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With this study, we aimed to improve solubility and provide enhanced evaluation of antimicrobial activity with the addition of glycerol as a co-solvent with DMSO. The antimicrobial activities of glycerol and DMSO were evaluated against *Staphylococcus aureus*, and glycerol was found to be cause less growth inhibition. Compounds with poor water solubility were selected for biological screening and solubility testing. The glycerol/DMSO solution improved the time to solubilize (at 10mM) in ten out of the twenty-one tested compounds. Apparent assay solubility was assessed using turbidity measurements with a spectrophotometer at 600 nm. Notable improvement in solubility (where less light was blocked by lack of particulate matter) was observed for six compounds with glycerol/DMSO in media. To gauge our spectrophotometric analysis of solubility, we subjected six compounds to analysis with liquid-chromatography coupled to mass spectrometry. These analyses confirmed the solubility results obtained spectrophotometrically. Improved bioactivity with glycerol/DMSO/media against *S. aureus* was observed for four of the seven active compounds, resulting in minimum inhibitory concentrations (MIC) less than that of our standard water/DMSO/media solution. This study provides a rapid and effective way of assessing solubility in standard antimicrobial assay conditions and offers new solutions for improving solubility *in-vitro* by employing glycerol as a co-solvent.

GLYCEROL FOR IMPROVING SOLUBILITY AND EVALUATION OF ANTIMICROBIAL ACTIVITY FOR NATURAL PRODUCTS

by

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Approved by

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CHAPTER I

GLYCEROL FOR IMPROVING SOLUBILITY AND EVALUATION OF ANTIMICROBIAL ACTIVITY FOR NATURAL PRODUCTS

1.1 Introduction

An estimated 30% of all potential drug leads fail due to some type of pharmacokinetic issue, with solubility (falling under the absorption category of ADME) often being the hardest to overcome.[1] These solubility issues frequently present themselves early in bioassay screenings, where assay compositions are often limited to low concentrations of dimethyl sulfoxide (DMSO) in media to solubilize the target compound or extract.[2] DMSO has long been used in both academic and industrial practices for its ability to dissolve a diverse array of organic and inorganic compounds.[3] [4] However, its decomposition products (water, formaldehyde, methyl mercaptan, dimethyl sulfide, and dimethyl disulfide) can prove to be quite harmful in bioassay screenings and make long term storage of compounds dissolved in this solvent problematic.[3] Furthermore, there are a large number of compounds which are poorly soluble in DMSO (a common solvent for high throughput screening assays) that could potentially have interesting biological activity.[5] [6] [7] Frequently, compounds which are poorly solubilized by DMSO fall into two categories[3]: 1. The compound is composed of a strong crystalline lattice making it hard for DMSO to penetrate and solubilize. 2. The compound has a high molecular

weight (strong intermolecular forces, typically >500 MW), a high number of rotatable bonds[8] and high LogP values (a partition coefficient between octanol and water), of which violate Lipinski's rule of 5 for potential drug canidates.[2, 9]These potential downfalls make the addition of a co-solvent to help solubilize advantageous in biological assays. With improved solubility, compounds that previously may have been deemed inactive due to poor solubility might show new activity.

One potential co-solvent that could be used to improve solubility is glycerol. Glycerol, a naturally occurring sugar alcohol, has long been used as a solvent for preparing dietary supplements because of its sweet flavor and low toxicity. Glycerol is also used to preserve bacterial and mammalian cells prior to storage at low temperatures, and appears to be well tolerated at high concentration.[10] Despite these appealing attributes, glycerol is rarely, if ever, used as a solvent for *in vitro* biological assays. With these experiments, we set out to explore the potential effectiveness of glycerol as a solvent for improving solubility and biological activity of antimicrobial agents *in vitro*. In addition, we sought to develop a rapid strategy of identifying situations in which test compounds demonstrate poor solubility.

We chose to use *Staphylococcus aureus* as our model organism for bioassay screenings due to its medical relevance.[11] *S. aureus* is one of the leading causes of skin infections each year, infecting 80,000 people and killing 11,000 in 2011 alone, according to the CDC. In evaluating activity against *S*. aureus, we expect that some

chemical classes will demonstrate poor solubility in standard assay conditions, and as a result to present poor minimum inhibitory concentration (MIC) values. Our expectation is the glycerol will improve the solubility of these compounds, resulting in improved biological activity.

Often with these bioassay screenings, the small plates and well sizes can make it hard to visually see particulate. Thus, the application of a robust analytical method to diagnose solubility issues would be very valuable in a high throughput screening scenario. The two standard methods of determining solubility rely either on high performance liquid chromatography (HPLC) or spectrophotometry.[12] HPLC offers the ability to distinguish contaminates and degradation products that spectrophotometers cannot, but at the cost of efficiency.[12] Due to the speed at which spectrophotometers can generate data, they are an optimal choice for high throughput screenings. Work done by Chen using a 96-well plate format in a multi-wavelength plate reader offered a rapid method of determining solubility in plates already used for biological testing, but still required filtering of the plate before reading the absorbance values.[13]

We sought out a slightly different approach of determining solubility using turbidimetric studies that are typically done in nephelometry[14], but in a 96-well format with a plate reader concurrent with our biological assay readings. Lipinski has shown turbidimetric approaches using wavelengths at 600 nm or greater can be used to quickly identify compounds with poor solubility, though his methods

differed by looking at solubility in a buffered solution using a dedicated diode array UV detector. [9] Absorbance at a wavelength of 600 nm is already widely used to monitor bacterial growth in antimicrobial assays.[15] We propose that a similar approach (monitoring absorbance at 600 nm) could be effective for evaluating compound solubility. Compounds that have poor solubility (i.e crashing out) should result in a high absorbance due to light scattering by undissolved particles. The experiments described herein explore the application of absorbance readings in a 96 well plate format as a means to diagnose poor solubility, and demonstrate the effectiveness of this approach for comparing the ability of various solvent combinations to dissolve biologically active natural products.

CHAPTER II

RESULTS AND DISUCSSION

2.1 Growth Inhibition by DMSO and Glycerol

As a first step in these studies, a comparison was made between the effects of DMSO and glycerol on growth of a *Staphylococcus aureus* culture. Figure 2.1 shows a comparison of solvents (DMSO and glycerol) with various percentage compositions in broth grown with *Staphylococcus aureus*. Overall, the inhibitory effect of glycerol in comparison to DMSO was found to be much lower (Figure 2.1). At low concentrations (<4%) of glycerol, a slight increase in bacterial growth in comparison to the vehicle (broth with bacteria). As concentrations of glycerol were increased bacterial growth was inhibited and at 32%, glycerol was found to be completely inhibitory. By contrast, DMSO was found to be inhibitory at levels as low as 1%, and completely inhibitory at 16%. The data presented in Figure 2.1 confirm our assumption that glycerol is much less antimicrobial than DMSO, making it an appealing co-solvent.

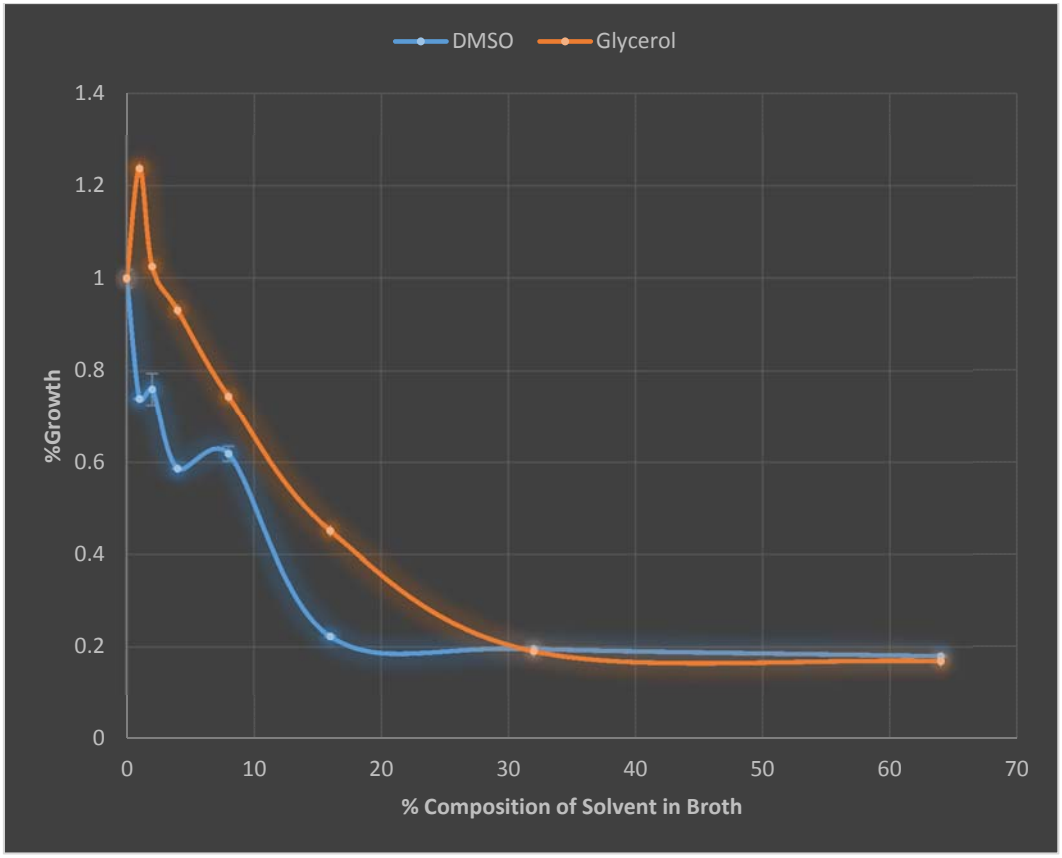

Figure 2.1 Comparison of Growth Inhibition by Glycerol and DMSO. A comparison of the effects of DMSO and glycerol on growth of *Staphylococcus aureus* in broth. The vehicle control containing neither solvent was normalized to 100% growth and subsequent solvent compositions were compared to this number. Glycerol caused less growth inhibition than DMSO making it an ideal co-solvent.

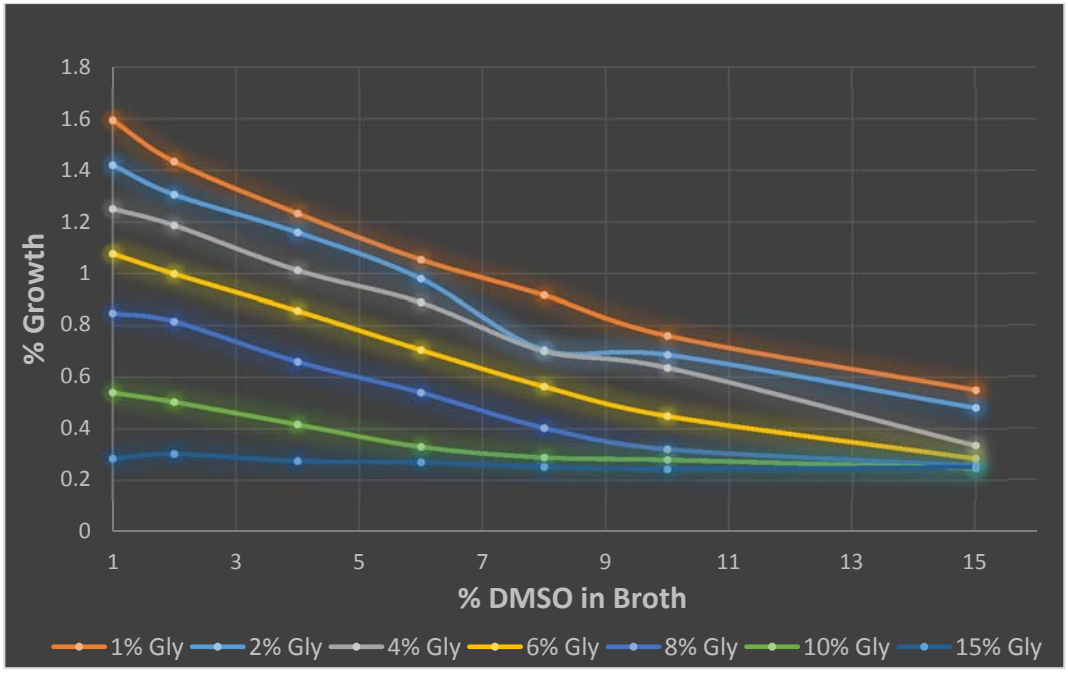

Figure 2.2 %DMSO in Varying Concentrations of Glycerol vs *S. aureus.* Combination studies of glycerol and DMSO at varying concentrations versus *S. aureus*. As DMSO levels in glycerol increased, we see a decrease in the growth of the bacteria.

Additional experiments were conducted to evaluate the combined effects of glycerol and DMSO on growth of *Staphylococcus aureus* (Figure 2.2). At low levels of either solvent combined, bacterial growth was mostly uninhibited. At combined concentrations totaling 8% (e.g. 2% glycerol with 6% DMSO) or more, a decline of growth is observed, until complete inhibition at 16% or more. To ensure there was no synergistic effect of the solvents against the bacteria, an isobologram was plotted as shown in Figure 2.3.

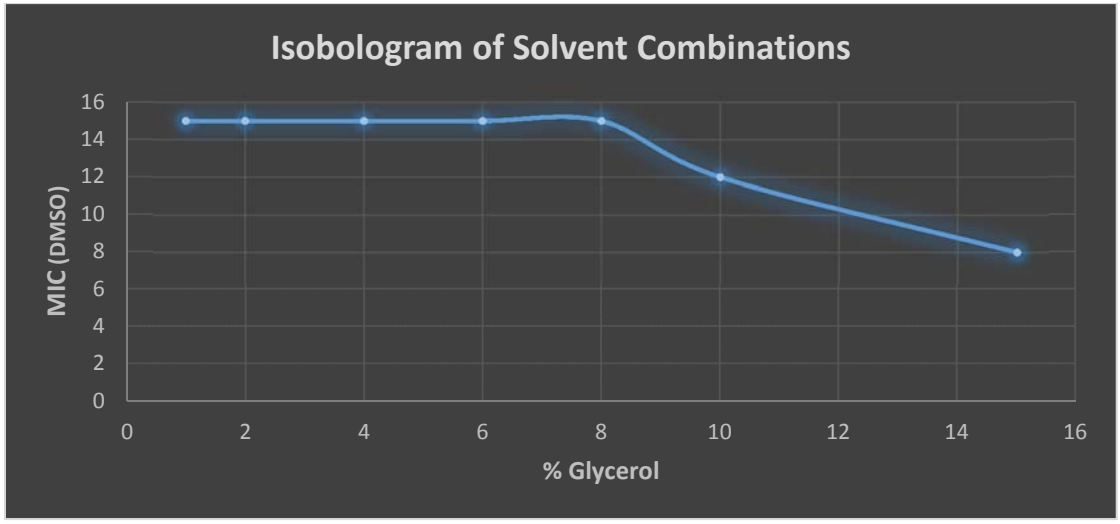

Figure 2.3. Isobologram of Solvent Combinations. An isobologram of the combinatorial studies of glycerol and DMSO inhibition of *S. aureus*.

Isobolograms are frequently used to determine if potential drugs (or solvents in this case) yield greater inhibition of the bacteria in combination than their individual effects could achieve.[16] This could be problematic as any deviation in assay conditions could yield results not indicative of bacterial suppression by a compound but instead of the solvents. At low concentrations of glycerol, high amounts of DMSO were required to completely inhibit the bacteria, as indicated by the isobologram. This was true up to 10% glycerol where we begin to see a linear decrease in the amount of DMSO required. This linear trend in the isobologram demonstrates that there is no synergistic effect between the two solvents and that their influence on bacterial growth is additive.[16] Using the results from Figures 2.2 and 2.3, a concentration of 2% DMSO and 2% glycerol was chosen for our assay, which is a standard amount of DMSO for antimicrobial studies.[11, 17] To compare the

effectiveness of this new co-solvent composition to the commonly employed broth composition, we chose a control containing 2% DMSO with 2% water.

2.2 Solubility Analysis

A series of compounds representing common natural products were chosen as the focus of these studies (Figure 2.4). Primarily, compounds known in the literature to be poorly soluble[18], (1, 2, 4, 5, 12, 14, 15, and 17) were chosen with the intention of evaluating how solubility could be improved with the addition of glycerol as a co-solvent. Additional compounds were selected to reflect common classes of natural products (3, 6, 7, 8, 9, 10, 11, 12, 13, 16, 18, 19, 20, 21). We aimed to test from as many families as possible to ensure our method of detecting solubility problems was more conclusive and not limited to one set of compounds. The experiments described in the next sections show comparisons of various parameters related to solubility of these compounds between 2% DMSO/2% glycerol and 2%DMSO/2% water (in Müeller-Hinton broth).

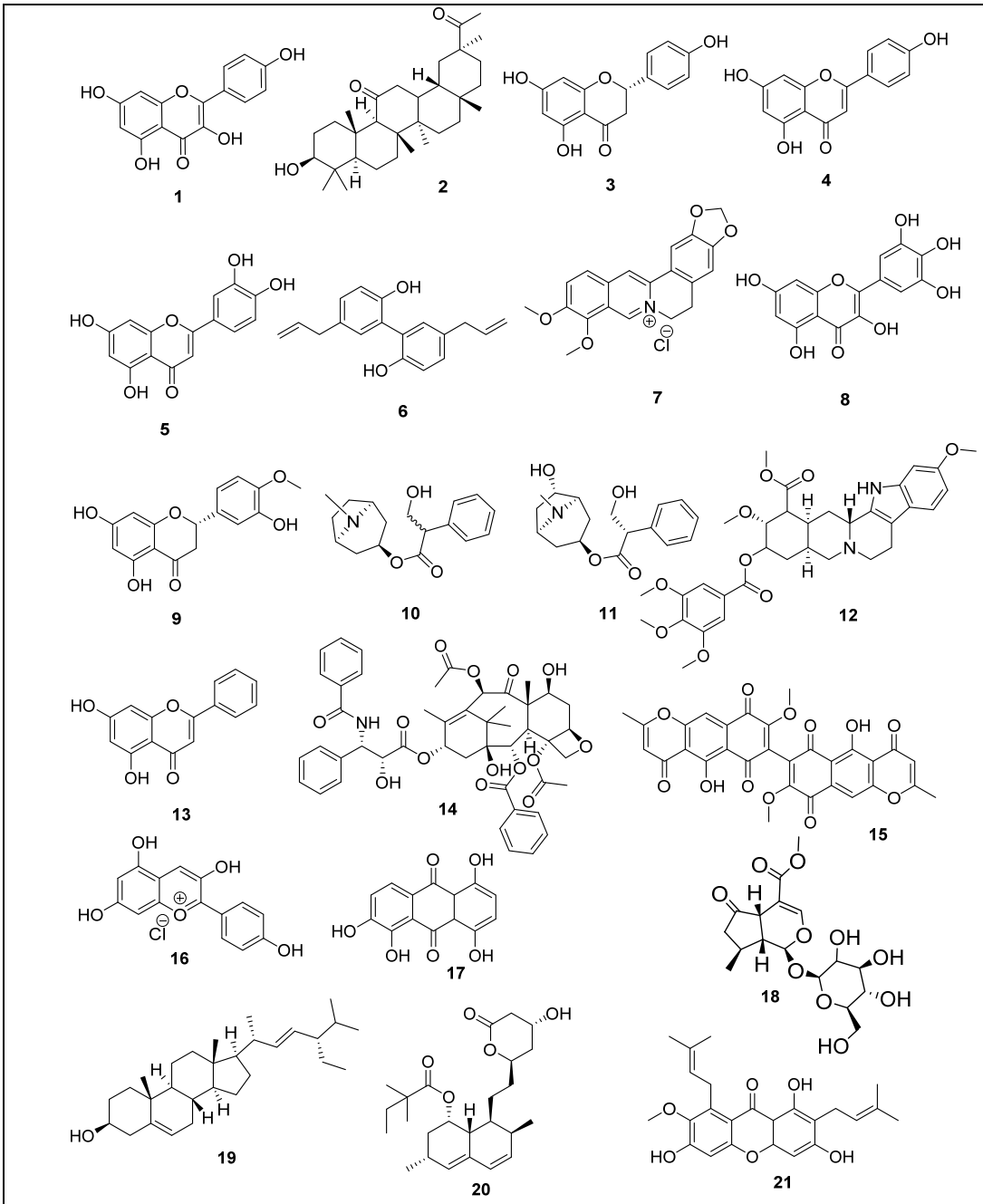

Figure 2.4. Tested Compounds. kaempferol (**1**) enoxolone (**2**) naringenin (**3**) apigenin (**4**) luteolin (**5**) magnolol (**6**) berberine (**7**) myricetin (**8**) hesperetin (**9**) atropine (**10**) anisodamine (**11**) reserpine (**12**) chrysin (**13**) Taxol (**14**) aurofusarin (**15**) pelargonidin (**16**) quinalizarin (**17**) cornin (**18**) stigmasterol (**19**) simvastatin (**20**) α-mangostin (**21**)

2.2.1. Time to Solubilize

To begin comparison of solubility, we chose to examine the length of time it took each compound to solubilize in each test solution. Time to dissolve compounds prior to biological assay can be very lengthy, and we expected that the DMSO/glycerol solution might speed this up in comparison to DMSO/water.

Our findings in Table 2.1 show DMSO/glycerol decreased the amount of time required to solubilize 10 out of the 21 standards employed here by >20 seconds. While many of these compounds did eventually solubilize in the DMSO/water solvent, the amount of time and effort (heating, sonicating, vortexing) was usually less with the glycerol/DMSO solvent composition. It was also noted that in compounds with poor solubility in standard preparation (i.e. time to solubilize >9minutes) in both solvents (12, 13, 15, 19), the glycerol aided in dispersing the compound throughout the vial, while in water/DMSO, the compounds tended to aggregate in clumps (Figure 2.5).

Table 2.1 Comprehensive Solubility Data

Structure	Family	MIC in Glycerol /DMSO[A]	MIC in Water/ DMSO[A]	Dissolving Time Glycerol/ DMSO	Dissolving Time Water/ DMSO	OD600 Glycerol/ DMSO[A] (400µM)	OD600 Water/ DMSO[A] (400µM)
(1)	Flavonol	75 µM	75 µM	1.30 min	3.30 min	0.273 ± 0.019*	0.204 ± 0.012
(2)	Terpenoid	250 µM	>400 µM	52 sec	9+ min	0.039 ± 0.016*	0.506 ± 0.002
(3)	Flavanone	>400 µM	>400 µM	46 sec	47 sec	0.001 ± 0.002	0.005 ± 0.003
(4)	Flavone	400 µM	>400 µM	50 sec	9+ min	0.292 ± 0.144	0.412 ± 0.076
(5)	Flavone	200 µM	350 µM	1.50 min	1.56 min	0.050 ± 0.011*	0.399 ± 0.010
(6)	Lignan	100 µM	100 µM	24 sec	1.06 min	0.000 ± 0.000	0.003 ± 0.002
(7)	Alkaloid	400 µM	400 µM	1.06 min	1.15 min	0.002 ± 0.001	0.000 ± 0.001
(8)	Flavonol	>400 µM	>400 µM	1.10 min	1.20 min	0.008 ± 0.000	0.008 ± 0.002
(9)	Flavanone	>400 µM	>400 µM	37 sec	1.05 min	0.000 ± 0.000	0.001 ± 0.002
(10)	Alkaloid	>400 µM	>400 µM	51 sec	1.1 min	0.000 ± 0.000	0.001 ± 0.002
(11)	Alkaloid	>400 µM	>400 µM	35 sec	40 sec	0.003 ± 0.001	0.004 ± 0.004
(12)	Alkaloid	>400 µM	>400 µM	9+ min	9+ min	0.056 ± 0.018	0.021 ± 0.006
(13)	Flavone	>400 µM	>400 µM	9+ min	9+ min	0.418 ± 0.018*	0.102 ± 0.008
(14)	Isoprene	>400 µM	>400 µM	3.50 min	9+ min	0.360 ± 0.063	0.479 ± 0.025
(15)[B]	Dimeric naphthoquinone	>100 µM	>100 µM	9+ min	9+ min	0.056 ± 0.010*	0.243 ± 0.006
(16)	Anthocyanidin	>400 µM	>400 µM	30 sec	33 sec	0.015 ± 0.000*	0.035 ± 0.003
(17)	Anthroquinone	>400 µM	>400 µM	2.02 min	2.35 min	0.326 ± 0.064*	0.467 ± 0.015
(18)	Iridoid	>400 µM	>400 µM	35 sec	34 sec	0.002 ± 0.001*	0.007 ± 0.002
(19)	Steroid	>400 µM	>400 µM	9+ min	9+ min	0.019 ± 0.002	0.033 ± 0.010
(20)*	Statin	>400 µM	>400 µM	1.07 min	2.10 min	0.072 ± 0.027	0.010 ± 0.003
(21)	Xanthone	6 µM	12 µM	1.17 min	5.50 min	0.056 ± 0.008	0.052 ± 0.004

* -Statistically significant difference in OD600 between DMSO/water and DMSO/glycerol at the 90th percentile

[A] - Müeller-Hinton broth included

[B] – Aurofusarin was tested at 100 µM due to limited supply

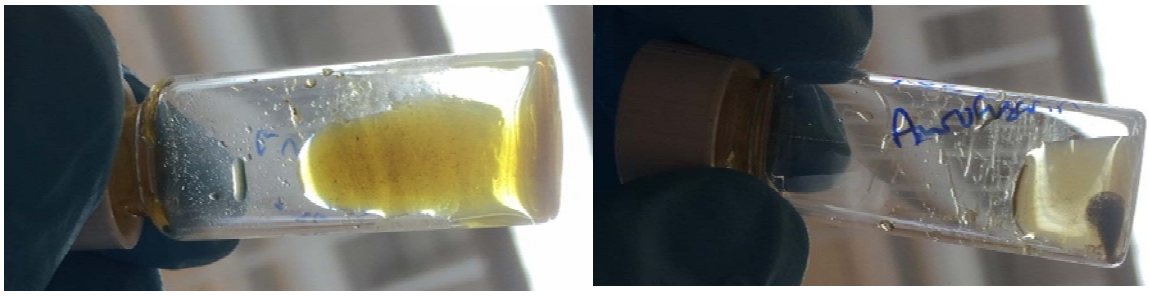

Figure 2.5. Comparison of Aurofusarin in Solvents. – Aurofusarin standard dissolved in glycerol/DMSO (left) and water/DMSO (right).

2.2.2. Spectrophotometric Method to Compare Solubility

We evaluated the effectiveness of a spectrophotometric method to compare the solubility of each compound in the two solvent systems. Particulate matter (due to poor solubility) should cause light scattering, and lead to an apparent increase in absorbance at 600 nm (OD600).

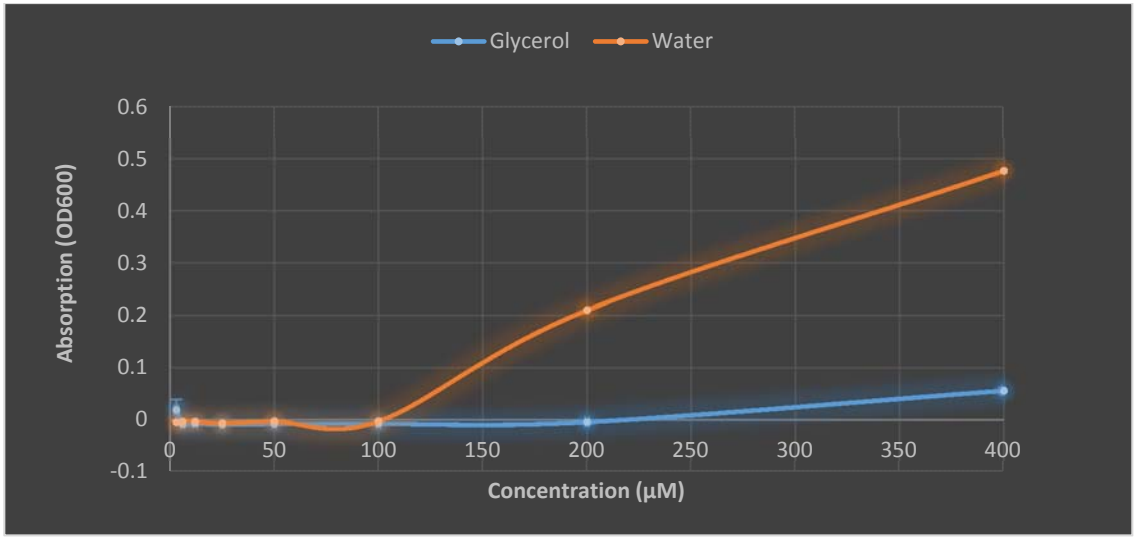

Figure 2.6. Solubility Analysis of Enoxolone. As concentration increased, we see an increase in absorbance for the DMSO/water solvent indicating poor solubility.

One such example is shown in Figure 2.6, where the 2% DMSO/water solution of enoxolone resulted in absorbance readings that were significantly higher than that of the same concentrations dissolved in 2% DMSO/glycerol. We attribute these high readings to the compound crashing out of solution, thus partially blocking some light resulting in a higher absorbance reading. We chose to record absorbance values at the highest concentration in Table 2.1 as this point tended to vary the most and would allow for an easy comparison in solvent composition. Absorption data comparison at the highest concentration tested (400μM) revealed the general trend that the glycerol/DMSO combination improved the solubility of six (2, 5, 15, 16, 17, 18) of the compounds and reduced solubility for three compounds (1, 13, 20).

One potential downside of using a spectrophotometric approach to compare solubility is the chance of potential false positives. It is important to note the non-linear form of the absorbance for the compound crashing out in the solubility graph (Figure 2.6). It is possible that linear increases in absorbance versus concentration could occur not because of particulate matter in the solution, but due to actual absorbance of light by the compound under investigation. This would be true if the compound absorbs light at 600 nm.

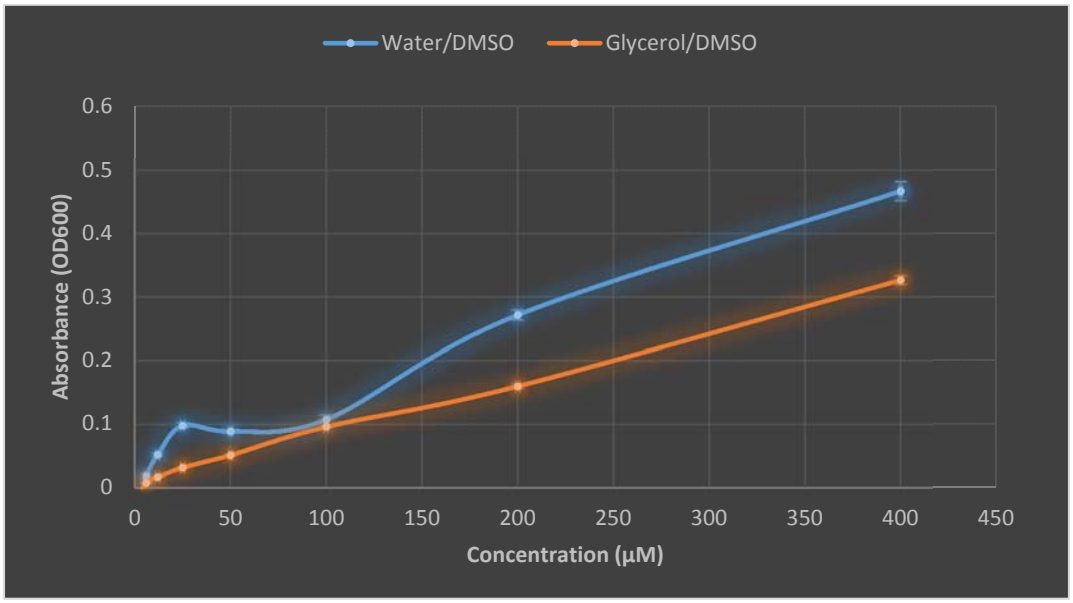

Figure 2.7. Solubility Analysis of Quinalizarin. Both solvent compositions resulted in a linear increase with respect to concentration. We theorize the compound is absorbing the light and following Beer's law.

Quinalizarin was one compound tested that we hypothesize gives a false solubility reading due to light absorbance (Figure 2.7). Quinalizarin has a deep red color, and absorbs in the 600 nm range (Figure 2.8). Thus, the data suggesting

solubility issues with this compound (Figure 2.7) are suspect. In order to minimize false reports in our absorbance analysis, readings from ~260 to 800 nm were collected at 400µM for each solvent condition to ensure there was no evident peak at or near 600 nm. A peak here could cause higher absorbance readings which could be misconstrued to be a poorly soluble compound such as with quinalizarin. In most cases however (for example, enoxolone in Figure 2.9), no absorbance peak at 600 nm was observed.

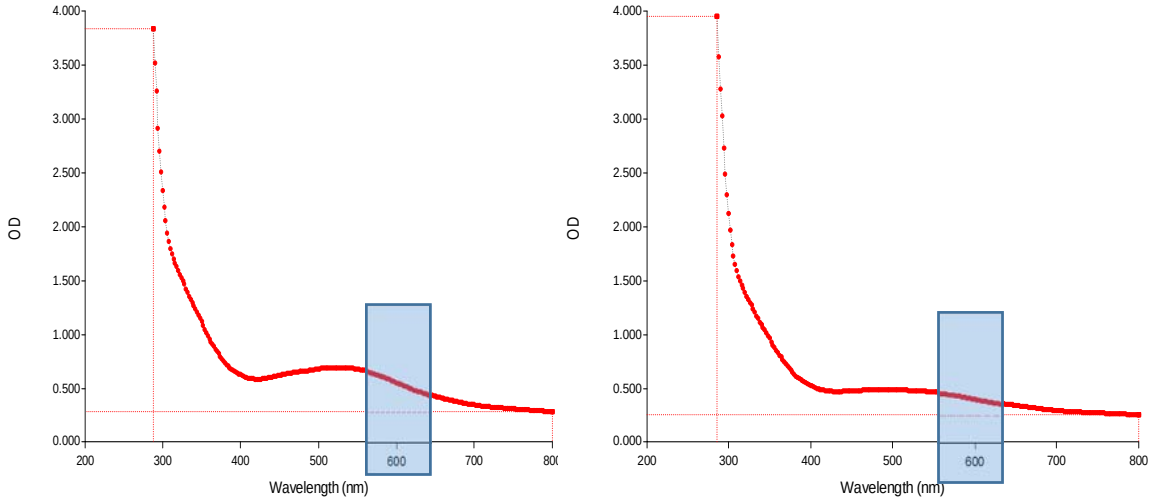

Figure 2.8. Quinalizarin Absorbance Spectrum. DMSO/glycerol/media left and DMSO/water/media right – a small increase in absorbance is found near 600 nm where plates are read.

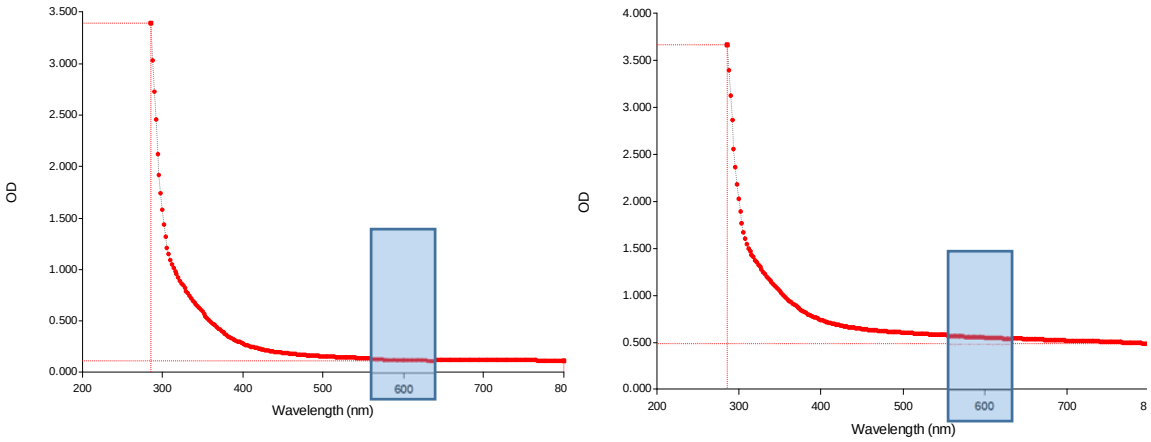

Figure 2.9. Enoxolone Absorbance Spectrum. DMSO/glycerol/media left and DMSO/water/media right – no peaks were found near the 600 nm region.

2.2.3. Comparison of Solubility with LC-MS

To confirm that our spectrophotometric approach of testing solubility was effective, standard practice of filtering the samples and subjecting them to LC-MS to compare overall peak areas of each compound in solution was conducted.

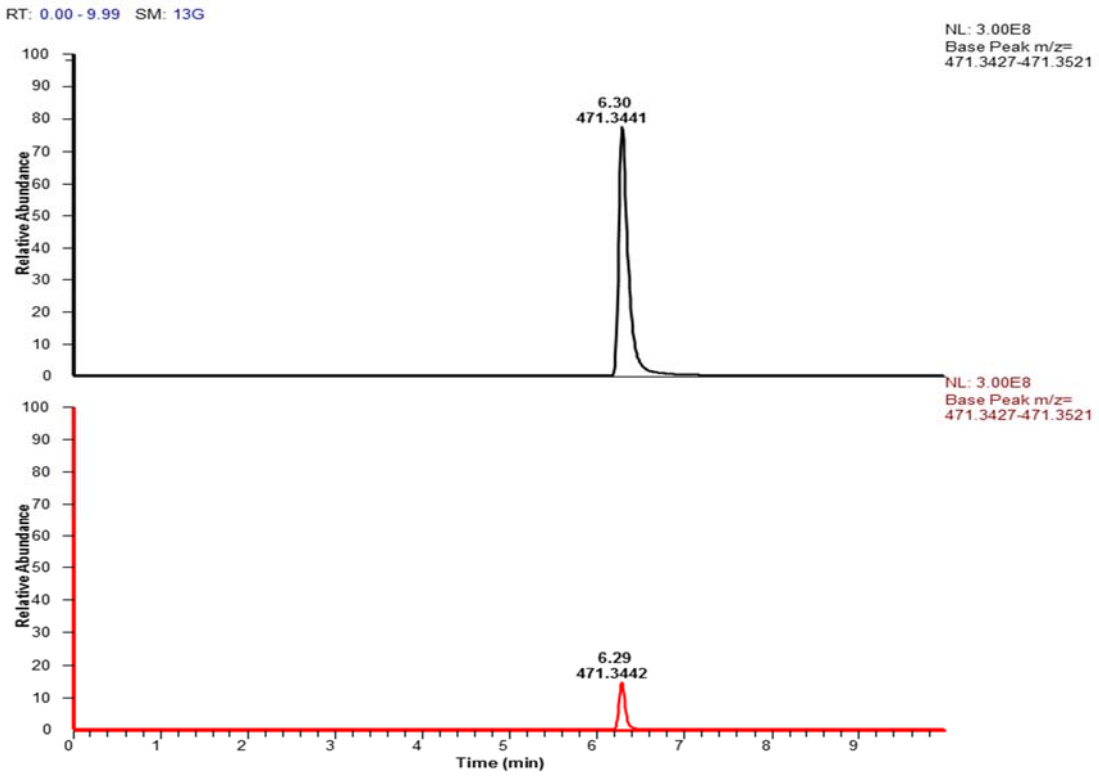

Figure 2.10. LC-MS Selected Ion Chromatogram of Enoxolone. DMSO/glycerol (top) and DMSO/water (bottom) at 400µM. The DMSO/glycerol peak area was 7.8 times larger than that of the DMSO/water, indicating the compound was more soluble in the glycerol solution.

In the case of enoxolone (Figure 2.10), the DMSO/water absorbance was higher than that of the DMSO/glycerol solution (0.506 vs 0.039 respectively) (Table 2.1). The high absorbance well readings are a direct result of insoluble compound. The insoluble portions were then filtered off and the filtrate was compared using LC-MS. The peak areas of these same wells show that enoxolone is more concentrated in the DMSO/glycerol solution, as the compound was in solution and not crashed out. These results are comparable to the spectrophotometric results for enoxolone and select compounds tested in Table 2.2.

Table 2.2 LC-MS Selected Ion Chromatogram Peak Area Comparisons with Standard Error			
Compound (400μM)	**Glycerol/DMSO Peak Area**	**Water/DMSO Peak Area**	**Analogous Results to Spectral Data**
Apigenin	$1.12 \times 10^7 \pm 2.79 \times 10^5$	$1.18 \times 10^7 \pm 2.13 \times 10^5$	Yes
Magnolol	$2.22 \times 10^7 \pm 2.82 \times 10^6$	$1.85 \times 10^7 \pm 3.57 \times 10^6$	Yes
Anisodamine	$1.69 \times 10^{10} \pm 7.83 \times 10^8$	$1.89 \times 10^{10} \pm 2.65 \times 10^8$	Yes
Simvastatin*	$2.47 \times 10^7 \pm 7.28 \times 10^5$	$4.53 \times 10^7 \pm 1.98 \times 10^6$	Yes
Enoxolone*	$1.76 \times 10^9 \pm 4.39 \times 10^7$	$2.07 \times 10^8 \pm 3.85 \times 10^6$	Yes
Aurofusarin[a]*	$7.62E \times 10^5 \pm 2.83 \times 10^4$	$5.08 \times 10^5 \pm 3.84 \times 10^4$	Yes
*Statistically significant difference between peak areas in the two solvents at the 90th percent confidence interval based on student's t-test. a- Tested at 100 μM due to supply			

These parallel results confirm that our spectrophotometric readings are a viable way of determining solubility of compounds in our assay. One potential downside of LC-MS verification of solubility as opposed to spectrophotometric studies is the requirement that your compound is ionizable. We expected quinalizarin would have had a slight difference in peak areas between solvents but were unable to ionize with electrospray ionization (ESI). Compounds that don't ionize well with standard ESI practices require different sources and method development which could hinder high throughput screenings. Additionally, reading with a plate reader takes approximately 30 seconds as compared to a 10 minute LC method required for separation using MS.

2.3 Antimicrobial Testing

Antimicrobial activity was evaluated against *S. aureus* with all of the compounds in Table 2.1 in both a DMSO/glycerol/broth and DMSO/water/broth solution. We expected that with improved solubility of the compounds, we would observe enhanced activity of bioactive compounds against the bacteria.

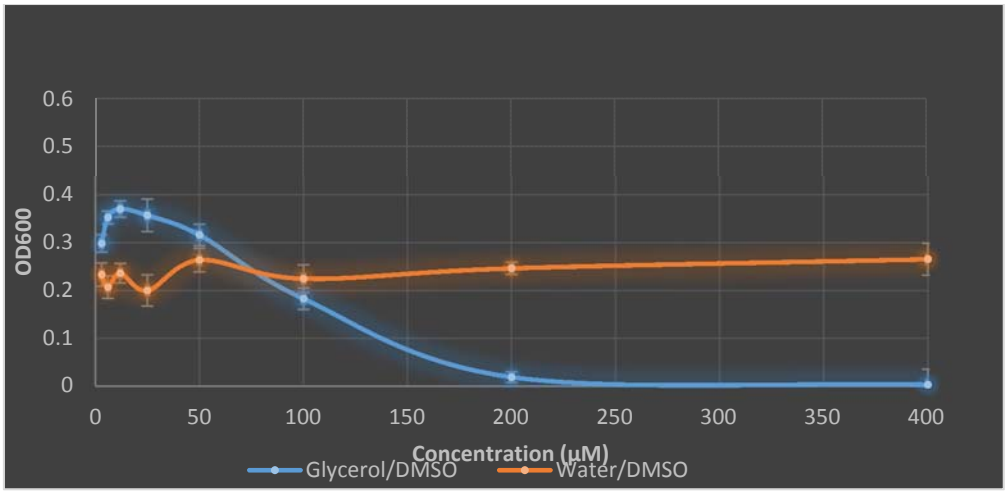

Figure 2.11. Minimum Inhibitory Concentration Studies of Enoxolone. We demonstrate that with poor solubility in water/DMSO, no activity was found for the compound. When solubilized with glycerol/DMSO, we see a standard MIC curve with a reported MIC of 250 µM.

Using enoxolone as an example (Figure 2.11), no activity was observed for the DMSO/water solution of the compound as indicated by the flat line of bacteria optical density. Without solubility testing, one would mistakenly report this compound as inactive. By contrast, the DMSO/glycerol solution demonstrated a standard minimum inhibitory concentration (MIC) of 250 µM proving the compound is bioactive. Our studies found that of the bioactive compounds,

DMSO/glycerol improved the ability to observe biological activity (i.e. reduced MIC values) for 4 (2, 4, 5, 6) out the 7 active compounds.

CHAPTER III

EXPERIMENTAL

3.1 Standard Preparation

Standards were ordered from from Sigma Aldrich (3, 7, 8, 9, 10, 11, 12, 13, 16, 17, 18, 19, 20, 21), Selleckchem (1, 2, 4, 5, 6), Acros-organics (14), Bioviotica (15) and were suspended to 10 mM using either 50/50 (v/v) DMSO/glycerol (Fisher Scientific) or DMSO/nanopure water in vials. A timer was then started and the standards were vortexed first to see if the compound would easily dissolve. In the event of poor solubility, the compounds were placed in sonicator to break up clumps, then a hot water bath for a short period and vortexed again. After solubilizing to the best of our ability, serial 2-fold dilutions were performed from 10 mM to 0.78 μM for each standard.

3.2 Antimicrobial Testing

Overnight seed cultures were diluted 1:3 with Müeller-Hinton broth and shaken for 2 hours at 300 rpm. An additional dilution to normalize the colony forming units (CFU) for each plate was performed to give a final dilution of 1.0×10^5 CFU/ml based on OD_{600} of 0.118 *S. aureus* after the 2 hours of shaking. Evaluation of antimicrobial testing was performed utilizing a 96-well sample plate setup in which 10 uL of each compound was transferred into a well containing 190 uL of broth and

50 uL of bacteria (SA1199 *Staphylococcus aureus*). Final concentrations of compound ranged from 400 μM to 3μM in triplicate. The plates were then shaken at 1000 rpm at 37 ˚C in a Stuart S1505 microtitre plate shaker for at least 18 hours. Following incubation, plates were read using a Synergy H1 Mutli-Mode Reader at 600 nm to obtain absorption spectra. A blank plate's optical density readings containing no bacteria (240 uL broth and 10 uL compound) was then subtracted from the sample plate to give the OD600 of the bacteria alone.

3.3 Solubility Analysis

Using the blank plate, solubility of each well concentration (400μM to 3μM) was evaluated by subtracting out the absorbance values of a 2% DMSO/glycerol or 2% DMSO/water control to find the absorbance value of the compound in question. These OD600 values were then plotted against concentration for comparison of solvents. The blank plate was also analyzed at 400 μM across multiple wavelengths from 280 nm to 800 nm to determine if there was an observable absorbance peak near the 600 nm range. The 400 μM wells were then vacuum filtered and analyzed via LC-MS (CH3CN/H2O (with 0.1% formic acid) 15-100% CH3CN over 10 minutes) using a Q Exactive Plus Hybrid Quadropole-Orbitrap coupled to an Acuity ultra-high performance liquid chromatography (UPLC) system. Selected ion chromatograms for each compound were then compared.

CHAPTER IV

CONCLUSION

In conclusion, this project was developed in order to quickly assess compound solubility within our bioassays as well as improve solubility with trouble compounds with the addition of glycerol as a co-solvent. Our studies found glycerol was suitable co-solvent with DMSO for improving solubility in bioassay screenings of *S. aureus*. It also found of the 21 compounds tested, 10 which showed improvement in the time it took to solubilize, 6 showed solubility enhancements in bioassays, and 4 showed improvements in MICs. These findings show we now have a rapid way to assess our solubility and offer a novel solution to solubility problems which may arise.

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