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Bioactive Secondary Metabolites in Cave Dwelling Fungi

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BIOACTIVE SECONDARY METABOLITES IN CAVE DWELLING FUNGI

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Abstract

Water samples were collected in May 2022 from WTH lake and silent lake located in Wind Cave National Park, South Dakota. From the water samples collected, 8 pure fungal colonies were isolated. All samples underwent chemical extraction, growth inhibition testing, proton-nuclear magnetic resonance spectroscopy (^{1}H NMR), and high-pressure liquid chromatography (HPLC). Three pure colonies also underwent DNA extraction, and sequencing of the ITS region for genus level identification. These samples were able to be identified to the genus level, and for these three samples were: *Talaromyces, sp., Linnemannia, sp., and Gliomatsix, sp.* The secondary metabolite that showed antimicrobial activity from *Talaromyces, sp.* was determined to be flavomannin A. The secondary metabolites of samples were of interest due to the increasing demand for novel compounds obtaining antimicrobial properties for use in the production of new antibiotics to combat the increasing issue of antibiotic resistance.

Introduction

Living organisms produce both “primary” and “secondary” metabolites. Primary metabolites are necessary to an organisms survival, as well as ubiquitous to life and include proteins, carbohydrates and nucleic acids. Secondary metabolites however, are unique to various species and help to catalyze reactions for particular organisms. These secondary metabolites are naturally produced by an organism however, they are not linked to reproduction or growth of the organism. Secondary metabolites can serve as an organism’s competitive weapons, metal transporting agents, or as sexual hormones among other functions[1]. Secondary metabolites often have differing uses for humans than typically seen in the organism they originate from.

A common example of a secondary metabolite with a differing use for humans than from where it originates, is nicotine. Nicotine comes from the plant *Nicotiana tabacum* and accumulates majorly in the plants leaves by means of a chemical barrier against herbavores[2]. This chemical is poisonous to small organisms such as bugs that might try to consume the plant leaves. However, nicotine is typically used as a stimulant drug and is the main psychoactive ingredient in tobacco products such as cigarettes.

The discovery of new secondary metabolites is increasingly more important due to the issue of antibiotic resistance. Antibiotic resistance is a pathogens ability to resist antibiotic treatment towards bacterial infections. The reasoning for the ongoing increase in antibiotic resistance today is majorly due to misuse of antibiotics, not taking the full number of antibiotics prescribed to rid you of a bacterial infection[3]. This increases the development of mechanisms for bacteria to use in order to decrease the medication’s efficiency

1. Demain, Arnold L., and Aiqi Fang. "The natural functions of secondary metabolites." History of modern biotechnology I (2000): 1-39.

2. Shitan N, Hayashida M, Yazaki K. Translocation and accumulation of nicotine via distinct spatio-temporal regulation of nicotine transporters in Nicotiana tabacum. Plant Signal Behav. 2015;10(7):e1035852. doi: 10.1080/15592324.2015.1035852. Erratum for: doi: 10.1371/journal.pone.0108789. PMID: 26251879; PMCID: PMC4622871.

3. Bush, K., Courvalin, P., Dantas, G. et al. Tackling antibiotic resistance. Nat Rev Microbiol 9, 894–896 (2011). https://doi.org/10.1038/nrmicro2693

over time. Bacteria becoming increasingly more resistant to antibiotics makes treatment costs higher as well as viruses more dangerous to human health. The discovery of new secondary metabolites would lead to the development of new, more productive antibiotics that these pathogens aren't resistant towards. This research study focused on the discovery and identification of novel secondary metabolites that originate from fungal species that could aid in the production of new antibiotics that aren't as resistant to strains of infections, ideally these new compounds could act through novel mechanisms to which the microbe lacks a pathway to resistance. More specifically, gram-negative bacteria are known to be more resistant against antibiotics than gram-positive bacteria making the discovery of secondary metabolites resistant to gram-negative bacteria the most crucial to this research[3]. Previously established research deals with the identification of these bacteria resistant metabolites, however this research project is important because it deals with a unique ecosystem not being actively focused on by other groups currently.

Identification of fungal samples have been previously accomplished by use of PCR and sequencing of the fungal DNA. In this method, the ITS region of the fungal DNA is amplified during PCR and used as a unique "fingerprint" for identification of the fungal genus[4]. Among the regions of the ribosomal cistron, the internal transcribed spacer (ITS) region has the highest probability of successful identification for the broadest range of fungi, with the most clearly defined barcode gap between the inter- and intraspecific variation[5]

4. Schoch, Conrad L., et al. "Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi." Proceedings of the National Academy of Sciences 109.16 (2012): 6241-6246.

5. Schoch, Conrad L., et al. "Nuclear Ribosomal Internal Transcribed Spacer (ITS) Region as a Universal DNA Barcode Marker for *Fungi*." *Proceedings of the National Academy of Sciences*, vol. 109, no. 16, 2012, pp. 6241–6246., https://doi.org/10.1073/pnas.1117018109.

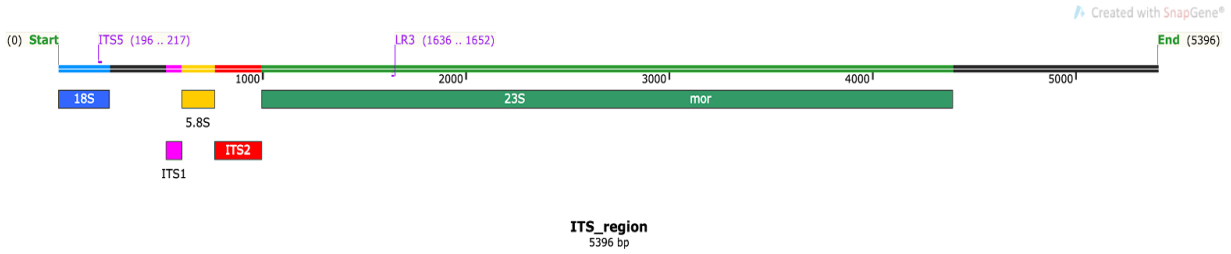

Figure 1. ITS region sequenced for fungal identification[4]

The amplified sequenced DNA "fingerprint" is put into a database of a collection of websites used to gather most likely genus "hits" to previously identified DNA chains. These are great at narrowing down a sequence of DNA to the genus due to the fact that there is a lot of data out there for known fungal species and their DNA in the ITS region, so you get long chains of sequences that can exactly match a chain of a known fungus.

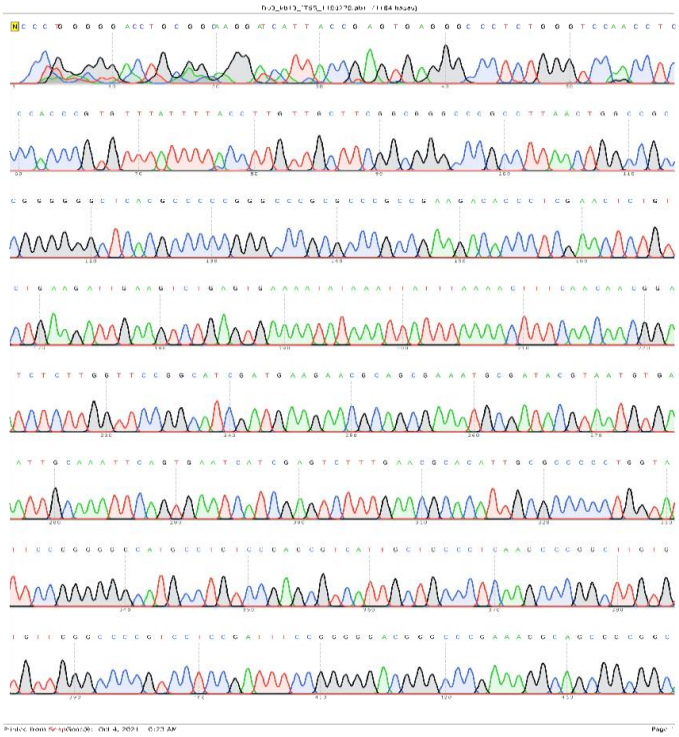

Image 1. A raw chromatogram of a fungal DNA sequence

PCR and sequencing are great tools for identification of a fungus to the genus level; however, there are limitations to definitive species identification. One study suggests that these limitations exist since up until recently it was common practice in mycology to name both the asexual and sexual stages of the same fungus with a different name (termed dual nomenclature), which caused confusion[5]. It is an important tool for fungal identification, but more specifically in fungal colonies that have been previously identified. Previous identification of fungus following PCR and sequencing have been accomplished via similar methods but this research aimed for the identification of novel fungal genus and species from the location of Wind Cave, South Dakota due to the limited amount of research previously done on fungal samples from within this cave. The ideal goals for this research were the growth of purified fungal colonies that went through DNA amplification and sequencing but came back with no or minimal matching hits in the databases meaning the discovery of a novel genus and/or species.

Sample collection

Wind Cave National Park, South Dakota is used for the sample collection location in this research due to an ongoing funding for the project by the Iowa Space Grant Consortium, a division of the National Aeronautics and Space Administration (NASA)[*]. This project spans a large range of majors at University of Northern Iowa including the Chemistry and Biochemistry, Biology, Digital Media, Earth and Environmental Science departments. Wind Cave, South Dakota is of interest for these projects due to the unique ecosystem not preciously discovered leading to the possibility of the discovery of novel fungal species and antimicrobial compounds. Wind Cave is longer than 150 miles in total and the samples for research collected

prior to December 2022, have been taken from less than 1% of this entire cave length as only about 10% of the entire predicted caves passageways have even been charted to date.

This research is a continuation of a project started in May of 2021 which successfully collected and identified 18 unique fungal samples from within Wind Cave. These fungal samples collected in 2021 were obtained from "Calcite Lake" in Wind Cave, SD. Of these fungal samples, DNA isolation allowed for the identification of the two fungal samples belonging to the fungal genus *Pseudogymnoascus*[6]. This fungal genus is one known to cause white nose rot in bat species. Of the samples analyzed in 2021, the structure of one secondary metabolite was identified to be of the genus *Penicillium*[5]. Certain species of the fungal genus penicillium are commonly found in the antibiotic penicillin.

The location WTH Lake where three water samples were collected for this project is a relatively isolated location within the cave, with limited contamination as less than 200 people have ever made it to the site of this sample location. This leaves a lot of opportunity for uncontaminated samples to grow fungal colonies that have been within the cave for many years potentially leading to the discovery of new fungi, and secondary metabolites that have never been isolated from other locations. A map of the full passageways known within Wind Cave, South Dakota can be seen below in image 1.

6. Raja, Huzefa A., et al. "Fungal identification using molecular tools: a primer for the natural products research community." Journal of natural products 80.3 (2017): 756-770.

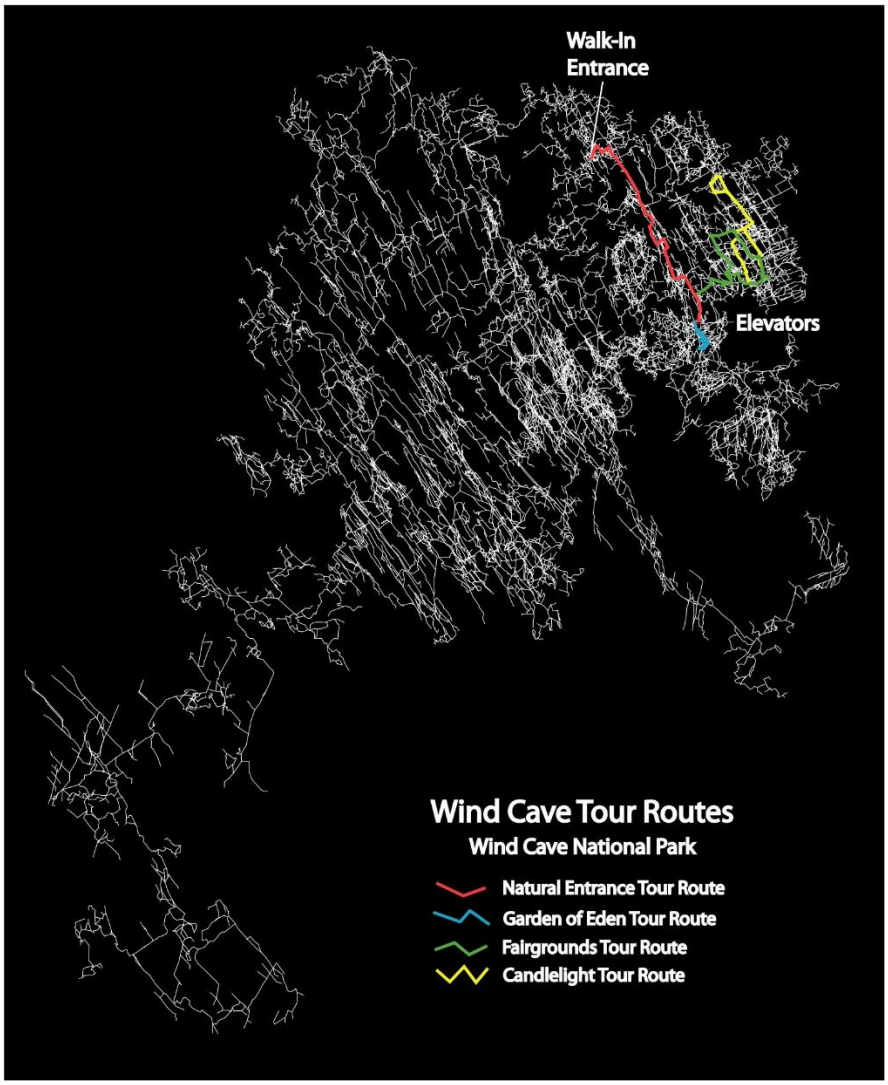

Image 2. A map of the current passageways known within Wind Cave, National. The colored lines depict the routes open to tour groups.

Experimental

To begin this research, a team of researchers traveled to Wind Cave, South Dakota in May of 2022 to collect water samples. Water samples were collected using sterile technique from two locations within Wind Cave: 3 samples from WTH Lake and 2 samples from Silent Lake. These water samples were collected without disturbing the sediment around the water collection site. After collection of the water samples, they were transported back to the lab in Cedar Falls, Iowa for plating. Each water sample was plated on 2 different mediums to ensure fungal growth, Potato Dexter Agar (PDA) and Yeast Peptone Dextrose (YPD). Duplicates of each sample and plate will be created in order to grow one fungal sample on each medium, one in light and one in the dark.

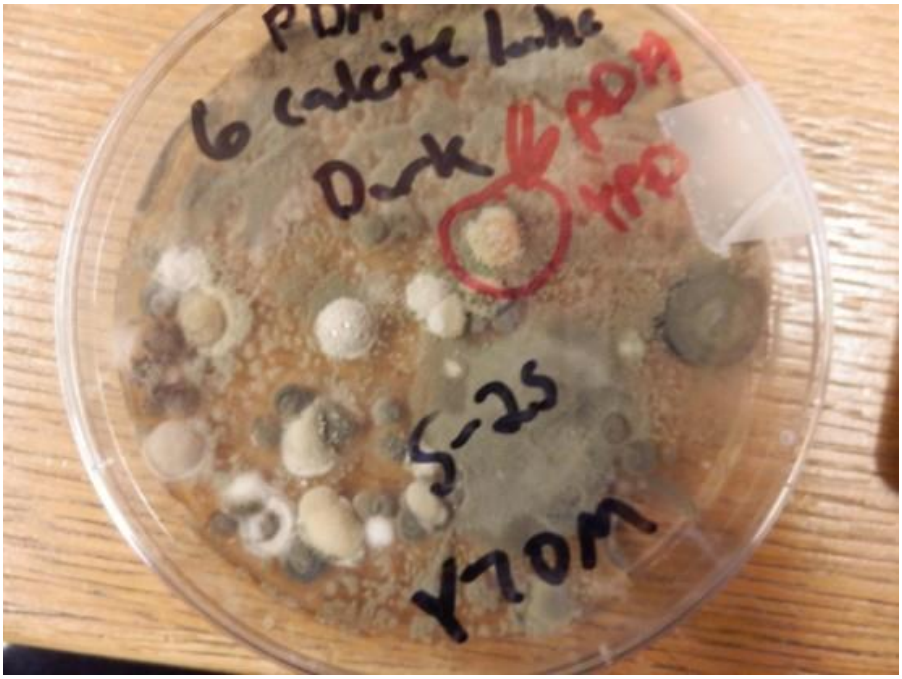

Image 3. A PDA plate with multiple fungal colonies needing to be isolated and purified.

These plates were set aside for 1-3 weeks to allow all types of fungi to grow. Once fungus had grown on the PDA and YPD plates, there were multiple types of fungus and bacteria on each plate that needed to be individually isolated. These multiple fungi were separated out by color, growing pattern and texture of the fungus itself. To separate the fungi, a sterile inoculating loop was used to transfer fungal spores from individual fungal colonies to new PDA and YPD plates in order to isolate pure fungal colonies which were again grown in duplicates, light and dark. The process was repeated until each set of PDA and YPD had pure fungal colonies with no signs of visible contamination, and could then move onto further isolation steps.

Image 4. Purified fungal samples NASA 30-46 grown on PDA and YPD plates that are ready to be grown on a rice culture.

Once the pure fungal samples were grown on PDA and YPD, the next step was to prepare the samples for extraction of the secondary metabolites by growing them on a rice culture. The rice was soaked in water overnight in a large Erlenmeyer flask, before being placed in the autoclave

for sterilization. Once the rice had been sterilized and cooled, a sterile inoculating loop was used to cut out a piece of either PDA or YPD with the pure fungal colony before adding the piece to the rice bed, and giving it a gentle shake to spread the fungal spores. The openings of the flasks containing the rice colonies were then covered with aluminum foil and wool, and allowed to grow for another 3 weeks, or until the fungus had covered the entire bed of rice.

Image 5. Purified fungal colonies grown on rice cultures for extraction

Post- rice growth, the fungal colonies were ready for the secondary metabolites to be extracted by means of an extraction sequence, a modified version of the Kupchan scheme[6]. To begin this extraction sequence, a 1:1 ratio of 50mL chloroform and 50mL methanol was added to the rice colony before using a large metal spatula to break up the rice and fungus to have the mixture in

contact with the secondary metabolites. This mixture was then placed on a shaker overnight. Once the mixture was taken off the shaker, 45 mL of chloroform and 75mL of water were added prior to vacuum filtration of the mixture. The remaining fungus and rice were discarded, and the filtrate was added to a separatory funnel. The work flow strategy can be seen below.

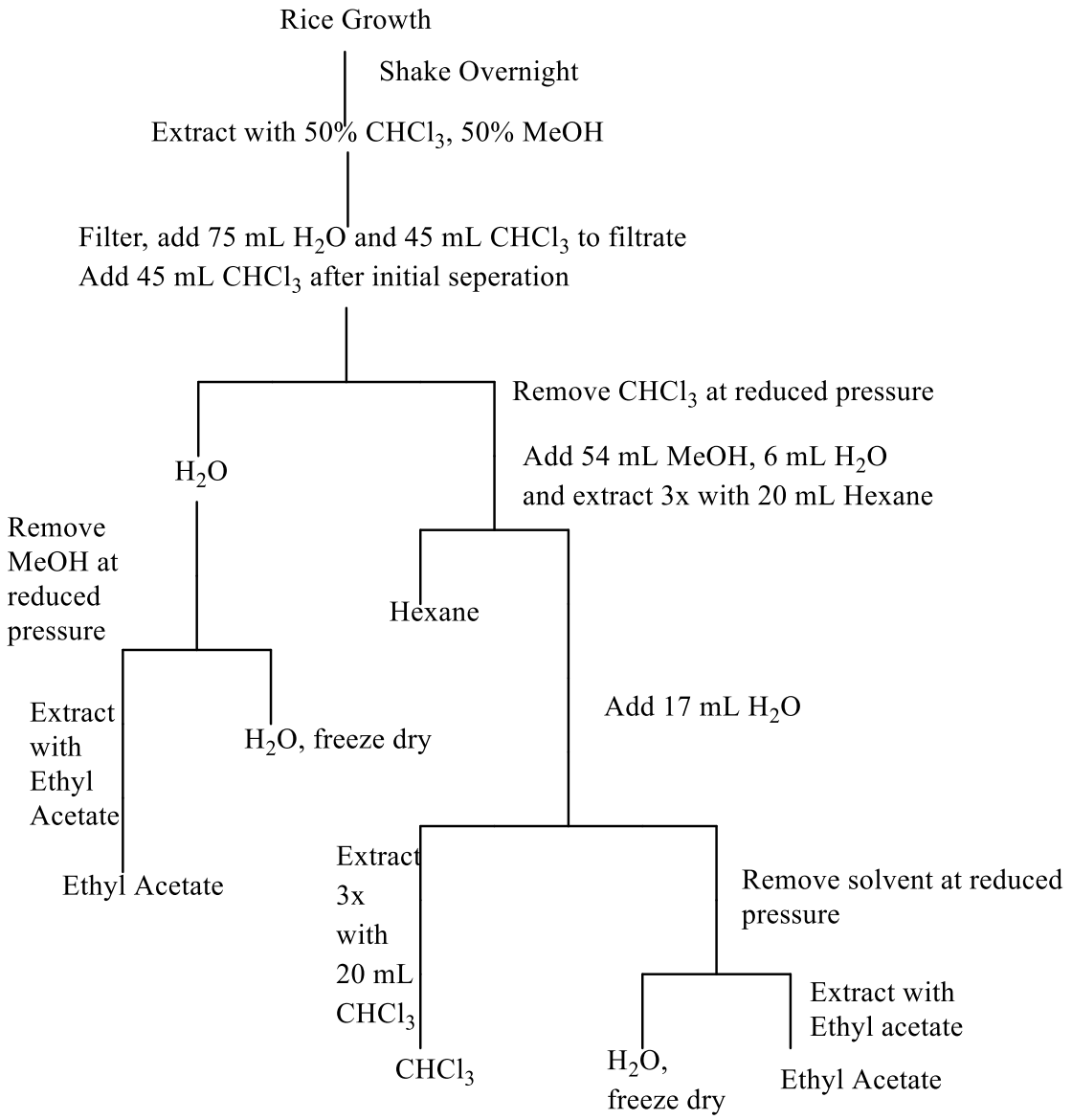

Figure 2. Work flow strategy for the full extraction sequence used to obtain sample fractions in $CHCl_3$, Ethyl Acetate, Hexane, and H_2O

The bottom layer of the filtrate was collected and labeled the crude organic layer. This filtrate was extracted once more with another 45mL of chloroform and the second chloroform fraction was added to the crude organic. The remaining water from this extraction was labeled the crude aqueous. The crude aqueous layer was then placed on the rotary evaporator to evaporate the methanol solvent until only water remained in the sample before placing it back into a separatory funnel. This crude aqueous was then extracted twice with 50mL of ethyl acetate and the fraction obtained was weighed, and a small amount (around 2mg) was saved for biological assay testing and the rest was stored for future testing.

The weight of this crude organic layer was recorded before a small amount (2mg) of this layer was then saved for biological assay testing. The rest was then dissolved back into a solution of water and hexane and extracted. The purpose of the hexane fraction is to remove a majority of the lipids or fats produced by the fungus due to their low probability of having antimicrobial activity. 2mg of the hexane fraction was collected for biological assay and the rest was stored in a vial for future testing. The remaining solution after the hexane fractions was combined with 17mL water and extracted with chloroform. The weight for the chloroform fractions was then collected, and a small amount of sample was set aside for biological assay testing. Lastly, the remaining fraction was then extracted twice with ethyl acetate each time to give a second ethyl acetate fraction as seen above in the work flow sequence. As seen above, the extraction sequence resulted in 5 extractions, 3 of which were tested for antimicrobial activity. The crude testing of these fractions were done at a concentration of 50 µg/µL DMSO.

After the extraction sequence, high-pressure liquid chromatography (HPLC) was performed on the ethyl acetate fraction as well as the chloroform fraction for each sample of interest. This chromatography gives a separation in the form of different peaks roughly for each unique compound present in the fraction. To prepare the sample, there were roughly 2mg of each sample dissolved into 300 microliters of methanol before being placed into an HPLC vial. The method by which each sample was ran changed the solvent percentages from 80% methanol and 20% water to 100% methanol over the course of the 14 minutes run. During the beginning of the run for each molecule, the polar molecules are the quickest to come off and show peaks on the chromatogram. At the very end is when the nonpolar molecules come off in the form of peaks on the chromatogram, and the area where most interesting antimicrobial compounds are shown on these diagrams, is in the center section of the chromatogram.

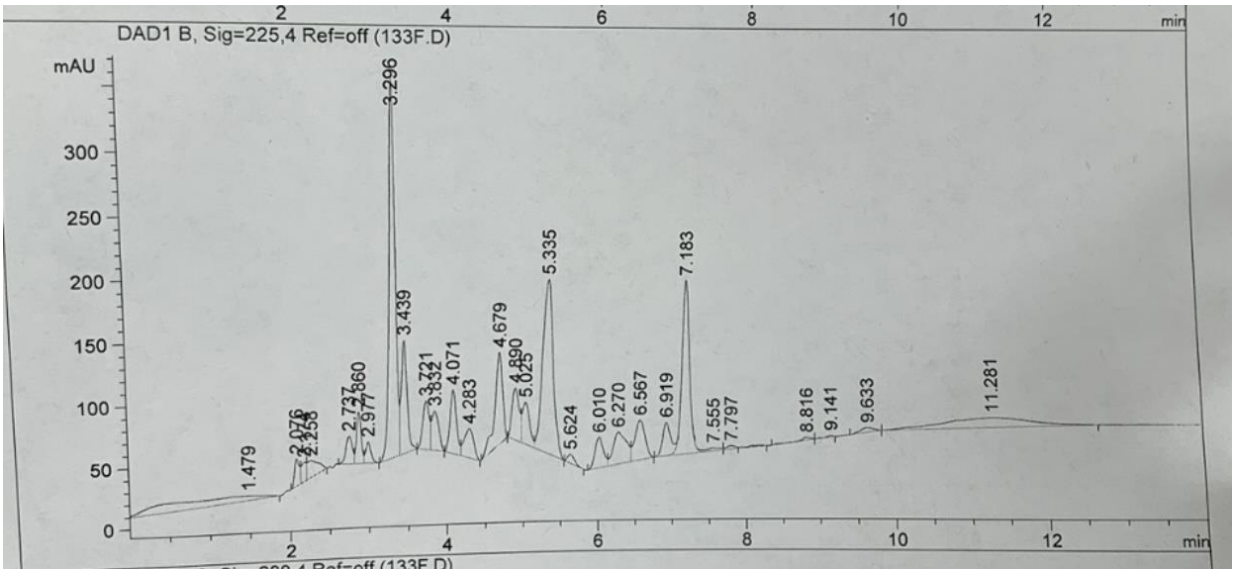

Image 6. An example of an HPLC chromatogram for sample 133F.D

Antimicrobial Assay

The next step was to prepare the bacteria plates for a biological assay. The four plates that were created for testing were: the gram-negative bacteria E. coli, gram positive bacteria Staphylococcus aureus, gram positive bacteria Bacillus cereus and the yeast Candida albicans.

Preparation of Assay Agar Plates

In preparation of these plates, 20mL of soy broth were placed into 4 different conical tubes and a sterile inoculating loop was used to swab each bacteria and mix it into its respective tube. Each tube is placed into the incubated shaker overnight. The next day, 9mL of fresh soy broth was placed into 4 new conical tubes and by use of a sterile pipette, 1mL of each grown bacteria broth was added to the soy broth to give a volume of 10mL in each conical tube. These were then placed back into the incubated shaker for a minimum of 3-4 hours to ensure that the bacteria are in their log stage for plating. After they had grown for the 3-4 hours, they were ready to be plated on soy plates and labeled with each bacteria. This was done by pouring the broth over the plates in a sterile environment and disposing of the excess broth in a bleach solution to kill the remaining bacteria in solution. These plates could be placed in the fridge until you were prepared for the biological assay testing.

All the samples from the prior extraction sequence then underwent a simple plate bioassay to be tested for antimicrobial activity against the gram-negative bacteria E. coli, gram positive bacteria Staphylococcus aureus, and Bacillus cereus and the yeast Candida albicans. To plate each of the samples, they were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 0.2mg/4 microliters for a crude test. Once the samples were dissolved to the desired concentration, four 4

microliter samples were pipetted onto small circular pieces of filter paper. One paper circle of each sample was placed on a plate of each bacteria to test for antimicrobial activity. To make sure that the tests are accurate, each plate had a positive control and a negative control, the positive control will show a ring of growth inhibition around the sample and can be used to compare whether the samples also provide growth inhibition. For the positive control, a known bacterial antimicrobial, tetracycline was used to give a growth inhibition ring of comparison. Growth inhibition in these tests showed that the samples contained antimicrobial components.

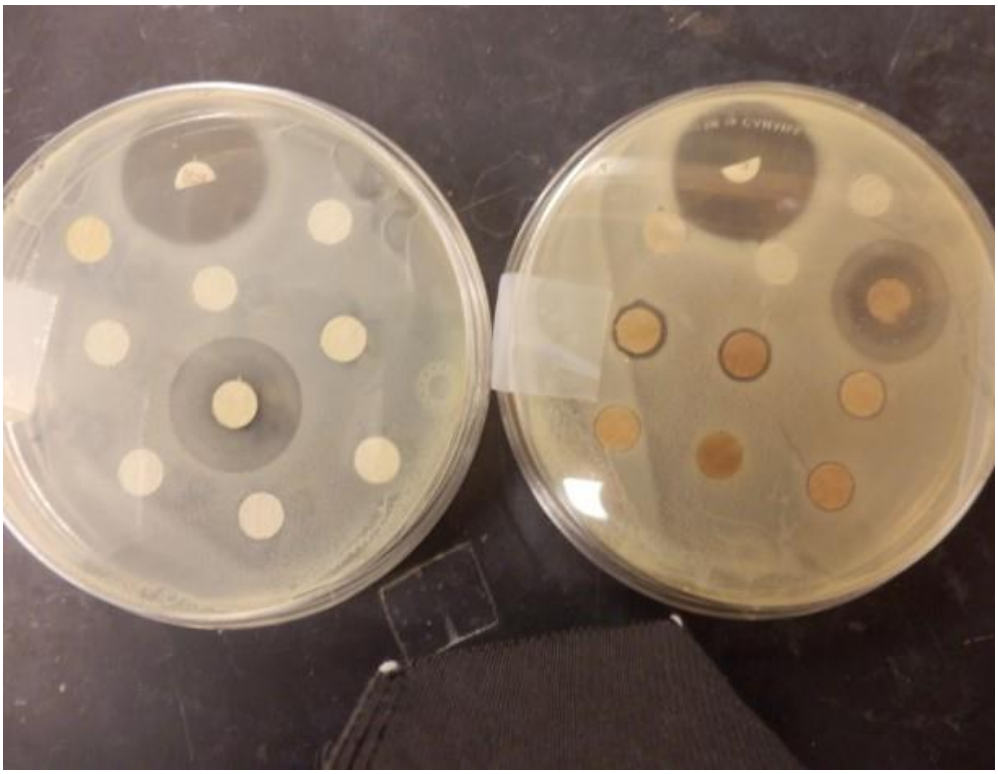

Image 7. Two PDA plates showing growth inhibitor tetracycline at the top and samples showing growth inhibition (antimicrobial activity)

Next, the HPLC spectra of the compound were observed to note the various peaks of different components in the compound. They were then run through a C-18 column to separate out

different molecules in the compound based on retention time and polarity. Once the compound is separated out, the various components were labeled with new letters to keep them separated, and a new biological assay test was performed in order to identify the molecule that has the antimicrobial activity.

Identification of Fungal Samples

Simultaneously, while determining antimicrobial compounds from the purified fungal colonies, the fungal taxonomy will be determined by use of fungal DNA extraction, and sequencing. To begin this process, the E.Z.N.A. Fungal DNA Mini Kit by Omega Norcross was used for DNA extraction. The method followed from the variety of options within this kit was that of the "fresh and frozen" methods and the samples that were extracted were fresh fungal colonies that had been grown for 3 weeks prior. For each unique sample, DNA was extracted following this procedure and then the DNA concentration could be measured by use of the NanoDrop. This helped determine the purity of the fungal samples used and determined if there was a high enough DNA concentration for PCR and then sequencing of the DNA.

The next step was to have the DNA undergo polymerase chain reaction (PCR) to amplify the targeted ITS region of the fungal DNA. For the PCR, the initial denaturation step occurred at 94° for two minutes. This was followed by 35 cycles at 50° and then a final hold at 72°. After PCR was complete, gel electrophoresis was performed to confirm that the DNA was successfully amplified, and a successful amplification would produce bands on the agarose gel that were about 1,500 base pairs in length. Once the DNA was successfully amplified, the

samples and primers were sent to the Iowa State Biotech Facility in Ames, Iowa for sequencing. Once the sequences were returned for both the forward and reverse primers, we were able to match up the longest chain of sequences to fungal sequences in multiple databases for the “longest hit” or matching chain. These longest hits of chains helped with the identification, especially when all of the top hits for matching sequences belonged to the same genus of fungi.

Once the fungi were identified at the genus level, pure fungal colonies of interest were labeled and grown in slant tubes that could be saved to grow future pure colonies of the same fungus for future testing. This was done by use of a sterile inoculating loop transferring the pure fungal spores into the slant tube, and allowing them to grow for 1-2 weeks before placing them in a cooler for storage and future testing.

Results

Five water samples were initially obtained from 2 separate bodies of water within Wind Cave, South Dakota. Two of these water samples were taken from Silent Lake, which is just off the public tour routes within the cave. The other three water samples were collected at WTH Lake which was a 5 hour round trip for sample collection in an area within Wind Cave that less than 200 people have ever been able to access. These initial samples grown were able to be purified into 8 unique fungal colonies that were used for the testing throughout this research project.

These were used as the starting materials for both the DNA extraction and sequencing, as well as the secondary metabolite identification and isolation.

Figure 3 below depicts in chart form the 3 samples from these that were able to be identified down to the genus level. Confident identification of these samples to the species level would require further sequencing of different portions of the DNA.

Sample Name	**Genus**
NASA 46A	*Talaromyces, sp.*
NASA 48	*Linnemannia, sp*.
NASA 53	*Gliomastix, sp.*

Figure 3. Three samples identified down to the genus level

NASA 48 and 53 were shown to have minimal amounts of antimicrobial activity however, NASA 46A showed antimicrobial activity against both gram-positive and gram-negative bacteria, so it was the sample focused on to determine the structure and nomenclature of the antimicrobial component.

Structural Elucidation

NASA 48 and 53 both showed limited antimicrobial activity in comparison to NASA 48, so the taxonomy of this fungal sample was prioritized in this research. NASA 46A was a sample taken during previous research in 2021 and was grown, isolated and identified with structural elucidation during this research project due to its antimicrobial activity against Staphylococcus aureus in the biological assays. NASA 46A was determined to be of the genus *Talaromyces,sp.*

with a likely species *wortmannii.* A pure colony of 46A was grown, and the antimicrobials were extracted to run proton and carbon NMR on the sample as well as HMBC and HSQC.

To determine the structure of the compound containing the antimicrobial activity, the sample underwent nuclear magnetic resonance spectroscopy (NMR). The way the samples were prepared for NMR was by being dissolved in 600-700 microliters of deuterated chloroform and placing the sample into an NMR tube. Proton NMR was important in the structural elucidation of this antimicrobial compound as the spectra gives peaks for the different types of protons and has a relative intensity of each peak based on how many of each of the types of protons are involved in the structure. Carbon NMR shows peaks for each of the carbons located within the molecule, which gives the carbon frame of the molecule. The high-resolution HSQC spectra shows a 1 bond away correlation from respective protons and a carbon atom within the spectra, and the HMBC spectra shows a 2-3 bond away correlation between protons and respective carbon atoms. The figures below show the ^{1}H NMR, ^{13}C NMR, HMBC and HSQC spectra for the molecule of interest from NASA 46A.

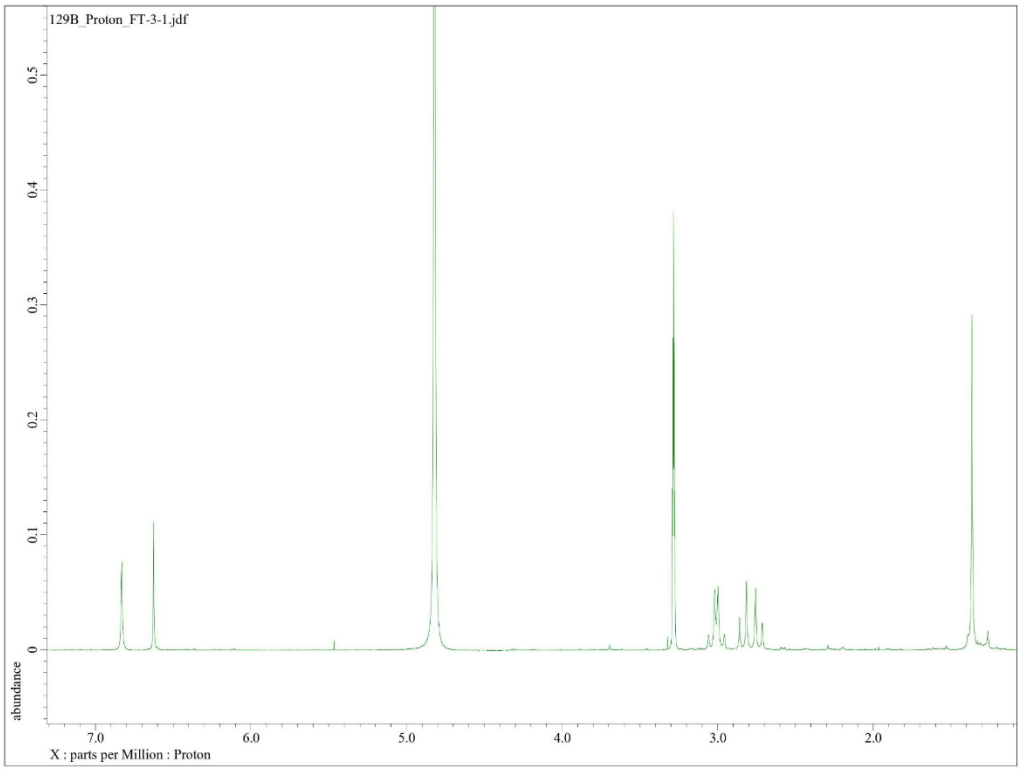

Figure 4. The ^{1}H NMR spectra for the antimicrobial compound originating from NASA 46A

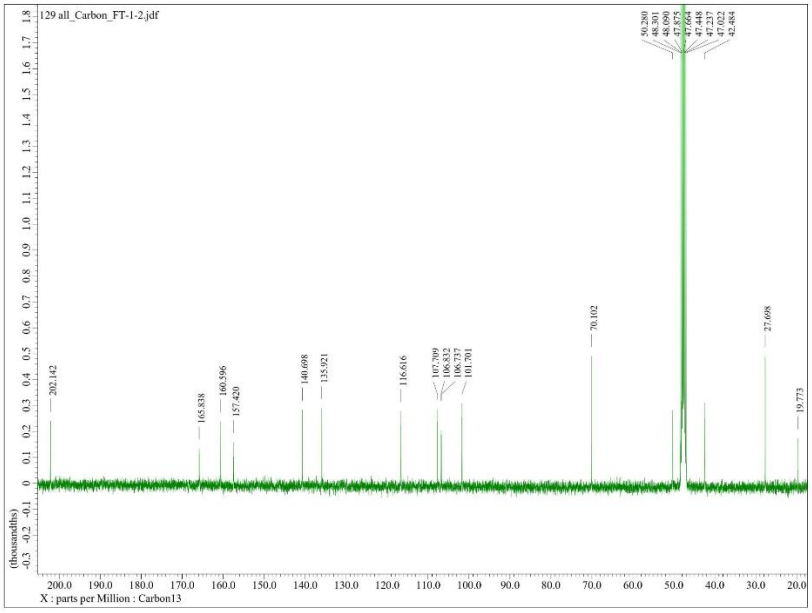

Figure 5. The ^{13}C NMR spectra for the antimicrobial compound originating from NASA 46A

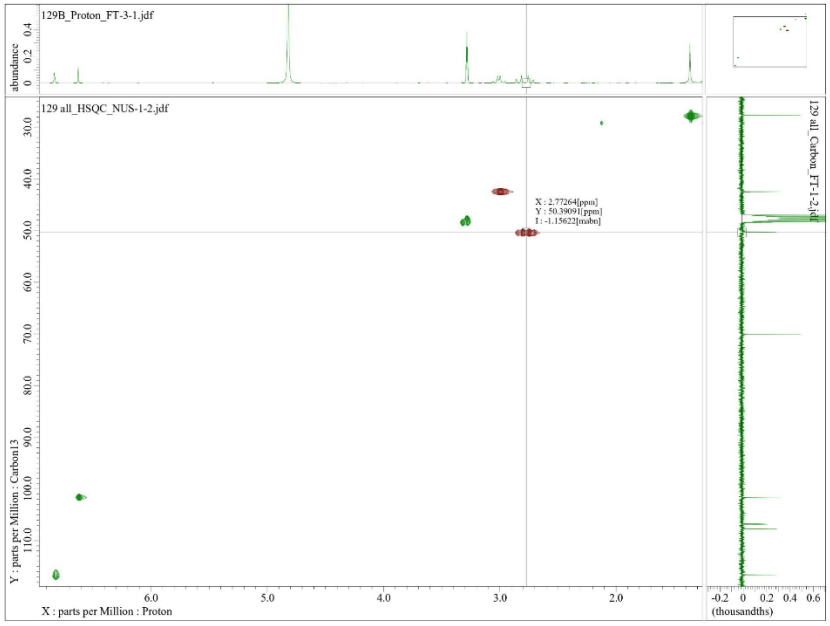

Figure 6. The HSQC spectra for the antimicrobial compound originating from NASA 46A

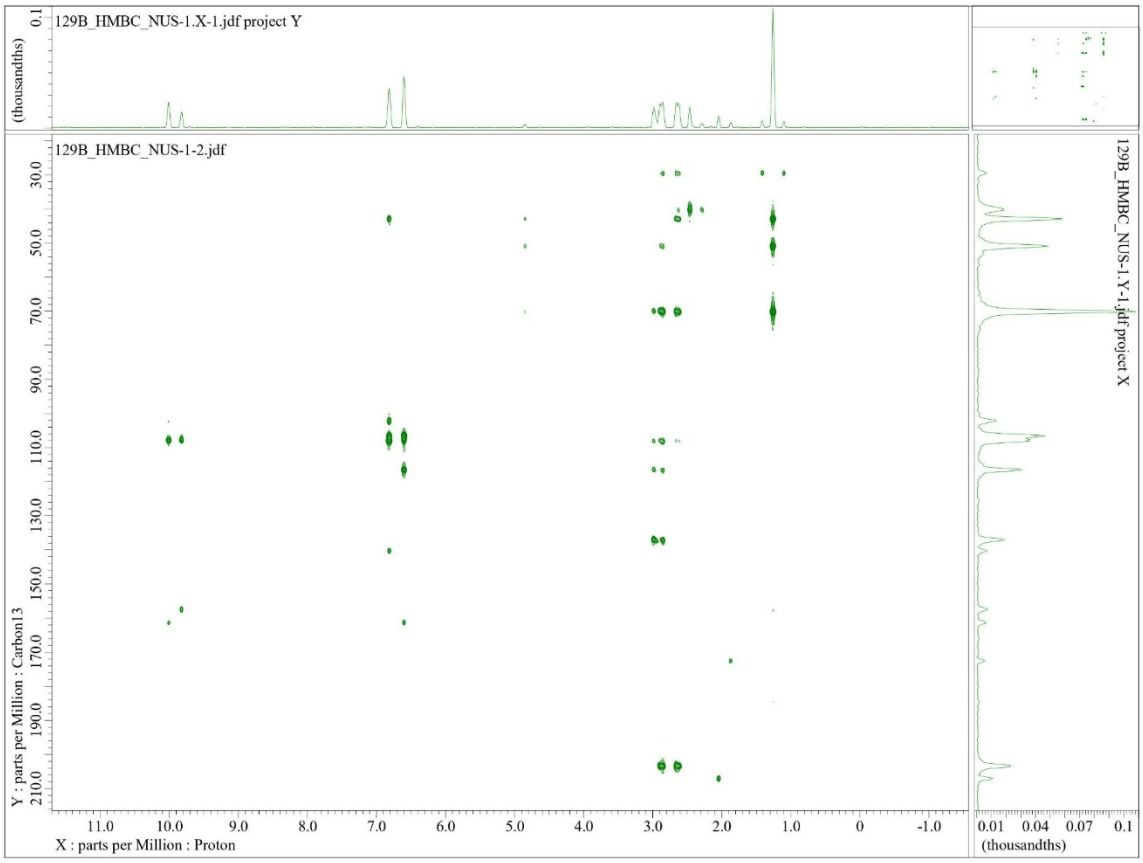

Figure 7. The HMBC spectra for the antimicrobial compound originating from NASA 46A

The final piece of data that helped conclude the structural elucidation of the molecule was the mass spectra for the sample. The mass spectra run on the compound of interest determined the exact chemical mass of the molecule to be 544.1363 atomic mass units (amu), which could be used to determine the number of non-proton and carbon atoms located within the molecule. The exact formula of the compound was determined to be $C_{30}H_{24}O_{10}$. The figure below depicts the determined structure of the antimicrobial component of NASA 46A based on each of the spectra shown above. This compound was determined to be known as flavomannin A. This compound has been determined to have been previously isolated from fungal samples, and due to its ability

to resist against the growth of Staphylococcus aureus, the original article from the Journal of Medicinal Chemistry notes the importance of this molecule and states that they encourage the further evaluation of this compound among others as potential starting points for antibacterial drugs[9]. This highlights the importance of the identification of the genus and species of NASA 46A, due to it's potential to produce these compounds of interest and gives the idea of the potential of related species under the same genus to contain similar antimicrobial compounds of interest.

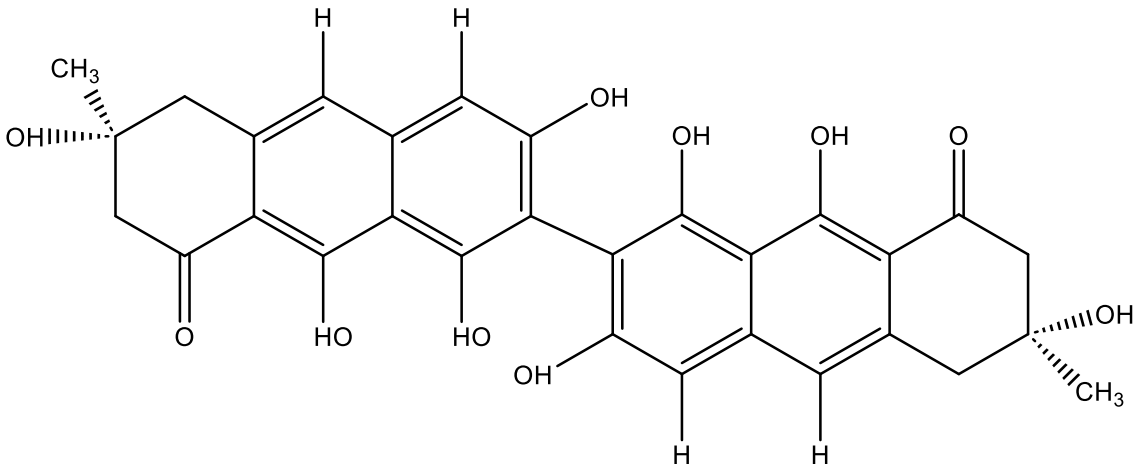

Figure 8. Structure of Flavomannin A from fungal sample NASA 46A

7. Kupchan, S. Morris. "Recent advances in the chemistry of terpenoid tumor inhibitors." Pure and Applied Chemistry 21.2 (1970): 227-246.

8. Horrocks, Rodney D., and Bernard W. Szukalski. "Using geographic information systems to develop a cave potential map for wind cave, South Dakota." Journal of Cave and Karst Studies 64.1 (2002): 63-70

9. Bara R, Zerfass I, Aly AH, Goldbach-Gecke H, Raghavan V, Sass P, Mándi A, Wray V, Polavarapu PL, Pretsch A, Lin W, Kurtán T, Debbab A, Brötz-Oesterhelt H, Proksch P. Atropisomeric dihydroanthracenones as inhibitors of multiresistant Staphylococcus aureus. J Med Chem. 2013 Apr 25;56(8):3257-72. doi: 10.1021/jm301816a. Epub 2013 Apr 16. PMID: 23534483.

Conclusions

As a result of this project, 8 purified fungal samples were isolated originating from 5 water samples collected in May 2022 at Wind Cave, South Dakota. These water samples were collected from 2 differing locations within Wind Cave, Silent Lake and What the Hell Lake. The goals of this research project were to isolate and identify both the fungal colonies grown from samples collected at Wind Cave, and to identify antimicrobial compounds produced by these fungal samples that could provide benefit to the medicinal production of antibiotics to combat antibiotic resistance which is increasingly more important to the scientific field as viral mutations multiply.

In the antimicrobial compound aspect, all samples collected were able to be tested for antimicrobial activity by means of biological assays. However, only enough sample of NASA 46A was able to be obtained for structural analysis. Several of the other NASA samples tested did not show any promising signs of antimicrobial activity that would be worth pursuing. NASA 46A was determined to contain the compound Flavomannin A which belongs to the genus *Talaromyces, sp.*

As for the fungal identification portion of this research, 3 of the 8 samples were able to be determined at the species level to a high level of certainty, and NASA 46A was able to be determined as a most likely species of *wortmanii* as well through other methods involving a database search of the secondary compounds the species produces. All three of the samples identified come from differing genus but were also not new fungi to discover. The three genus that the samples were determined to originate from are *Talaromyces, sp., Linnemannia, sp.,* and *Gliomastix, sp.*

With this project there is a lot of potential with future research when it comes to the replication of procedures followed in this research. The fungal samples are individually in slant tubes in hopes that the secondary metabolites isolated here could be isolated again using the same procedures later on. Within Wind Cave in South Dakota, samples for this research in years past as well as this project have only been obtained from 3 water sources within the cave spanning about 1% of the caves entire known pathways[7]. Future researchers could venture further into the cave into places that have been less influenced by human activity and are therefore more sterile and untouched. This could lead to the discovery of more secondary metabolites that have existed within the cave ecosystem for many years. NASA 46A could also be further explored down the road as a potential for new antibiotics that viral mutations aren't resistant against.

Another potential relating to future research for this project would be the incorporation of new mediums for fungal growth. The two mediums used for this research were YPD and PDA, however there are a multitude of growth medium options for fungal growth, and the use of a new medium could lead to the growth of differing novel fungal colonies that didn't previously grow on either YPD or PDA.

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