactericidal effects at the 26 µg/ml and 52 µg/ml concentrations (within 48 hr) and had inhibitory effects at the 6 µg/ml and 13 µg/ml concentrations (Figure 4.19). In the presence of Tween 80 at 0.05% v/v concentration, no effect from Hfn, bactericidal or inhibitory, was seen (Figure 4.20).

The MBC values shown here are at least 65 times higher than the Hfn concentrations (approximately 0.4 µg/ml) found in rat and human blood after a high dose (1200 mg) of SJW extract taken orally (Biber et al., 1998). Therefore, oral administration of the SJW herb does not reach adequate Hfn concentrations for mycobacterial death. Studies involving Hfn administration techniques that bypass the digestive system for direct treatment, such as the use of inhalants (Gelperina et al., 2005; Shoyele and Cawthorne, 2006; Chow et al., 2007; Sung et al., 2007; Jaafar-Maalej et al., 2009; Salama et al., 2009), merit investigation.

When keeping the Hfn concentration constant at 46 µg/ml (concentration in 1:50 dilution (2% v/v) of 1X crude extract) and varying the Tween 80 concentration from 0.003-0.1%, a decreasing ability of Hfn to inhibit mycobacterial growth was seen with increasing Tween 80 concentration (Figure 4.21). The 0.1% Tween 80 concentration had the same approximate CFU count as the no-Tween80/Hfn control. A Tween 80 dose-
dependent CFU count reduction was observed with the 0.025% and 0.05% concentrations, and the more dilute Tween 80 concentrations reached CFU counts of 100 CFU/ml or less over the 72-hr study.

*Effect of Tween 80 on MeOH Crude Extract.* The crude extract controls showed a similar pattern as Hfn. In the absence of Tween 80, cell death was seen in all concentrations assayed: 0.33 (1/8X), 0.67 (1/4X), 1.33 (1/2X), and 2.66 (1X), mg extract/ml, which contained 6, 11, 23, and 46 µg/ml Hfn, respectively (Figure 4.22). In the presence of Tween 80 (0.05%), the crude extract caused bactericidal effects in the 1.33 mg extract/ml (23 µg/ml Hfn) only (2.7 mg extract/ml not tested) (Figure 4.23). Slight inhibitory effects (10-fold less) were seen in the 0.67 mg extract/ml (11 µg/ml Hfn).

The difference in inhibitory effect seen in the absence and presence of Tween 80 is significant. This finding is important for any antimicrobial study for mycobacteria involving Tween 80; it may invite a reinvestigation of reported MIC/MBC values of other antimycobacterial agents if Tween 80 was used in the studies. Whether the cause of this effect is an interaction between Tween 80 and Hfn or between Tween 80 and the bacterial cell wall remains to be studied. It is known that Tween 80 affects the outermost layer of the cell wall (Parish and Stoker, 1998), which understanding could help in determining the mechanism of action for Hfn. Another possible explanation for this effect is the formation of Tween 80 micelles around Hfn molecules, preventing the otherwise inhibitory Hfn from working. Also, the fact that inhibition was seen in the crude extract samples containing 23 µg Hfn/ml, but not in the pure Hfn (23 µg/ml) samples when in the presence of Tween 80, suggests that the crude extract contains
compounds which either stabilize Hfn, interact with Tween 80, or interact with the bacterial cell wall to allow inhibition to occur at a higher degree than pure Hfn.

*Effect of Hypericin and Pseudohypericin.* No bactericidal or inhibitory effects were seen in the Hpn or Phn samples at any assayed concentrations (Figures 4.24 and 4.25). However, a synergistic effect of these compounds with each other or with Hfn was not studied and merits investigation.

*Effect of Light on Inhibition.* No significant effect of light on Hfn inhibition of *M. KMS* was seen. Though the Hfn concentrations were not high enough to achieve MBC values, dose responses were seen in both the light-exposed tubes (Figure 4.26) and the tubes kept in the dark (Figure 4.27); these responses were remarkably similar. Due to an experimental error in the 24-hr CFU count for the control of the light samples, the value for the control from the dark samples was used for this particular time point. From the results of these studies, it was decided to not investigate the effects of light any further.

**SEM Imaging**

The results from SEM imaging are shown in Figures 4.28 – 4.32. Figure 4.28 shows untreated images of all the bacteria used in the MBC experiments. Figure 4.29 shows the untreated image of *M. KMS* again along with its response to the 1.33 mg extract/ml crude extract treatment at times 4, 8, and 24 hr. Figure 4.30 shows the progressive response of *M. smegmatis* to 2.66 mg extract/ml crude extract treatment at times 12, 24, and 48 hr in addition to its control. Figure 4.31 shows the *B. subtilis* response to 1.33 mg extract/ml treatment at times 30 min, 1 hr, and 2 hr in addition to its
control. Figure 4.32 shows the response of *E. coli* to 2.66 mg extract/ml treatment after 72 hr in addition to its control.

While no visible or experimental effect can be seen in the response of *E. coli* to SJW (other than a darkening of the media beyond the darkness of the extract addition, which effect was not studied), there appears to be a trend in the response of the SJW-susceptible organisms to the crude extract: (1) an increase in field debris - whether or not this is cell debris or extract debris (<0.2 μm because of filtered extract) is uncertain, but it is suspected to be cell debris, as it is not seen in the treated *E. coli* images; (2) a “beading,” or blistering effect in the cell wall; and (3) cell wall splitting, cracking, or rupturing, which leads to spilling of the cell contents. Cell death is known to occur before the third part of the cell degradation trend, based on times from the MBC results. It is suspected that cell death occurs closer to the second phase. These results support the MBC results, which show *B. subtilis* as having the most rapid SJW response. These results also suggest that there is either a direct chemical interaction with the cell wall or an interference with genetic cell wall maintenance.

**Conclusions**

The results from this chapter show a *M. tuberculosis* homology comparison to all nonpathogenic, genetically sequenced mycobacteria for *M. MCS* (72%), *M. smegmatis* (69%), *M. KMS* (69%), *M. vanbaalenii* (67%), *M. JLS* (66%), and *M. gilvum* (65%). SJW compounds are effective at inhibiting every currently sequenced, nonpathogenic *Mycobacterium* isolate, as well as *M. phlei*, which has not yet been sequenced. There is no visible difference in the level of growth of mycobacteria between LB media and MB
media, but LB media produces more vibrant mycobacterial pigmentation, which makes colonies easier to see and quantify. The five *Mycobacterium* isolates assayed in the MBC studies are resistant to ampicillin, but cell pigmentation is reduced when grown on Amp agar media. EtOH has a higher toxicity to *M. KMS* than MeOH, though only in reducing CFU population 10-fold at the highest concentration tested (4% v/v). MeOH has no toxicity to any of the assayed strains at the concentrations most often used in this study (2-4% v/v). No inhibition difference has been seen between SJW extracts of different solvents, though this has only been assayed at a 4% (v/v) concentration of an extract from a 0.36 mg herb/ml solvent solution. Depending on concentration, the MeOH SJW extract is inhibitory and bactericidal to (mg extract/ml culture) *M. JLS* (0.33-0.67), *M. KMS* (0.33-0.67), *M. MCS* (0.33-0.67), *M. smegmatis* (1.33-2.66), *M. phlei* (0.33-0.67), and the Gram-positive control *B. subtilis* (0.67-1.33); it has no effect on the viability of *E. coli* at the concentrations assayed.

An MIC value for the SJW EtOH extract has been determined for *M. KMS* (0.5-1% v/v of an extract from a 0.36 mg herb/ml solvent solution) using the dilution-in-agar method. In the absence of Tween 80, Hfn has a MBC of 6-13 µg/ml for *M. JLS*. Tween 80 represses Hfn inhibition of *M. JLS* at Tween 80 concentrations of ≥ 0.025% (v/v) at 46 µg/ml Hfn (Hfn concentration in 2.66 mg extract/ml); this may be due to micelle formation around Hfn molecules or an interaction of Tween 80 with the cell wall. Tween 80 also raises the MBC of *M. JLS* when used with the crude extract, but not to the degree seen with pure Hfn, even if both solutions have the same Hfn concentration; this may be due to a synergistic inhibitory effect of another SJW compound with Hfn, protection of Hfn from the effects of Tween 80, or a direct interaction of crude extract compounds with
the cell wall. Tween 80, however, helps to reduce clumping and solubilize the SJW extract in culture and makes the cell dilution CFU quantification method more accurate. Hpn and Phn do not have an inhibitory effect on M. JLS at the concentrations assayed. Light does not appear to affect the inhibitory effect of Hfn. SEM imaging suggests that SJW has an effect on the cell wall integrity/maintenance of the susceptible organisms, which knowledge should help in future studies of determining the mechanism of action.
Table 4.1 Inhibition demonstration of SJW extracts of various solvents and various SJW brands after one week of growth on crude-extract-containing plates (4% extract, v/v). Degree of growth is indicated by number of plus signs, and no growth is indicated by a minus sign. Some responses were not determined (ND) due to fungal contamination. Controls (shown at bottom) contained solvent only.

<table>
<thead>
<tr>
<th>Nature's Way</th>
<th>Acetone</th>
<th>Ethanol</th>
<th>Ethyl Acetate</th>
<th>Methanol</th>
<th>Chloroform</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. JLS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>M. KMS</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>M. MCS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>M. van. PYR-1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>M. gilvum PYR-GCK</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>P. putida</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

NOW

<table>
<thead>
<tr>
<th>Nature's Way</th>
<th>Acetone</th>
<th>Ethanol</th>
<th>Ethyl Acetate</th>
<th>Methanol</th>
<th>Chloroform</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. JLS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>M. KMS</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>M. MCS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>M. van. PYR-1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>M. gilvum PYR-GCK</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>P. putida</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

GNC

<table>
<thead>
<tr>
<th>Nature's Way</th>
<th>Acetone</th>
<th>Ethanol</th>
<th>Ethyl Acetate</th>
<th>Methanol</th>
<th>Chloroform</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. JLS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>M. KMS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>M. MCS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M. van. PYR-1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>M. gilvum PYR-GCK</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>P. putida</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

Controls (solvent only)

<table>
<thead>
<tr>
<th>Nature's Way</th>
<th>Acetone</th>
<th>Ethanol</th>
<th>Ethyl Acetate</th>
<th>Methanol</th>
<th>Chloroform</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. JLS</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>M. KMS</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>M. MCS</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>M. van. PYR-1</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>M. gilvum PYR-GCK</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>P. putida</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>
Table 4.2  Inhibition demonstration of SJW extracts of various solvents and various SJW brands after two weeks of growth on crude-extract-containing plates (4% extract, v/v).  Degree of growth is indicated by number of plus signs, and no growth is indicated by a minus sign.  Some responses were not determined (ND) due to fungal contamination.  Controls (shown at bottom) contained solvent only.

<table>
<thead>
<tr>
<th>Nature’s Way</th>
<th>Acetone</th>
<th>Ethanol</th>
<th>Ethyl Acetate</th>
<th>Methanol</th>
<th>Chloroform</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. JLS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>M. KMS</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>M. MCS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>M. van. PYR-1</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M. gilvum PYR-GCK</td>
<td>+</td>
<td>ND</td>
<td>++</td>
<td>ND</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>P. putida</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

NOW

<table>
<thead>
<tr>
<th>Nature’s Way</th>
<th>Acetone</th>
<th>Ethanol</th>
<th>Ethyl Acetate</th>
<th>Methanol</th>
<th>Chloroform</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. JLS</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>M. KMS</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>M. MCS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>M. van. PYR-1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M. gilvum PYR-GCK</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>P. putida</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

GNC

<table>
<thead>
<tr>
<th>Nature’s Way</th>
<th>Acetone</th>
<th>Ethanol</th>
<th>Ethyl Acetate</th>
<th>Methanol</th>
<th>Chloroform</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. JLS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>M. KMS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>M. MCS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>M. van. PYR-1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M. gilvum PYR-GCK</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>P. putida</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

Controls (solvent only)

<table>
<thead>
<tr>
<th>Nature’s Way</th>
<th>Acetone</th>
<th>Ethanol</th>
<th>Ethyl Acetate</th>
<th>Methanol</th>
<th>Chloroform</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. JLS</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>M. KMS</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>+++</td>
</tr>
<tr>
<td>M. MCS</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>M. van. PYR-1</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>M. gilvum PYR-GCK</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>P. putida</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>
Table 4.3  Minimum bactericidal concentrations for the seven MBC-assayed strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>SJW MBC (mg extract/ml culture)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. JLS</em></td>
<td>0.33-0.67</td>
</tr>
<tr>
<td><em>M. KMS</em></td>
<td>0.33-0.67</td>
</tr>
<tr>
<td><em>M. MCS</em></td>
<td>0.33-0.67</td>
</tr>
<tr>
<td><em>M. smegmatis</em></td>
<td>1.33-2.66</td>
</tr>
<tr>
<td><em>M. phlei</em></td>
<td>0.33-0.67</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>N/A</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>0.67-1.33</td>
</tr>
</tbody>
</table>
Figure 4.1 Venn diagram for genetic similarity of all currently sequenced, nonpathogenic mycobacteria to \textit{M. tuberculosis}. Comparisons are made using amino acid sequences. Non-percentage numbers indicate number of genes. Outer percentages indicate genome unique to each \textit{Mycobacterium} isolate. Middle percentages indicate percentage of each isolate genome similar to \textit{M. tuberculosis}. Inner percentages indicate percentage of \textit{M. tuberculosis} genome incorporated in each isolate (of greatest interest for this study).

Figure 4.2 The five \textit{Mycobacterium} strains used in the MBC studies: (starting in top right, going counterclockwise) \textit{M. JLS}, \textit{M. KMS}, \textit{M. MCS}, \textit{M. smegmatis}, and \textit{M. phlei}. 
Figure 4.3 Growth plates for demonstration of inhibitory effect and comparison of extracts of different solvents and different SJW brands. Only the plates for the GNC brand are shown here. Organisms on plate are as follows (starting at top, going counterclockwise): *P. putida* (G- control), *M. vanbaalenii*, *M. gilvum*, *M. MCS*, *M. JLS*, *M. KMS*. Because this particular extract used was not filtered, fungal growth occurred in several of the *M. KMS* sections; these were classified as “not determined.”
<table>
<thead>
<tr>
<th></th>
<th>LB Media</th>
<th>Middlebrook Media</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>M. JLS</strong></td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td><strong>M. KMS</strong></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td><strong>M. MCS</strong></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td><strong>M. smegmatis</strong></td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
<tr>
<td><strong>M. phlei</strong></td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**Figure 4.4** Comparison of mycobacterial growth on LB and MB media. Dark background behind plates is to show contrast. For the USU isolates (top three), growth on LB media provided higher bacterial visibility.
Figure 4.5  Growth comparison of five *Mycobacterium* isolates on antibiotic-free LB (left) plates and ampicillin-containing LB (50 µg/ml, right) plates. Organisms assayed were (starting at top and going clockwise) *M. JLS, M. KMS, M. MCS, M. smegmatis*, and *M. phlei*. A higher degree of cell pigmentation was seen on the antibiotic-free plates, so antibiotic-free media was used in all studies for consistency and to enable the easier use of the cell dilution method for CFU quantification.

Figure 4.6.  Typical example of CFU quantification results using the cell dilution method (*M. JLS*). The CFU count for this sample would be $1.1 \times 10^8$ CFU/ml according to the most dilute spot and would be $6.1 \times 10^7$ CFU/ml according to the next least dilute spot, yielding an average of $8.55 \times 10^7$ CFU/ml with a standard deviation of $3 \times 10^7$. 
**Figure 4.7** Effect of MeOH on *M. KMS* viability. Concentrations assayed (v/v) were at final working volumes of 0.5%, 1.0%, 2.0%, and 4.0%, with a no-MeOH control included. Error bars are standard deviation (SD).

**Figure 4.8** Effect of EtOH on *M. KMS* viability. Concentrations assayed (v/v) were at final working volumes of 0.5%, 1.0%, 2.0%, and 4.0%, with a no-EtOH control included. Error bars are SD.
Figure 4.9 Growth plates from dilution-in-agar MIC study of SJW EtOH extract. Starting from upper left two plates and reading in columns, concentrations (% of 1X stock solution) were 8%, 4%, 3%, 2%, 1%, 0.5%, 0.25%, 0.13%, 0.07%, 0.03%, and 0%, performed in duplicate plates. Organism key is on bottom right.
Figure 4.10 Typical cell dilution plates used for CFU quantification in MBC studies. Concentrations assayed were 2X, 1X, 1/2X, 1/4X, 1/8X, and 1/16X, representing 4.5, 2.7, 1.3, 0.7, and 0.3 mg extract/ml, respectively. The bottom right figures show zoomed in views of colonies from the dilutions to show cell morphology. MeOH-only controls (denoted “MeOH”) and no-MeOH/SJW (denoted “None”) are the bottom two sections of the bottom plate of each isolate image.
Figure 4.11 Typical growth/death results of *M. JLS* from the crude extract MBC studies. SJW MeOH extracts of various concentrations were used at 2% (v/v) the total volume: 0.17, 0.33, 0.67, 1.33, and 2.66 mg extract/ml culture. The MBC was between 0.33-0.67 mg extract/ml. Error bars are SD.

Figure 4.12 Typical growth/death results of *M. KMS* from the crude extract MBC studies. SJW MeOH extracts of various concentrations were used at 2% (v/v) the total volume: 0.17, 0.33, 0.67, 1.33, and 2.66 mg extract/ml culture. The MBC was between 0.33-0.67 mg extract/ml. Error bars are SD.
Figure 4.13  Typical growth/death results of *M. MCS* from the crude extract MBC studies. SJW MeOH extracts of various concentrations were used at 2% (v/v) the total volume: 0.17, 0.33, 0.67, 1.33, and 2.66 mg extract/ml culture. The MBC was between 0.33-0.67 mg extract/ml. Error bars are SD.

Figure 4.14  Typical growth/death results of *M. smegmatis* from the crude extract MBC studies. SJW MeOH extracts of various concentrations were used at 2% (v/v) the total volume: 0.33, 0.67, 1.33, 2.66, and 4.46 mg extract/ml culture. The MBC was between 1.33-2.66 mg extract/ml. Error bars are SD.
Figure 4.15 Typical growth/death results of *M. phlei* from the crude extract MBC studies. SJW MeOH extracts of various concentrations were used at 2% (v/v) the total volume: 0.17, 0.33, 0.67, 1.33, and 2.66 mg extract/ml culture. The MBC was between 0.33-0.67 mg extract/ml. Error bars are SD.

Figure 4.16 Typical growth/death results of *E. coli* from the crude extract MBC studies. SJW MeOH extracts of various concentrations were used at 2% (v/v) the total volume: 0.33, 0.67, 1.33, 2.66, and 4.46 mg extract/ml culture. There was no SJW MBC for this strain. Error bars are SD.
Figure 4.17  Typical growth/death results of *B. subtilis* from the crude extract MBC studies. SJW MeOH extracts of various concentrations were used at 2% (v/v) the total volume: 0.17, 0.33, 0.67, 1.33, and 2.66 mg extract/ml culture. The MBC was between 0.67-1.33 mg extract/ml. Error bars are SD.

Figure 4.18  The time course of SJW effect on *B. subtilis*. In early MBC studies, the “time 0” CFU count was taken immediately after adding the SJW extract, which would have been within about 1 hr. Cell growth rebound was seen in the 0.67 mg/ml and 1.33 mg/ml concentrations. The *Mycobacterium* isolates did not see such a rapid inhibition response. Error bars are SD.
Figure 4.19 Growth/inhibition results for *M. JLS* treated with Hfn in the absence of Tween 80. Concentrations assayed were 6, 13, 26, and 52 µg/ml culture. The MBC was 6-13 µg/ml. Error bars are SD.

Figure 4.20 Growth/inhibition results for *M. JLS* treated with Hfn in the presence of Tween 80 (0.05% v/v). Concentrations assayed were 3, 6, 11, and 23 µg/ml culture. There was no MBC for these samples. Error bars are SD.
Figure 4.21 Tween 80 repression of Hfn inhibition of *M. JLS* with all samples except the control containing 46 µg/ml Hfn. Decreasing inhibition occurred with increasing Tween 80 concentration. The 0.1% concentration had similar CFU counts as the No-Tween 80/Hfn control. Decreasing CFU counts were seen in the 0.05% and 0.025% concentrations, and all lower Tween 80 concentrations reached CFU counts of 100 CFU/ml or less over the 72-hr study. Error bars are SD.

Figure 4.22 Growth/inhibition results for *M. JLS* treated with crude extract (for comparison in the pure compounds MBC studies) in the absence of Tween 80. Concentrations assayed were 0.33, 0.67, 1.33, and 2.66 mg extract/ml culture. Inhibition was seen in all crude extract concentrations in this study. Error bars are SD.
Figure 4.23  Growth/inhibition results for *M. JLS* treated with crude extract (for comparison in the pure compounds MBC studies) in the presence of Tween 80 (0.05% v/v). Concentrations assayed were 0.17, 0.33, 0.67, and 1.33 mg extract/ml culture. The MBC was between 0.67-1.33 mg extract/ml. Error bars are SD.

Figure 4.24  Growth/inhibition results for *M. JLS* at various concentrations of Hpn without Tween 80 added to the culture. Concentrations assayed were 3, 7, 13, and 27 µg Hpn/ml culture. No concentrations reached MBC levels. Error bars are SD.
**Figure 4.25** Growth/inhibition results for *M. JLS* at various concentrations of Phn without Tween 80 added to the culture. Concentrations assayed were 3, 6, 13, and 25 µg Phn/ml culture. No concentrations reached MBC levels. Error bars are SD.

**Figure 4.26** Growth/inhibition results for the light-exposed samples (*M. KMS*) of the light/dark Hfn study. Concentrations assayed were 0.1, 1, and 5 µg Hfn/ml culture. The samples in light and dark had nearly identical results. Error bars are SD.
Figure 4.27 Growth/inhibition curves for the foil-wrapped samples (*M. KMS*) of the light/dark Hfn study. Concentrations assayed were 0.1, 1, and 5 µg Hfn/ml culture. The samples in light and dark had nearly identical results. Error bars are SD.
Figure 4.28  SEM images of untreated strains used in the SJW inhibitory/bactericidal assays.  (a)  M.  JLS, (b)  M.  KMS, (c)  M.  MCS, (d)  M.  smegmatis, (e)  M.  phlei, (f)  E.  coli, (g)  B.  subtilis
Figure 4.29 SEM images of the progression of *M. KMS* response to SJW MeOH crude extract (1.33 mg extract/ml). Exposure at times (a) 0, (b) 4 hr, (c) 8 hr, and (d) 24 hr.
Figure 4.30  SEM images of the progression of *M. smegmatis* response to SJW MeOH crude extract (2.66 mg extract/ml). Exposure at times (a) 0, (b) 12 hr, (c) 24 hr, and (d) 48 hr.
Figure 4.31 SEM images of the progression of *B. subtilis* response to SJW MeOH crude extract (1.33 mg extract/ml). Exposure at times (a) 0, (b) 4 hr, (c) 8 hr, and (d) 24 hr.
Figure 4.32  SEM images of the response of *E. coli* to SJW MeOH crude extract (2.66 mg extract/ml). Exposure at times (a) 0 and (b) 72 hr.
CHAPTER 5
EFFECT OF HYPERICUM PERFORATUM ON HUMAN ALVEOLAR EPITHELIAL CELLS

Introduction

The aim of this study was to investigate the potential of using one or more of the SJW compounds as an inhalant for TB. Therefore it was decided to investigate the effect of these compounds on the human carcinomic alveolar epithelial lung cell line A549. Studies were performed that investigated the effects of MeOH, SJW MeOH crude extract, Hpn, Phn, Hfn, and M. JLS cells on A549 cell viability.

Materials and Methods

A549 Media (Ham’s) Preparation

The protocol for A549 media (Ham’s) preparation is in the Appendix.

A549 Cell Culturing

A549 Culturing from Freezer Stock. Freezer stocks of A549 (1-ml stocks), about 16th subculture, were obtained from Dr. Soonjo Kwon from the USU Biological Engineering Tissue Engineering Laboratory. Cell cultures were started from -80°C freezer stocks by adding the cells to a tissue-culture-treated T25 (25 cm²) or T75 flask (75 cm²) (BD, Fisher Scientific, Pittsburgh, PA). Warmed (37°C) media was then added to the cells (5 ml for T25 or 12 ml for T75). If the flask did not have a vent cap, the lid was left slightly open to allow gas exchange. Cells were incubated in a CO₂ incubator
(model 3531, Fisher Scientific, Pittsburgh, PA) at 37ºC and 5% CO₂. Media was changed every 2 or 3 days.

Subculturing. To subculture the cells, the old media was removed, and 0.05% trypsin EDTA (Invitrogen, Carlsbad, CA) was added in volumes of 50-250 μl/cm². The flasks were then incubated at 37ºC for 5-15 min until the cells had completely detached from the surface. Visual confirmation of cell detachment was done using an inverted microscope. About 5 ml media was added to inhibit the trypsin, and this cell solution was transferred to a 15-ml centrifuge tube. Another 5-ml media was added to rinse any remaining cells from the flask and was also added to the centrifuge tube. The tube was centrifuged for 10 min at 1000 RPM (834 RCF), and supernatant was discarded. A 4-ml volume of warmed media was added, and the cells were gently resuspended. A simple subculture consisted of splitting this volume evenly between two or more flasks and bringing the culture volume to the correct level with media.

Cell Seeding at Specific Densities in Well Plates. If a specific cell seeding density was desired, a Hausser Scientific Bright-Line hemacytometer (Fisher, Pittsburgh, PA) was used to determine cell concentration (Figure 5.1). After cleaning the cell chamber surface and cover slide, the cover slide was placed firmly over the two chambers, and 10 μl of resuspended cell solution was added to one of the chambers via an injection slot. Capillary action filled the chamber. Using an inverted microscope, four cell counts were taken from four 1 mm² x 0.1 mm deep squares (divided into 16 smaller squares for easier counting). The counts were averaged and multiplied by 10⁴ to obtain cells/ml. Cell concentrations were adjusted by diluting with media and adding to a 6-well (9.6 cm²/well) or 48-well (0.75 cm²/well) tissue culture plate (BD Falcon, Fisher, Pittsburgh,
Seeding densities ranged from $6.6 \times 10^4$ to $2.6 \times 10^5$ cells/cm$^2$ in the A549 viability studies (approximately $1.3 \times 10^4$ cells/ml would reach confluency in 48 hr).

**Freezer Stock Preparation.** Freezer stocks of the A549 cells were made by preparing a 5% (v/v) dimethyl sulfoxide (DMSO) (Fisher Bioreagents, Pittsburgh, PA) cell solution after the centrifugation and resuspension steps of subculturing. The solution was aliquoted into 1.5-ml Bio Plas microcentrifuge tubes (Fisher, Pittsburgh, PA) and stored at -70°C. Cells were stored in cryopreservation tubes in liquid nitrogen (-196°C) for long-term storage.

**Treatments of A549 Cell Cultures**

Various treatments were assayed for effect on A549 cell viability: MeOH, SJW MeOH crude extract, Hpn, Phn, Hfn, and *M. JLS* cells. Treatments were always added to confluent cultures after the first (“time 0”) sample was taken. The MeOH treatment was assayed in 6-well plates, and all other treatments were assayed in 48-well plates. MeOH was added at four concentrations in triplicate two weeks after seeding ($2.6 \times 10^4$ cells/cm$^2$). SJW MeOH crude extract (in triplicate) and Hfn (in duplicate) were added at various concentrations (always $\leq 2\%$ v/v) three days after seeding ($2.6 \times 10^5$ cells/cm$^2$) for one study and one week after seeding ($6.6 \times 10^4$ cells/cm$^2$) for another study using glass syringes (Hamilton, Reno, NV). Hpn and Phn were added in triplicate at various concentrations three days after seeding ($2.6 \times 10^5$ cells/cm$^2$) using glass syringes. *M. JLS* cells were added in duplicate at $5 \times 10^6$ cells/cm$^2$ to A549 cultures one week after seeding ($6.6 \times 10^4$ cells/cm$^2$); cell concentration was determined using an average of four 0.04 mm$^2$ squares (Figure 5.1) of the hemacytometer.
**A549 Viability Testing**

*Pierce Bicinchoninic Acid (BCA) Total Protein Assay.* To harvest cell material for the Pierce BCA total protein assay, media was removed from the plate wells, and each well was rinsed with phosphate-buffered saline (PBS) (HyClone, Fisher, Pittsburgh, PA). A 1% (v/v) Triton X-100 (Fisher, Pittsburgh, PA) solution was added to the cells in consistent volumes, at least enough to cover the cells (650 µl/cm² was typical), for cell lysis. The cells were then incubated at 37°C for 45 min, then thoroughly scraped off using a 1-ml pipette tip and resuspended in the Triton solution. The cells were transferred to 1.5-ml microcentrifuge tubes and stored at -80°C until a Pierce BCA total protein assay (Thermo Scientific, Rockford, IL) was performed. The protocol used was supplied with the kit (Thermo, 2010).

*Direct Cell Counting.* To harvest cells for direct cell counting, cells were rinsed with PBS, detached with 200 µl of 0.05% trypsin EDTA (in like manner as was done when subculturing), and added to 300 µl Ham’s media for trypsin inactivation. Direct cell counts were then taken using the hemacytometer (Figure 5.1). Brightfield images were taken for many of the samples before cell removal (in wells) and after cell counting (while on the hemacytometer) using a Nikon Fluorescence Microscope (Eclipse Ti-DH, Nikon, Melville, NY) with attached camera (Cool Snap HQ², Photometrics, Tucson, AZ).

*Statistical Analysis.* The direct cell counting results of the various A549 treatments were analyzed for statistically significant differences between each other using the General Linear Modeling (GLM) Procedure with SAS statistical analysis software. The results were analyzed as a 4x10x2 factorial design, with 4 levels of time, 10 levels of “additives” (including the controls), and 2 replicates (each replicate value was taken by
averaging the four hemacytometer counts of each cell sample). Results were then grouped by statistically significant differences using the Ryan-Einot-Gabriel-Welsh q (REGWQ) test.

Testing Mycobacterial Growth in Ham’s Media

The five Mycobacterium strains assayed in the MBC studies were tested for ability to grow in Ham’s media by preparing two sets of 12-ml culture tubes, each tube containing 5-ml Ham’s media. One set contained no antibiotics, and the other set contained penicillin-streptomycin (100 U/ml and 100 µg/ml, respectively). Each tube was inoculated with 100 µl freezer stock of bacteria and incubated at the proper temperatures. Growth was observed after eight days.

Results and Discussion

Effects of Treatments on A549 Cell Viability

Statistical Analysis. The residuals vs. normal percentiles plot of the A549 cell counts from the direct cell counting study showed adequate homoscedasticity. Partial R^2 values of the treatments (proportion contribution to the final outcome, which all add to 1), as well as their degrees of freedom (DF) (number of values that are free to vary in the final statistical calculation), F-values (used to calculate p-values) and p-values (used to show statistical significance if under 0.05), are shown in Table 5.1. SJW additive had the greatest effect (partial R^2 0.441), followed by the interaction between additive and time (partial R^2 0.282), followed by time (partial R^2 0.240). REGWQ grouping for the
additives is shown in Table 5.2, with means of different letters being significantly different from each other. The groupings are as follows: (A) the MeOH control, no-MeOH control, M. JLS, and Hfn 3 µg/ml samples; (B) the Hfn 6 µg/ml, Hfn 11 µg/ml, and Hfn 23 µg/ml samples; (C) the Hfn 11 µg/ml, Hfn 23 µg/ml, and Hfn 46 µg/ml samples; (D) the Hfn 46 µg/ml, crude 1.33 mg extract/ml, and crude 2.66 mg extract/ml samples.

MeOH Treatment. Results for the effect of MeOH on A549 viability are shown in Figure 5.2. The higher protein concentrations from these plates (due to suspending the cells in a smaller relative volume of Triton X-100 than the assays that used 48-well plates) were corrected by dividing by the fold-size difference in the 6-well and 48-well surface area (9.6/0.75 = 12.8). The order of total protein concentration (high to low) was 0%, 1%, 3%, and 2%, with a mean of 48.5 µg/ml +/- 2.2 (std. dev.). There was no pattern seen in the treatment responses. No statistically significant differences were seen between the concentrations using the GLM procedure in the SAS (3 DF, F-value: 4.00, p-value: 0.0518) program. Therefore, MeOH concentrations up through 3% did not have an effect on A549 viability.

SJW MeOH Crude Extract Treatment. The Pierce BCA protein assay was not effective at quantifying protein from the crude extract treatment due to the brown cell staining that the cells received from the crude extract. This caused OD readings that were much higher than the controls (Figure 5.3). Therefore, efforts to quantify A549 cells treated with crude extract with colorimetric assays were abandoned, and the direct cell counting method was used.
Results showing the use of the direct cell count method are shown in Figure 5.4. Cell death in both of the crude extract samples (1.33 and 2.66 mg extract/ml) was seen after one day. Cell death was interpreted to be the absence of cells after PBS rinsing or a non-round, shredded, cell morphology (different from all other viable cells, which were round and intact). Such morphology is seen in the crude-extract-exposed A549 cell images in Figures 5.5-5.7. Viable cells (as seen in the controls) were flat, adhered to the plate, and tessellated; nonviable cells were round and detached (at least to some degree).

It should be noted that the cells treated with crude extract did not detach from the well surface or disaggregate with trypsin treatment, even after rinsing off the media with PBS. Mild scraping was required to remove the cells from the wells. This same effect was seen with the A549 samples in the protein assay after crude extract treatment; they also did not detach with PBS rinsing. It is unknown why this occurred, but it is possible that the crude extract contains a trypsin inhibitor. It is also possible that the crude extract contains one or more compounds that change the properties of the A549 cell membranes, causing them to adhere to the well surface and each other, even after cell death.

_Hyperforin Treatment._ The Hfn-treated cells were the only cells with a conclusive effect from the Pierce BCA protein assay (Figure 5.3). After 2 days of exposure, rinsing with PBS completely removed the cells. The direct cell count study revealed a dose-dependent response of the cells to Hfn, with the 11, 23, and 46 µg/ml concentrations causing complete cell death. The half maximal lethal dose (LD$_{50}$) (concentration that kills half the cell population) for the A549 cells in response to Hfn was 3-6 µg/ml (5.6-11.2 µmol/L). There was usually no cell debris left behind after rinsing dead cells with PBS; however, cell debris was found in some Hfn samples and
were included in the images (see Figures 5.5-5.7). Contrast between live and dead cells is easily apparent in the 3 and 6 µg Hfn/ml images in Figure 5.7.

Thus far, no study has been found to investigate the effect of Hfn on the A549 cell line. Though the results from this study do not show a lack of Hfn toxicity to primary human lung cells, the LD$_{50}$ values found for Hfn in this study place the A549 cell line in line with a list of several other known human carcinomic (or immortal) cell lines to which Hfn is antiproliferative. Thus, the A549 cell line may be added to this list and likely shares the same mechanism of action in Hfn-induced apoptosis (induction of mitochondrial-mediated pathway) (Schempp et al., 2002; Medina et al., 2006).

**Hypericin, Pseudohypericin, and M. JLS Treatments.** The Hpn and Phn treatments were only assayed with the Pierce BCA protein assay, which showed no significant viability effect at 28 µg/ml (Hpn) and 61 µg/ml (Phn). The Hpn treatment did reach a LD$_{50}$ value (based on the “time 0” value) at 72 hr, though this value had a relatively high standard deviation. The Phn treatment reached a LD$_{50}$ value at 48 hr, but cell numbers were above that value the following day. However, the error in these ranges makes further testing of these compounds necessary, but merited, as hypericin is known to cause photosensitization and cell death in mammalian cells through high singlet oxygen yield (Agostinis et al., 2002). Also, the effect of combinations of the hypericins (with each other and with Hfn) on A549 viability merits investigation.

No statistically significantly different effect from the controls was seen in the A549 samples treated with *M. JLS* (at 5x10$^6$ bacteria/cm$^2$). The bacteria were added to media containing penicillin/streptomycin (10K U/ml and 10K µg/ml, respectively) and did not produce colonies after plating onto antibiotic-free LB agar plates. The bacteria
were introduced in the same range as was used in similar studies (Lin et al., 1998). As the M. JLS cells were nonviable, these results should be similar to the effect that would have been gained from adding mycobacterial cell components.

**Mycobacterial Growth in Ham’s Media**

The results of the growth test for all the bacterial strains from the MBC studies are shown in Table 5.3. No strains grew in media containing Pen-Strep, and only *M. JLS* and *E. coli* grew in the antibiotic-free media. These results may be of use for future studies on the effects of these strains on A549 cells when alive in culture, since the presence of dead *M. JLS* cells did not have a significant viability effect.

**Conclusions**

The studies performed on the effect of SJW MeOH extract and compounds show that MeOH does not have a significant effect on A549 cell viability at or below 3\% (v/v). Hpn and Phn concentrations of 28 µg/ml and 61 µg/ml, respectively, do not permanently or consistently lower the A549 cell count below LD$_{50}$ values. Hfn has a statistically significant effect on A549 cell viability at concentrations from 6 µg/ml to 46 µg/ml and does not at 3 µg/ml or lower. Hfn also causes cell death at concentrations ≥11 µg/ml, with an LD$_{50}$ of 3-6 µg/ml; this value is similar to a list of other carcinomic cell lines known to be sensitive to Hfn-induced apoptosis through a mitochondrial-mediated pathway, and these results justify the addition of the A549 cell line to that list. The repeated failure of trypsin to detach/disaggregate crude extract-treated A549 cells from the plate surface and from each other suggests that either the crude extract contains a trypsin-inhibiting compound or that the extract somehow prevents cell detachment after
death. The presence of nonviable *M. JLS* cells, at a concentration of $5 \times 10^6$ cells/cm$^2$, does not show a statistically significant viability effect on A549 cells. *M. JLS* and *E. coli* grow in antibiotic-free Ham’s media.
Table 5.1  Statistical analysis parameters for the A549 samples from the direct cell count study. Degrees of freedom show how many values are free to vary in the final statistical calculation. Partial $R^2$ values show what proportion a treatment or interaction has on the final outcome, and these values add to 1. Additive (what was added to the A549 cells) had the greatest effect, followed by the interaction of additive and time, followed by time. The F-value is used to determine the p-value, which if under 0.05, is considered statistically significant.

<table>
<thead>
<tr>
<th></th>
<th>Deg. Freedom</th>
<th>Partial $R^2$</th>
<th>F-value</th>
<th>Pr&gt;F (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>3</td>
<td>0.24</td>
<td>86.36</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Additive</td>
<td>9</td>
<td>0.441</td>
<td>53.02</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Time*Additive</td>
<td>27</td>
<td>0.282</td>
<td>11.31</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Table 5.2  Ryan-Einot-Gabriel-Welsh q (REGWQ) test for A549 cell treatments that received direct cell counting. Treatments are listed in descending order by mean. Groups of each letter are statistically significantly different from other groups.

<table>
<thead>
<tr>
<th>Additive</th>
<th>Mean</th>
<th>N</th>
<th>REGWQ Grouping</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeOH control</td>
<td>45.9</td>
<td>8</td>
<td>A</td>
</tr>
<tr>
<td>No-MeOH control</td>
<td>45.2</td>
<td>8</td>
<td>A</td>
</tr>
<tr>
<td>M. JLS</td>
<td>44.2</td>
<td>8</td>
<td>A</td>
</tr>
<tr>
<td>Hfn 3 µg/ml</td>
<td>39.1</td>
<td>8</td>
<td>A</td>
</tr>
<tr>
<td>Hfn 6 µg/ml</td>
<td>24.2</td>
<td>8</td>
<td>B</td>
</tr>
<tr>
<td>Hfn 23 µg/ml</td>
<td>22.0</td>
<td>8</td>
<td>C, B</td>
</tr>
<tr>
<td>Hfn 11 µg/ml</td>
<td>21.0</td>
<td>8</td>
<td>C, B</td>
</tr>
<tr>
<td>Hfn 46 µg/ml</td>
<td>13.8</td>
<td>8</td>
<td>C, D</td>
</tr>
<tr>
<td>Crude 2.6 mg extract/ml</td>
<td>11.5</td>
<td>8</td>
<td>D</td>
</tr>
<tr>
<td>Crude 1.3 mg extract/ml</td>
<td>9.9</td>
<td>8</td>
<td>D</td>
</tr>
</tbody>
</table>
Table 5.3  Growth results in Ham’s media of all *Mycobacterium* isolates used in the MBC studies. Growth is marked by a “Y” or “N” for yes or no.

<table>
<thead>
<tr>
<th>Strain</th>
<th>No Antibiotic</th>
<th>Containing Pen-Strep (100 U/ml, 100 µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. JLS</em></td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td><em>M. KMS</em></td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td><em>M. MCS</em></td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td><em>M. smegmatis</em></td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td><em>M. phlei</em></td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>
Figure 5.1 The improved Neubauer Bright-Line Hemacytometer setup (www.hausserscientific.com). The surface is 100 µm deep. The four 1-mm² sections (highlighted in red) were used to calculate A549 cell concentration, and each section gives cells/100 nl. The four smaller, 0.04-mm² sections (highlighted in blue) were used to calculate bacterial concentration, and each section gives cells/4 nl.
Figure 5.2  Pierce BCA total protein assay for the effect of MeOH on A549 viability. No statistically significant viability differences were seen. Cells were assayed at concentrations from 0-3% and harvested at 72 hr exposure. Error bars are SD.

Figure 5.3  Pierce BCA total protein assay results for Hpn, Hfn, Phn, and SJW MeOH crude extract at concentrations found in the 4.46 mg extract/ml crude extract. Due to cell staining by the crude extract, the protein assay gave OD readings that were much higher than the controls. Imprecise results were seen with the other samples. Cell death was seen, however, in the Hfn sample. Error bars are SD.
Figure 5.4  Direct cell count results (2 replicates, 4 counts each) for effect of dead *M. JLS* cells, SJW MeOH crude extract, and Hfn on A549 cell viability. Cell death occurred with the 1.33 and 2.66 mg extract/ml crude extract concentrations and in the 11, 23, and 46 µg/ml Hfn concentrations. Cell death did not occur in the no-MeOH/SJW control, MeOH control, or *M. JLS* samples. The Hfn LD$_{50}$ value was 3-6 µg/ml. Error bars are SD. Corresponding images are seen in Figures 5.5-5.7.
Figure 5.5 24-hr exposure of A549 cells to dead *M. JLS* cells (5×10⁶ bacteria/cm²), crude extract (1.33 and 2.66 mg extract/ml), and Hfn (3, 6, 11, 23, and 46 µg/ml). Cell death occurred in the crude extract samples and in the three highest-concentrated Hfn samples, with a Hfn LD₅₀ of 3-6 µg/ml (5.6-11.2 µmol/L). Cell morphology shown in bottom right of each image.
Figure 5.6 48-hr exposure of A549 cells to dead *M. JLS* cells (5x10⁶ bacteria/cm²), crude extract (1.33 and 2.66 mg extract/ml), and Hfn (3, 6, 11, 23, and 46 µg/ml). Cell death occurred in the crude extract samples and in the three highest-concentrated Hfn samples, with a Hfn LD₅₀ of 3-6 µg/ml (5.6-11.2 µmol/L). Cell morphology shown in bottom right of each image.
Figure 5.7 72-hr exposure of A549 cells to dead *M. JLS* cells (5x10^6 bacteria/cm^2), crude extract (1.33 and 2.66 mg extract/ml), and Hfn (3, 6, 11, 23, and 46 µg/ml). Cell death occurred in the crude extract samples and in the three highest-concentrated Hfn samples, with a Hfn LD$_{50}$ of 3-6 µg/ml (5.6-11.2 µmol/L). Cell morphology shown in bottom right of each image.
CHAPTER 6
ENGINEERING APPLICATIONS AND FUTURE CONSIDERATIONS

Several potential engineering applications or considerations of this research, as well as possible future directions, are listed below:

**Engineering Applications and Considerations**

1. Investigating the optimal droplet size of Hfn aerosols for delivery to the lungs.

2. Micro- or nano-encapsulating SJW compounds in a dissolving suspension, looking into the possibility of a time-released treatment.

3. Looking into the design of encapsulation materials, taking into consideration lipophilicity, dissolving rate, and surface wetting (for cell adhesion) parameters. A polymeric material such as poly (lactide-co-glycolide) (PLGA) could be used.

4. Undertaking a direct focal delivery study of SJW-compound-encapsulation particles, engineering the surface of the particles to preferentially adhere to the cell surfaces of TB granulomas. This would enable the use of higher medication concentrations and minimize toxicity to healthy cells.

5. Investigating mass transfer rates of SJW compounds in cell culture, developing mass transfer vs. penetration depth charts.

6. Determining values for exposure time and frequency of treatment with SJW compounds to adequately repress or kill *Mycobacterium* strains in lung cell models.

7. Investigating the surface-coating rates of nebulized liquid, microparticles, and nanoparticles containing SJW compounds. This investigation could also look into
developing models which would predict necessary medication flow rates to coat only a given percentage of cell surface area at a time; the idea of this would be to allow oxygen transfer to the lung cells while still uniformly coating the cell surface over time.

8. Employing the use of fluorescent particles (such as quantum dots that fluoresce in the tissue-transmission range) that are attached to mycobacterial-specific antibodies for locating mycobacterial infection sites in the lungs. Successes in this area could reduce or eliminate the need for using X-rays in TB screening.

9. Using mycobacterial-specific antibodies in materials used in targeted drug delivery (such as the above-mentioned nanoparticles) to mycobacteria in the lungs. Lung-cell models treated with dead *Mycobacterium* cells (or live if using non-antibiotic-containing media), which have been shown to be a viable option as a result of this study, could make adequate models.

10. Developing a cancer-screening method that detects Hfn metabolites. It is known that Hfn affects carcinomic cells, and it may be possible that only these types of cells metabolize this compound.

11. Investigating systemic Hfn treatment parameters in animals (after appropriate *in vitro* screening) to investigate Hfn effectiveness in metastatic prophylaxis.

12. Looking into the viability (based on successes in the previously mentioned applications in this section) of a home-treatment option for TB patients.
Future Work Recommendations (In Order of Priority)

1. Performing viability and inflammatory assays of pure Hfn against primary lung cells and other primary human cells to determine if the Hfn levels at which inflammation or cell death occurs is sufficiently above the Hfn levels known to cause apoptosis in carcinomic cell lines. Such studies should also include other SJW compounds to investigate their effects. Viability assays would likely include direct cell count and live-dead fluorescence assays. Inflammatory assays would likely include cell membrane integrity, metabolic function, and interleukin-8 assays. Findings from such studies would be beneficial for both antimycobacterial and cancer research.

2. Narrowing in on the mechanism of action for Tween 80 repression of Hfn inhibition of mycobacteria. Such studies would include treating a non-mycobacterial, Hfn-susceptible strain (such as \textit{B. subtilis}) with the same Hfn concentration and variable Tween 80 concentrations (similar to the study shown in Figure 4.21). A variation of this study that would use mycobacteria but other known mycobacterial antibiotics instead of Hfn should also be done to see if the Tween 80 effect is Hfn-specific. Antibiotics chosen should include those that affect the cell wall more directly (INH, EMB) and those that affect the cell in other ways (RIF, SM). Findings from this study may also contribute to discovering the Hfn mechanism of action if the Tween 80 Hfn repression is due only to modification of outermost layer of the cell wall.

3. Investigating the surfactant that is known to be present in the lungs (Foster \textit{et al.}, 1998) and assaying this surfactant (or surfactants other than Tween 80) in SJW
inhibitory assays against mycobacteria to see if the Tween 80 Hfn-repression effect seen with \textit{M. JLS} is specific to Tween 80. This would help determine if Hfn treatment of TB through inhalation is a viable option.

4. Conducting further studies to investigate the mechanism of action for SJW crude extract and Hfn in both mycobacteria and A549 cell inhibition/toxicity. Such studies would likely employ the use of microarray technology to understand genetic induction/repression with SJW exposure. Studies that assay for free mycolic acids or several other specific cell wall components may also be employed; such techniques have been used in determining the mechanisms of action with other mycobacterial antibiotics (Dover \textit{et al.}, 2008). Another method may be to compare SEM images of bacteria treated with other mycobacterial antibiotics to images of bacteria treated with SJW compounds.

5. Including binary or tertiary combinations of SJW compounds in assays that investigate their synergistic effects on both bacteria and lung cells. It is possible that a combination of compounds has a more potent inhibitory or bactericidal effect than Hfn alone. The SJW inhibitory studies presented in Chapter 4 showed a more rapid inhibition response with the crude extract than with pure Hfn, even if both had the same Hfn concentration; combination studies would be a first step to discovering the reason for this. No such study has been seen in literature.

6. Investigating the stability of Hfn in blood. Such understanding would be critical for correct dosage administration. It would also be one of the first steps of investigation for the possibility of using Hfn for metastatic cancer treatment.
7. Comparing the effects of SJW extracts of different organic solvents in more detail than was done in this study. One such solvent would be ethyl acetate, which was shown in this study to produce a lighter-colored extract and may make colorimetric assay a more feasibly option. As colorimetric assays are often less laborious than direct CFU or cell counting methods, this may make many aspects of SJW research more efficient.

8. Investigating the cause of trypsin-EDTA ineffectiveness in detaching A549 cells after treatment with the SJW crude extract. It may be possible that the MeOH extract contains a trypsin inhibitor, which may be useful for other biotechnology applications.

9. Developing an HPLC preparative method to obtain greater quantities of SJW active compounds. This would make pure compound studies possible in greater volumes of media, making concentration and volume variability due to evaporation less of an issue. It would also allow the assaying of higher concentrations of the pure compounds against bacteria and mammalian cells.

10. Refining the photoconversion/photodegradation parameters for SJW crude extracts, narrowing in on the Hpn/Phn-converting and Hfn-degrading wavelengths. As seen from the Hfn degradation study in Chapter 5, using specific light conditions are important to maintaining accurate concentrations. Knowing specific wavelengths at which degradation is avoided may eliminate unnecessary, burdensome precaution (such as working in overly dark conditions).
REFERENCES


APPENDIX
Appendix. Protocols
Luria (LB) Broth and Agar

**LB Broth**

LB broth 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 covered in foil and autoclaved at 121°C for 30 min. The broth was stored at room temperature.

**LB Agar**

If preparing agar LB for plates, 15 g Difco agar (BD, Fisher, Pittsburgh, PA) was added to the mixture above before autoclaving at the same temperature and time. For agar plates, the agar was gently swirled immediately after autoclaving and allowed to cool in a laminar flow hood to approximately 55°C. The agar was then poured into 100x15 mm Petri dishes (Fisherbrand, Fisher, Pittsburgh, PA) in aliquots of approximately 25 ml. After cooling for about 30 min with the lid cracked open to allow the plates to dry, the plates were placed in a bag and kept at 4°C until use.
Middlebrook (MB) 7H9 Broth and 7H10 Agar

MB Broth

To make MB 7H9 broth, 4.7 g of Difco Middlebrook 7H9 powder (BD, Fisher, Pittsburgh, PA) was suspended in 900 ml ddH2O (containing 2 ml glycerol or 0.5 g Tween 80, if desired) and autoclaved at 121°C for 10 min. When media had cooled to 45°C, 100 ml Middlebrook ADC Enrichment (BD, Fisher, Pittsburgh, PA) was added aseptically.

MB Agar

To make MB 7H10 agar, 19 g of Difco Middlebrook 7H10 powder (BD, Fisher, Pittsburgh, PA) was suspended in 900 ml ddH2O containing 5 ml glycerol. After mixing, this solution was heated until boiling and boiled for 1 min to dissolve the powder. It was then autoclaved at 121°C for 10 min. The agar was gently swirled immediately after autoclaving to resuspend the agar. After cooling to 50-55°C, 100 ml Middlebrook OADC Enrichment (BD, Fisher, Pittsburgh, PA) was added aseptically. Agar was then poured into plates in aliquots of approximately 25 ml. After cooling for about 30 min with the lid cracked open to allow the plates to dry, the plates were placed in a bag and kept at 4°C until use.
A549 Media Preparation

Nutrient mixture F-12 Ham’s media in powder form was obtained from Sigma-Aldrich (N3520) (St. Louis, MO). Each bottle was formulated for 1 L media. The powder from one of these bottles was added to 900 ml purified H₂O, and the residual powder from the bottle was rinsed and added to the solution. Sodium bicarbonate (NaHCO₃), 2.5 g, was added, and the pH was lowered to between 7.1 and 7.2 using 1N hydrochloric acid (HCl). Another 100 ml purified H₂O was added. This solution was then 0.2-µm filtered and stored at 4°C if it was not going to be used within two months (could be stored up to six months). If media was needed soon, fetal bovine serum (FBS) (Invitrogen) was added at 10% the final media volume along with 100X Penicillin-Streptomycin solution (10K U/ml and 10K µg/ml, respectively, from Thermo Scientific – Fisher) at 1% the final volume prior to use.
SEM Fixation Protocol

The cell fixation protocol used by Drs. TC and FenAnn Shen for the SEM images is as follows:

Reagents:

1. 2% buffered glutaraldehyde

   23.83 grams HEPES in 40 ml 50% glutaraldehyde. Bring to 1L with DI water.

   -OR-

   23.83 grams HEPES in 250 ml 8% glutaraldehyde. Bring to 1L with DI water. Bring pH to 7.0.

   Note: Can use 30.24 g PIPES or 21.402 g sodium cacodylate trihydrate (toxic, contains arsenic) in place of HEPES. PBS can also be used but leaves salt crystals.

2. 1-2% osmium Tetroxide

   4 ml 4% osmium tetroxide, 4 ml 0.4M HEPES (95.32 g in 1L DI water), and 8 ml DI water. pH to 7.0.

   Note: If PIPES is used in 2% buffered glutaraldehyde, then use 120.96 g PIPES in 1 L DI water. If cacodylate used, then use 85.608 g sodium cacodylate trihydrate in 1 L DI water.

3. 0.1M HEPES Buffer

   23.83 grams HEPES in 1 L of DI water. pH to 7.0

   Note: If PIPES used in 2% buffered glutaraldehyde, then use 30.24 g PIPES in 1 L DI water. If sodium cacodylate trihydrate is used, then use 21.402 g sodium cacodylate trihydrate in 1 L DI water.

4. 50%, 70%, 95%, and 100% EtOH
Protocol

1. Fix sample with 2% buffered glutaraldehyde. Fixative should be 10 to 20 times the volume of the sample. Let sit for 2 hr minimum.

2. Rinse with 0.1 M HEPES buffer 3 times for 5 min each with gentle agitation.

3. Post fix in 1-2% osmium tetroxide for 1 hr.

4. Rinse with 0.1 M buffer 3 times for 5 min each.

5. Alcohol series dehydration:
   - 50 % EtOH, 2 times for 10 min each with agitation.
   - 70 % EtOH, 2 times for 10 min each with agitation.
   - 95 % EtOH, 2 times for 10 min each with agitation.
   - 100 % EtOH, 3 times for 15 min each with agitation.

6. Keep in fridge until ready to dry and sputter coat.

7. Dry.
   
   **Option A:** Use a critical point dryer.
   
   **Option B:** Chemical drying
   
   1. (2 parts 100% EtOH : 1 part HMDS) for 15 min.
   2. (1 part 100% EtOH : 1 part HMDS) for 15 min.
   3. (1 part 100% EtOH : 2 parts HMDS) for 15 min.
   4. HMDS alone for 15 min, 3 times.
   5. Let the last HMDS evaporate in a fume hood overnight.

8. Mount samples on specimen stubs, sputter coat and view.
Notes

1. HMDS, glutaraldehyde, and osmium tetroxide are all toxic. They need to be put in separate bottles to be disposed.

2. The solid container that touches HMDS, glutaraldehyde, or osmium tetroxide needs to be put in a solid chemical waste box to be disposed.

3. HEPES is a buffer solution to wash off other chemicals without changing the pH values.

4. Glutaraldehyde is to stop biological process in an organism – chemical fixing.

5. Osmium tetroxide is to keep structure integrity when the organism is fixed.

6. HMDS is a chemical drying agent to remove water. Thus, the final step does not need water in the HMDS solution.