Selective screening of natural antibiotics through lead discovery

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Abstract: The rise in drug resistant bacteria generates challenges for researchers, doctors, and patients. While drug and pharmaceutical companies continue to focus on developing synthetic drugs, a growing number of academic communities are beginning to revitalize a traditional lead discovery process for discovering new drug candidates. The work reported in this study establishes a two-path, parallel approach. We will assist in the search for unique active compounds. The first path incorporates extraction, isolation, and purification of known antibiotics from bacterial sources. Through various techniques, this phase of the project ensures the proper selection and characterization of the different classes of antimicrobial compounds. The second path creates a robust screening methodology for potentially active compounds. Through activity screens, such as zone of inhibition testing and phytochemical screening, these complementary procedures provide information toward the antimicrobial activity and chemical class of a compound. With the information gained from the second path, proper extraction methodologies may be constructed to promote selectivity of active compounds during each step in the isolation process of unknown samples. Although identification of novel compounds through lead discovery proves challenging, the development of systematic screening and isolation procedures provides a solid framework for identifying and isolating new drug candidates.

1 Introduction

The rise of bacterial resistant pathogens originates from the overuse of broad-spectrum antibiotics in human medicine, veterinary medicine, and agricultural settings.[1–3] Once a pathogen becomes resistant to an antibiotic, the antibiotic responsible for combating that pathogen exhibits no effect. In other words, that antibiotic is now useless. Common bacterial resistant pathogens known as ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter spp.*) cause the greatest number of infections in US hospitals because of their ability to "escape" antibacterial drugs.[4] In 2005, infection from methicillin-resistant *S. aureus* (MRSA) caused approximately 19,000 patient deaths in the US, surpassing the number of deaths caused by HIV/AIDS.[5] Later, in 2013, a report by the CDC estimated two million US citizens are infected by antibiotic-resistant pathogens, resulting in a minimum of 23,000 deaths. With the amount of effective antibiotics dwindling, the need for novel antibiotics continues to rise at a global crisis rate.

Alexander Fleming's discovery of penicillin in 1928 sparked the antibiotic age of medicine. Fleming found penicillin effective against all Gram-positive pathogens. These pathogens promote diseases, such as scarlet fever, pneumonia, gonorrhea, and meningitis.[6] As soon as the antibiotic age began, a number antibiotic families with an enormous range of structural and functional features have been discovered through both natural sources and synthetic methods.[7–9] But, with the ever increasing number of antibiotic resistant bacteria, communities from across the globe must resort beyond the traditional chemical families shown in **Figure 1**.[10]

Researchers continue the search for new antibiotics and with some success. One approach utilizes starting materials with no connection to known chemical families, in hopes of synthesizing new families of antibiotics. For example, Delamanid, from the nitroimidazole class of drugs containing a 5-nitroimidazole group, was developed by Otsuka Pharmaceutical and entered phase III of clinical trials to target mycolic acid biosynthesis.[11] Another approach derives new antibiotics from a semi-synthetic method. Using the core structure of known antibiotic classes, alteration of the supporting functional groups provides new antibiotic capabilities. Within the aminoglycoside family, Plazomicin proves effective against the Gram-negative bacteria carbapenem-resistant Enterobacteriaceae and MRSA.[12] Additional antibacterial agents approved for clinical use include, but are not limited to, Plazomicin (Achaogen, Inc.) from the aminoglycoside family, Cilastatin (Merck & Co. Inc.) from the beta-lactam family, and Nemonoxacin (TaiGen Biothechnology Co. Ltd.) from the fluoroquinolones family.[13] Although the synthesis of new antibiotics is possible, a large portion of active antibiotic microbials remain undiscovered in nature.

Herein, we report a lead discovery-based project focused on the process of collecting, screening, extracting, isolating, and charactering biologically active compounds from natural sources. To identify naturally occurring antibiotics, the chosen screening techniques include inhibition testing and phytochemical screening.[14,15] The combination of both inhibition and phytochemical screening provides two separate indications of antibiotic activity. First, inhibition screening relies on qualitative analysis to determine the effectiveness of an antimicrobial agent.[16] In a sterile agar plate, bacteria or fungi are spread evenly on the plate. Following an incubation period, which allows the pathogen and antimicrobial compounds to interact, the formation of an inactive zone around the antimicrobial reveals the effectiveness of the antimicrobial. The size of the zone suggests the strength and potency of the agent. Second, phytochemical screening serves as an additional qualitative analysis method. The phytochemical method uses a library of known chemical reactions to help identify specific chemical families, with examples of these families shown in **Figure 1**.[14,17] For example, after various preparation steps and the addition of sodium hydroxide, a solution containing a compound within the flavonoid family will appear pink. Beyond

flavonoids, different tests are necessary to identify different chemical families. Combining both inhibition and phytochemical screening ensures complementary analysis throughout the screening process, requires minimal cost, and can be performed quickly.

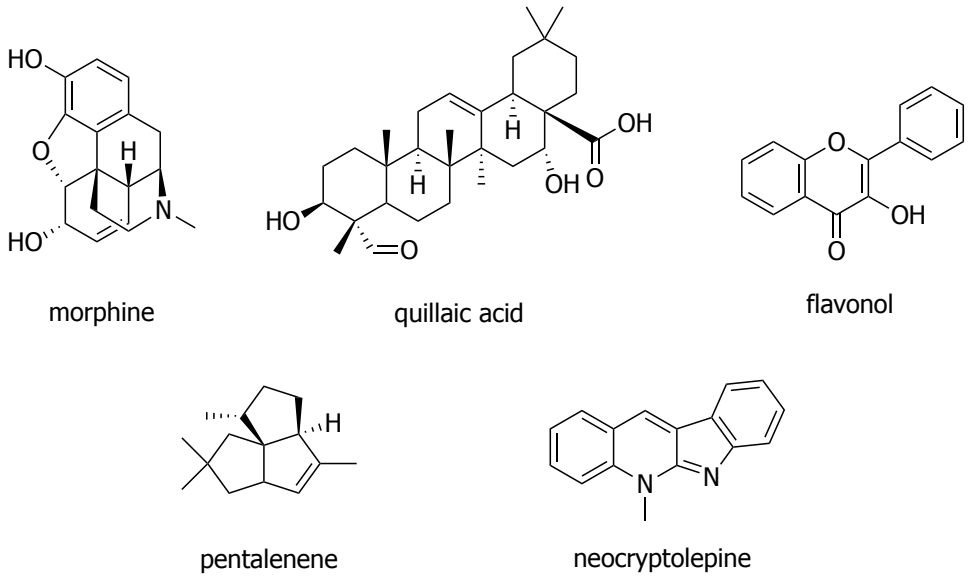

Figure 1. A sample of antibiotic compounds from the alkaloid (*morphine)*, saponin (*quillaic acid*), flavonoid (*flavonol*), terpenoid (*pentalenene*), and phenolic (*neocryptolepine*) families.

Having discussed the screening techniques that will assist in identifying active antimicrobial properties and chemical families, the process of extraction, purification, isolation, and characterization must now take place. Extraction typically relies on polarity, size, and chemical functionality of the compound in order to be separated from a complex solution. Based on the information provided from inhibition and phytochemical screening, extraction and purification steps can be determined. For example, an early process for extracting and purifying penicillin required liquid-liquid extraction using two immiscible solvents and pH adjustments in order to alter the functionality of the molecule, gradually isolating the compound.[18] Following isolation, the compound must be characterized based on its structure and chemical properties. Characterization relies mainly on mass spectrometry (MS), and other techniques include Nuclear Magnetic Resonance spectroscopy (NMR) and Fourier Transform Infrared spectroscopy (FT-IR). MS accounts for the most commonly used technique in regards to characterization of antimicrobial variants, meaning both NMR and FT-IR serve a largely supporting role in characterization.[19–22]

This study focuses on finding new drug candidates by leveraging the large number of antimicrobial compounds already being secreted by bacteria in nature. Although the characterization of a single active compound through lead discovery proves challenging, the development of systematic screening procedures will help guide researchers through the process of isolating unknown samples. A general outline for the lead discovery process is shown in **Figure 2**. The fight against antibiotic resistant bacteria will continue to be waged on many fronts. It is through the lead discovery process that the greatest source of novel antibiotics may be discovered in natural products, and that lead discovery screening may reveal the greatest implications against antibiotic resistant bacteria.

Figure 2. A five-step sequential outline of the lead discovery process for the collection, screening, extraction, and characterization of a possible drug candidate from an unknown source.

2 Materials and Methods

MATERIALS

Pseudomonas fluorescens, *Streptomyces griseus*, and *Bacillus subtillus* were purchased from Carolina Biological Supply Company. *Escherichia coli* was provided from a freezer stock sample in the Bethel University Biochemistry Department. Solvents, agar, petri dishes, and culture materials were also provided by Bethel University.

METHODS

Zone of inhibition methodology: The methods consisted of a two-system approach. The initial methodology developed was to establish a standard procedure to determine the antibiotic activity levels of known antibiotic containing compounds. This specific zone of inhibition method follows the well-known Kirby-Bauer guidelines.[23] The inhibition of *Escherichia coli* using *Pseudomonas fluorescens* as the active agent follows as an example.

Using a sterile cotton swab, *Escherichia coli* was collected from the culture media. The sample was then spread on an agar plate by streaking the plate three times, rotating the plate approximately 60 degrees with each application. The introduction of *Pseudomonas fluorescens* began with "hole punching" and sterilizing circular disks from filter paper. The disks were then impregnated with 10 μL *Pseudomonas fluorescens* after being cultured for 48 h and centrifuged to concentrate the active components in the solution. Once prepared, the *Pseudomonas fluorescens* disks were then transferred onto the *Escherichia coli* agar plates. Following incubation at 35 ºC for 24 h, the samples were assessed for antibiotic activity based on the absence of growth surrounding the filter paper disks.

Isolation methodology: The second path of the two-system approach involved the extraction and isolation of active compounds from the starter culture. The extraction and isolation of pseudomonic acid (mupirocin) will follow as an example.[24]

Pseudomonas fluorescens was grown in a submerged culture of nutrient broth. After 48 h of growth, the addition of 0.5% w/v barium chloride and centrifugation removed large solids from the solution. Using isobutylmethyl ketone (IBMK), the solution was concentrated through stepwise partitions. All acidic and basic partition steps were adjusted using concentrated and dilute HCL and NaOH. The pH was confirmed via litmus paper. On a 200 mL scale, the centrifuged solution was initially brought to a pH of 4.5. The solution was then partitioned with 40 mL of IBMK. The IBMK layer was then transferred to 160 mL of water at pH 8.5. The water layer was collected, brought to a pH of 4.5 and partitioned with 50 mL of IBMK. Following the third partitioning, the IBMK layer was evaporated under reduced pressure until approximately ¼ of the IBMK remained. A final partition with 50 mL of water at pH 8.5 was completed. The water was then brought to a pH of 7, and the sample was ready for concentration.

3 Results and Discussion

Various factors contribute to the antibiotic activity of a cell strain. Some strains naturally produce antibiotics under standard conditions. Natural antibiotic production typically arises as the cell secretes waste. Other strains produce antibiotics as a secondary response. Under conditions of high stress or limited resources, cells begin to compete for survival against other cell types. As either a defense mechanism, or an energy saving mechanism, cells will secrete antibiotics to inhibit competitors from consuming environmental resources. For this reason, researches have conducted studies assessing antibiotic production based on resource availability, competitive environments, and inhospitable conditions.[25] The culture media selected for cell growth is shown in **Table 1**.

Table 1. Recommended and selected cell growth media for various known antibiotic producing cell strains.

Culture Broth	Cell Strain	Cell Growth
Nutrient Broth	*S. greiges*	NO
Nutrient Broth	*P. fluorescens*	YES
Nutrient Broth	*B. subtillus*	YES
Nutrient Broth	*E. coli*	YES
ISP2	*S. greiges*	NO
ISP2	*B. Subtillus*	NO
Luria-Bertani Broth	*E. coli*	YES

Although recommended, *Streptomyces greiges* did not exhibit signs of cell growth in ISP 2 culture media. It was also did not grow in the nutrient broth medium, which was selected as the standard medium for growing cell cultures. The ISP 2 and nutrient broth media likely did not possess sufficient nutrients for the growth of the *Streptomyces greiges* strain. As a Gram-negative cell strain, similar to *Pseudomonas fluorescens*, it is unlikely the presence of competing factors contributed to the inability of *Streptomyces greiges* to survive.

The largest portion of this project focused on the development of a zone of inhibition procedure. As a measure of the presence and activity of antibiotic producing cell strains, the zone of inhibition protocol establishes a qualitative and quantitative approach for determining antibiotic activity. As displayed in **Table 2** and visually represented in **Figure 3**, zone of inhibition trials were conducted using the known antibiotic producing cell strains of *Pseudomonas fluorescens*, *Streptomyces griseus*, and *Bacillus subtillus*. From the data, only *Pseudomonas fluorescens* suggested possible antibiotic activity against *Bacillus subtillus*. Additionally, *Bacillus subtillus* and *Streptomyces griseus* displayed no signs of antibiotic activity against the *Escherichia coli* cell strain. These were again, known antibiotic producing cell strains that were expected to possess the ability to ward off the *Escherichia coli* genus. The difficulty in generating a clear zone of inhibition, as shown in **Figure 3,** likely stems from the inability to produce a concentrated sample of antibiotics secreted by the active cell strains. To achieve more conclusive results, steps are being made to introduce competitors in the culture media to initiate secondary response antibiotic production. Also, extraction and isolation steps may provide more concentrated samples.

Table 2. *Pseudomonas fluorescens* revealed slight signs of antibiotic producing activity, while *Streptomyces griseus* and *Bacillus subtillus* failed to display activity based on the zone of inhibition (ZOI) assessment.

Streptomyces griseus Activity (against *Escherichia coli*)

Plate	ZOI Method	Amount Added (µL)	ZOI Present
A	Direct	10	NO
B	Filter Paper	10	NO
C	Direct	25	NO

Bacillus subtillus Activity (against *Escherichia coli*)

Plate	ZOI Method	Amount Added (µL)	ZOI Present
D	Direct	10	NO
E	Filter Paper	10	NO

Pseudomonas fluorescens Activity (against *Bacillus subtillus*)

Plate	ZOI Method	Amount Added (µL)	ZOI Present
F	Direct	10	Slight
G	Filter Paper	10	Slight
H	Centrifuged & Filter Paper	10	Slight

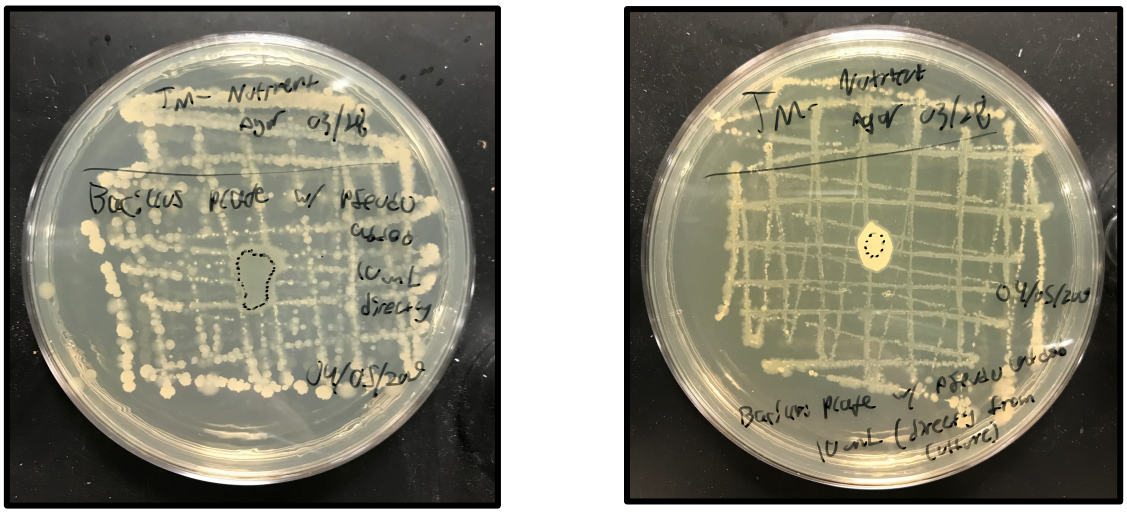

Figure 3. *Bacillus subtillus* agar plates exposed to *Pseudomonas fluorescens*. Image of 10 µL *Pseudomonas fluorescens* added directly to the agar plate (left). Image of *Pseudomonas fluorescens* added to filter paper prior to application (right).

The second main focus of this project was to initiate the process of creating a robust extraction protocol for various chemical families. As show in **Figure 4**, a general outline for the extraction, concentration, and isolation of antibiotic compounds from cell culture is depicted.

Proof of isolation through characterization is yet to be confirmed. In future work, confirmation methods will appear in the form of the already discussed zone of inhibition and phytochemical screening methods, as well as instrumentation methods such as MS, FT-IR, and NMR analysis.

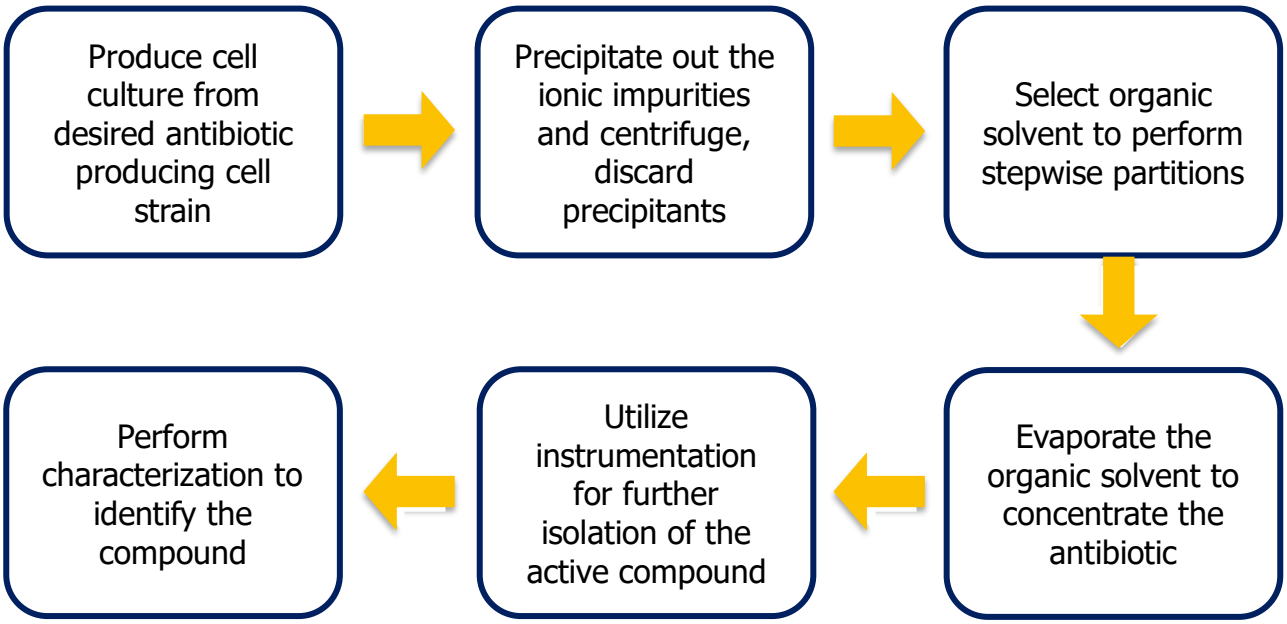

Figure 4. A general outline for the concentration, isolation, and characterization for antibiotic compounds from cell cultures. This outline constitutes the second path of the study.

4 Conclusions

The two goals of this project include (1) the development of a robust screening methodology and (2) the identification of a new active antimicrobial compound through extraction of an unknown sample. Although the discovery of a new antimicrobial may prove as the most rewarding piece of the project, the methods provide a larger impact. With the two-path approach, screening procedures generate key insight toward making well informed decisions regarding the activity, chemical family, and separation steps of the unknown sample. Improvements in zone of inhibition clarity and cell culture growth are required to continue the development of this project. And, the efforts from this project in obtaining a robust, in-house lead discovery system will be an important resource for any laboratory.

Although researchers face extreme challenges, it is essential that the scientific community continues the search for naturally occurring antibiotic compounds. In the face of the current COVID-19 pandemic, the possibility exists that nature has already adapted to combat the virus. Synthetic methods will struggle to keep pace with the evolution of antibiotic resistant microorganisms. Meaning, that nature will always be a source of inspiration for researchers dedicated to the discovery of antimicrobial compounds.

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