Jul 28th, 11:00 AM - 1:30 PM

Analysis of Biologically Active Secondary Metabolites from Fungi

Elizabeth E. Guevara
University of Northern Iowa

Kirk Manfredi
University of Northern Iowa

University of Northern Iowa
Chemistry & Biochemistry

Analysis of Biologically Active Secondary Metabolites from Fungi

Elizabeth E. Guevara, Kirk P. Manfredi
Department of Chemistry & Biochemistry, University of Northern Iowa, Cedar Falls, IA 50614

Introduction

Fungi produce secondary metabolites, which are organic compounds that allow the organism to be competitive in their environment. Secondary metabolites are not involved in growth and development, instead they affect ecological interactions to increase survivability. These compounds will often have antimicrobial properties, leading to their use in antibiotics. Isolating a compound with significant antimicrobial activity could lead to its use in therapeutic antibiotics. New antibiotics are needed to address the growing issue of antimicrobial resistant bacteria. This project explores the antimicrobial compounds produced by fungi collected Wind Cave National Park or carried by local insects. Accurate taxonomic identification of the fungal samples is essential to gain a full understanding of the fungus and its potential biochemical properties. Results to date are presented.

Research Goals

- Isolate antimicrobial compounds from fungus
- Quantify microbial growth inhibition of compounds
- Identify fungal specimens

Isolation & Sequencing

Fungi were collected from Wind Cave National Park and local insects. The samples were replated until a single fungus was isolated, which was grown on rice for a few weeks.

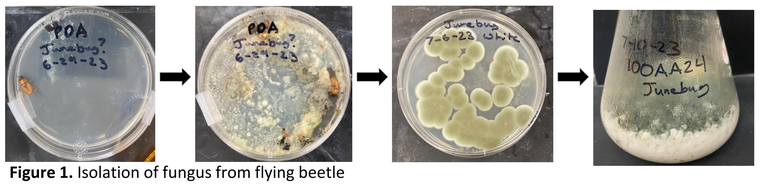

Figure 1. Isolation of fungus from flying beetle

The fungal DNA was extracted and the internal transcribed spacer (ITS) region was amplified with polymerase chain reaction (PCR) using the common ITS5 and LR3 primers. The ITS region is between the 18S and 28S rRNA genes. This region exhibits the largest amount of variation between species, making it the most useful region for identification[1].

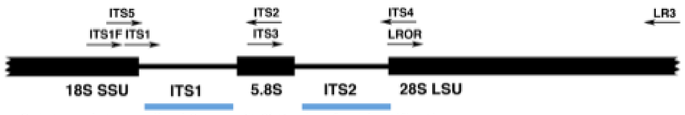

Figure 2. Internal Transcribed Spacer (ITS) Region for Identification

Following runs on 2% agarose gel to confirm the specificity of amplification, PCR samples were purified before sequencing. To identify each fungus, a NCBI-BLAST[2] search compared sample sequence to known fungal sequences.

Sample Name	Scientific Name	Match Length	Similarity (%)
NASA 36B	*Aspergillus amylovorus*	810	100
NASA 38	*Pseudogymnoascus pannorum*	802	100
NASA 46	*Pseudogymnoascus pannorum*	773	100
NASA 46 Brown	*Pseudogymnoascus pannorum*	719	100
Spider Y167G	*Penicillium brevicompactum*	806	100
Spider Y167H	*Trichoderma harzianum*	783	99.87
Mosquito Y169A	*Aureobasidium pullulans*	805	99.88
Mosquito Y170A	*Cladosporium cladosporioides*	795	100

Table 1. Sample Sequence Identification

Extraction & Biological Assay

Once the fungus was fully grown on rice, the rice culture was broken up and shaken overnight with a 1:1 mixture of $CHCl_3$ and MeOH, to extract the organic compounds. Then a modified Kupchan scheme was performed, giving three fractions, hexane, chloroform, and ethyl acetate.

Each of these fractions were tested for antimicrobial activity with a biological assay. Each sample was tested with *E. coli, B. subtilis, S. aureus,* and *C. albicans.* A 0.2 mg/µl solution of the sample in DMSO was prepared and 4 µl was pipetted onto a filter paper disc. The sample discs, a blank disc with 4 µl DMSO and half a disc of tetracycline were placed onto a plate inoculated with bacteria. The plates were incubated overnight and the samples were compared to tetracycline, a common antibiotic. If the sample inhibited bacterial growth, it was saved for further analysis.

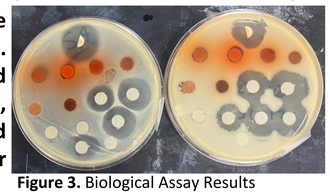

Figure 3. Biological Assay Results

Purification & Identification

Analysis and purification of the samples was carried out using high-performance liquid chromatography (HPLC) which separates compounds based on their polarity and retention time. Fractions were collected that corresponded to each peak on the chromatogram. These fractions were tested with a bioassay to determine which contained the active compound.

Liquid chromatography-mass spectrometry (LCMS) was used to identify the active compounds. LCMS is a method that physically separates molecules before analyzing their mass. The results include a mass spectrum with peaks that correspond to the molecular weight and fragmentation pattern of a molecule. Using LCMS, mycophenolic acid was identified from the chloroform layer of *Penicillium brevicompactum*.

Mycophenolic Acid ($C_{17}H_{20}O_6$)
321.1348 = $C_{17}H_{20}O_6$ + H
343.1165 = $C_{17}H_{20}O_6$ + Na
663.2441 = 2($C_{17}H_{20}O_6$) + Na

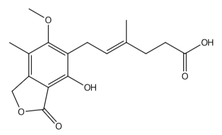

Figure 4. Structure of $C_{17}H_{20}O_6$

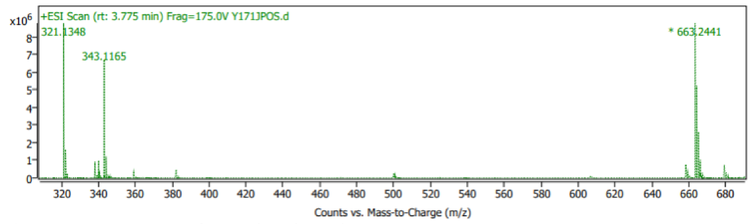

Figure 5. Mass spectrum of Mycophenolic Acid

Carbon and proton NMR were used to confirm the identity of the sample as mycophenolic acid, both spectra matched those published in literature[3]. Research on the secondary metabolites of *Penicillium brevicompactum* was conducted and it was found to commonly produce mycophenolic acid[4], a well known immunosuppressant used in medications today.

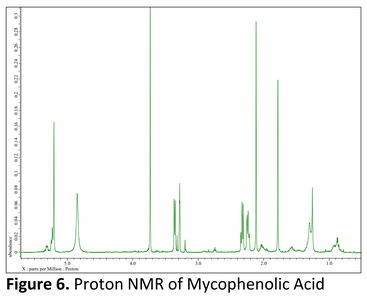

Figure 6. Proton NMR of Mycophenolic Acid

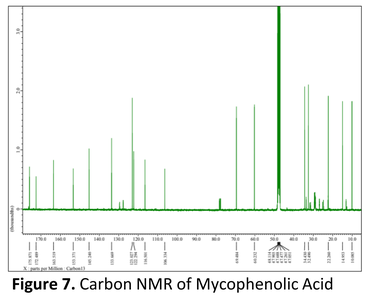

Figure 7. Carbon NMR of Mycophenolic Acid

Quantifying Antimicrobial Activity

A new compound ($C_{11}H_8Cl_2O_4$) with significant antimicrobial activity against *Staphylococcus aureus* was isolated from a *Pseudogymnoascus* species. LCMS and NMR were used to determine the structure of this dichloro compound. A bioassay using bacterial dilutions was performed to quantify the microbial growth inhibition of this compound.

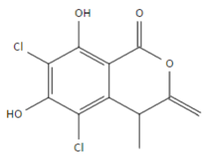

Figure 8. $C_{11}H_8Cl_2O_4$ (169D)

Initial serial dilutions of *S. aureus* were plated to estimate the number of bacterial cells in the sample, in colony forming units (cfu) per mL. It was determined that the sample contained 7.6 x 10^8 cfu/mL.

Figure 9. SA Dilutions: 10^{-7}, 10^{-6}, 10^{-5}

Six solutions of three increasing concentrations of the active compound (169D) and three of tetracycline were prepared in DMSO and placed in a broth sample of diluted *S. aureus*. These solutions were placed in a microtiter plate alongside DMSO with *S. aureus*, and DMSO with sterile broth. The samples were incubated and shaken for twenty hours. The absorbance at 600 nm was measured every minute to track bacterial growth.

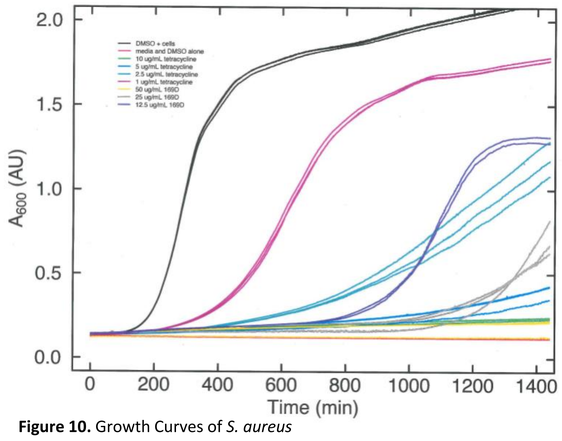

Figure 10. Growth Curves of *S. aureus*

The minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial that will inhibit visible bacterial growth, typically for 16-20 hours. The MIC of 169D was estimated to be between 12.5 µg/mL and 25 µg/mL.

Conclusions & Future Work

The minimum inhibitory concentration of $C_{11}H_8Cl_2O_4$ was estimated; more work is necessary to reproduce these results and determine an accurate value. The genus and species of eight fungi were identified. From these fungi, several samples with antimicrobial activity were extracted. Mycophenolic acid was isolated from *Penicillium brevicompactum*. More work is needed to purify and isolate the active compounds in the other samples. A plethora of samples were recently collected from Wind Cave which will take time to grow and extract.

Acknowledgements

- The University of Northern Iowa College of Humanities, Arts, and Sciences
- The University of Northern Iowa Summer Undergraduate Research Program
- Iowa Space Grant Consortium under NASA Award No. 80NSSC20M0107
- Justin Peters for help with PCR and DNA sequencing

Citations

[1]Raja, H. A., Miller, A. H., Pearce, C. J., & Oberlies, N. H. (2017). Fungal Identification using Molecular Tools: A primer for the Natural Products Research community. *Journal of Natural Products*, *80*(3), 756–770. https://doi.org/10.1021/acs.jnatprod.6b01085
[2]*BLAST: Basic Local Alignment Search Tool*. (n.d.). https://blast.ncbi.nlm.nih.gov/Blast.cgi
[3]Williams, R. W., Lively, D., DeLong, D. C., Cline, J. A., Sweeney, M. J., Poore, G. A., & Larsen, S. (1968). MYCOPHENOLIC ACID : ANTIVIRAL AND ANTITUMOR PROPERTIES. *The Journal of Antibiotics*, *21*(7), 463–464. https://doi.org/10.7164/antibiotics.21.463
[4]Lu, X., Zheng, Z., Zhang, H., Huo, C., Xiu, Z., Ma, Y., Ren, X., Aibing, K., He, J., Gu, Y., & Shi, Q. (2009). Two new members of mycophenolic acid family from Penicillium brevicompactum Dierckx. *The Journal of Antibiotics*, *62*(9), 527–529. https://doi.org/10.1038/ja.2009.54