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Dereplication and Prioritization of Natural Product Extracts for Antifungal Drug Discovery

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DEREPLICATION AND PRIORITIZATION OF NATURAL PRODUCT EXTRACTS FOR ANTIFUNGAL DRUG DISCOVERY

by

Kara Fowler

A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

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ABSTRACT

KARA FOWLER: Dereplication and Prioritization of Natural Product Extracts for Antifungal Drug Discovery
(Under the direction of Melissa Jacob)

The prevalence of opportunistic fungal infections in hospital settings are at alarming rates. Through technological advances in natural product research, plants have become a useful source for antifungal drug discovery. More samples can be screened at once, fractionated in less time, and biologically tested at smaller quantities. However, isolating antifungal compounds from these plant extracts and determining their specific chemical makeup can still take up a significant amount of time. Therefore, this thesis presents a prioritization technique to prevent the need to isolate every compound within an active fraction. With the guidance of literature research on the genus and species of each plant sample coupled with the biological activity data collected on each sample, plant extracts can be prioritized for isolation efforts or thrown out completely if found uninteresting. This method of prioritization also fuels dereplication efforts by preventing researchers from spending time and resources on plants that have already been worked on. Plants that have had little work done on them and that have highly active fractions will lead to the discovery of novel antifungal drugs.
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<tr>
<td>HTS</td>
<td>high-throughput screening</td>
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<td>NCNPR</td>
<td>National Center for Natural Products Research</td>
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<tr>
<td>LC-MS</td>
<td>liquid chromatography-mass spectrometry</td>
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<td>UV</td>
<td>ultra violet</td>
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<tr>
<td>ASE</td>
<td>Accelerated Solvent Extraction</td>
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<td>SPE</td>
<td>solid-phase extraction</td>
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<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<td>HPLC</td>
<td>high-performance liquid chromatography</td>
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<td>UPLC-MS</td>
<td>ultra performance liquid chromatography-mass spectrometry</td>
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<tr>
<td>QC</td>
<td>quality control</td>
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<tr>
<td>ESI</td>
<td>electrospray ionization</td>
</tr>
<tr>
<td>CLSI</td>
<td>Clinical and Laboratory Standards Institute</td>
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<tr>
<td>MIC</td>
<td>minimum inhibitory concentration</td>
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<td>MFC</td>
<td>minimum fungicidal concentrations</td>
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<tr>
<td>$IC_{50}$</td>
<td>half maximal inhibitory concentration</td>
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<td>PDA</td>
<td>photodiode array</td>
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Introduction

Natural products have been used for medicinal purposes dating back to 2600 BCE in Mesopotamia (Newman et al. 2007). Many plants and herbs were used for headaches, wounds, indigestion, and other everyday ailments. Records show that *Artemisia annua* was used in China, snakeroot plant in India, coca bush in Mesoamerica, opium poppy in Egypt, and many other natural products around the world. The most extensive record is the “Ebers Papyrus” from Egypt in 1500 BCE, listing over seven hundred drugs that were mostly derived from plants (Newman et al. 2007). Plants have been so widely used as medicines because they are so accessible, renewable, and plentiful (Mishra and Tiwari 2011). In recent decades, researchers have studied what components of these plants provide activity against diseases and infection. Natural products are an immense resource for compounds with a tremendous amount of functional and chemical diversity (Tu et al. 2010).

Opportunistic fungal infections can be fatal to patients with compromised immunities, including cancer patients, transplant recipients, and those who have contracted human immunodeficiency virus / acquired immunodeficiency syndrome (HIV/AIDS). The most common pathogenic fungal species is *Candida* which now makes up 8-9% of all bloodstream infections and has a high fatality rate of up to 40% which is unacceptable with today’s medicinal technology. The prevalence of these life-threatening infections in hospital settings has reached alarming rates and there is a growing need for more effective and extensive antifungal drugs. There are only three types of antifungal drugs in use today: those that that target the cell membrane (polyenes, e.g. Amphotericin B), those that inhibit ergosterol biosynthesis (azoles, e.g. Fluconazole), and those that
target biosynthesis of the cell wall (echinocandins, e.g. Caspofungin). However each of these classes of antifungal drugs has serious curative limitations. Amphotericin B has significant toxicity, some species of fungi can develop drug resistance to azoles, and echinocandins can only be administered to a patient intravenously (Roemer et al. 2011).

In the past twenty years, natural product research efforts in the discovery of new antifungal drugs have died down due to the complexity and significant time consumption of isolating active compounds. It can take up to several months to perform a primary screening of crude plant extracts, fractionate the extract based on bioassay results, purify the active compound(s), and determine the exact chemical structure of isolated compounds. However, new technological advances in high-throughput screening (HTS) have allowed for more samples to be screened at one time and at smaller quantities, saving time and resources. Recent developments in analytical and automation technologies have improved the speed at which samples are fractionated and processed. These innovations have allowed for a new opportunity to re-establish natural products as a source for novel drug discoveries (Tu et al. 2010).

Even though modern technology has improved drug discovery immensely, natural product extracts can cause complications themselves. First of all, polyphenols (vegetable tannins), can elicit false-positives in enzymatic and cellular screening methods because of their promiscuous enzyme inhibition and alterations in the cells’ redox potential. Polyphenols can be found at significant concentrations in ethanol extracts of plants. A second problem arises in the chemical diversity of a single plant extract. This extract’s chemical composition may contain various classes of chemicals that often display different biological activities which can sometimes oppose each other. Finally,
compounds that are biologically active may be present in crude plant extracts but may be at immeasurably low concentrations and therefore do not show bioactivity during screening. Various research teams have attempted to eliminate these problems with plant extracts (Tu et al. 2010).

Saint Jude Children’s Research hospital in Memphis, Tennessee, in collaboration with the National Center for Natural Products Research (NCNPR) at the University of Mississippi, has proposed a more efficient high-resolution and high-throughput fractionation strategy to limit natural product complications. Plants from around the world were collected, extracted, and fractionated using a high-throughput fractionation procedure to generate thousands of fractions. These fractions were also evaluated chemically by analyzing them via liquid chromatography-mass spectrometry (LC-MS), which determines the chemical make-up of the fraction by molecular weight and ultraviolet (UV) absorption characteristics. These fractions were then evaluated for their ability to inhibit the growth of a panel of pathogenic bacteria and fungi. Fractions that showed significant anti-infective activity were then selected for LC-MS analysis.

**Experimental**

Plants were collected, delivered to NCNPR, freeze dried, and ground. Once ground they were indexed by GPS, genus and species, geographic location, plant part, collector, date, and voucher species storage. Each ground sample was transferred to a specific Accelerated Solvent Extraction (ASE) cell was filled with 95% ethanol and pressurized to 1500 psi at 40°C. A ten minute long static period was used to extract plant compounds, and the sample was then flushed full with 95% ethanol. The cell was then
purged of all liquid for 120 seconds. This process was then repeated two more times for each sample. The combined plant extracts were dried completely with the use of a Speedvac or a Genevac. The dried extracts were then provided to St. Jude Children’s Research Hospital in Memphis, Tennessee.

Once the dried samples arrived at St. Jude, polyphenols were removed through the use of a 700 mg polyamide-filled cartridge and a 48-place positive-pressure solid-phase extraction (SPE) manifold. The ethanol extracts, which were each around 100 mg, were dissolved and transferred onto a polyamide SPE cartridge. Five column volumes of methanol were then used to rinse the column. A Zymark TurboVap LV Concentration Workstation produced a stream of nitrogen to dry the collected effluent.

Following prefractionation, samples were dissolved in 2 mL of dimethyl sulfoxide (DMSO). Each sample was separated into 24 fractions and collected in 16 x 100 mm preweighed glass tubes. The preparative high-performance liquid chromatography (HPLC) separations were performed on a Gemini 5 μm C18 110A column. A Shimadzu LC-8A binary preparative pump with a Shimadzu SCL-10A VP system controller was connected to the Gilson 215 auto sampler and Gilson 215 fraction collector. Detections were obtained with a Shimadzu SPD-M20A diode-array detector and a Shimadzu ELSD-LT II evaporative light scattering detector. The mobile phase was composed of water (A) and methanol (B): 0 min, 98:2; 0.5 min, 98:2; 6.5 min, 0:100; 12.3 min, 0:100; 12.5 min, 98:2; 12.95 min, stop. 25 mL/min was the flow rate.

A GeneVac HT series II high-performance solvent evaporation system was used to dry the collected fractions. The chamber was preheated to and remained at 35 °C. The
SampleGuard Control temperature was set at 40 °C, and the CoolHeat Enable pressure was set at 40 mbar.

The fraction-collection tubes were preweighed with a Bohdan BA-200 Balance Automator and were positioned on a custom Gilson 207 test tube rack. Then, tubes containing natural product fractions were reweighed with the Bohdan BA-200. A FWA program developed on a Pipeline Pilot platform was used to calculate the net weight of each fraction using the difference between the two weights.

The natural product plant fractions were plated using a Freedom Evo Tecan system. Samples in GeneVac racks were dissolved in the suitable plating solvent. The dissolved samples were transferred onto 384-well plates for ultra performance liquid chromatography-mass spectrometry (UPLC-MS) analysis.

The final quality control (QC) of the natural product fractions was achieved with the use of a Waters Acquity UPLC-MS system, using a Waters Acquity UPLC system and an SQ mass spectrometry detector. An Acquity UPLC BEH C₁₈ column was used. The mobile phase was composed of water consisting of 0.1% formic acid (A) and acetonitrile (B). Each analysis ran for a total of 3 minutes.

A Waters SQ mass spectrometer was used to ionize and detect natural product fractions. Both the positive and negative electrospray ionization (ESI) modes were used. The capillary voltage was set at 3.4kV and the extractor voltage was 2V. Nitrogen was used as the nebulizing gas. 130 °C was the set source temperature. The scan range was m/z 130-1400. OpenLynx was used for data processing by extracting all graphic information and converting it to text files to allow transfer to a database for storage and analysis (Tu et al. 2010).
Fractions were received from St. Jude Children’s Research Hospital at 2 mg/mL in DMSO and transferred to 384-well microplates along with drug controls. Organisms were obtained from the American Type Culture Collection in Manassas, VA and include the fungi *Candida albicans* ATCC 90028, *Cryptococcus neoformans* ATCC 90113, and *Aspergillus fumigatus* ATCC 204305. All organisms were tested using adjusted renditions of the Clinical and Laboratory Standards Institute (CLSI) methods. Optical density was used to monitor growth for all the organisms except for *A. fumigatus* (NCCLS 2002). Media augmented with 5% Alamar Blue™ was used for growth detection of *C. albicans* and *A. fumigatus* (NCCLS 2006).

The samples (1 μL), were serially-diluted and were transferred in duplicate to 384-well flat bottom microplates. Inocula were prepared by correcting the OD<sub>630</sub> of microbe suspensions in incubation broth [RPMI 1640/0.2% dextrose/0.03% glutamine/MOPS at pH 6.0 (Cellgro) for *Candida spp.*., Sabouraud Dextrose for *Cryptococcus neoformans*, and 5% Alamar Blue™/RPMI 1640 broth (0.2% dextrose, 0.03% glutamine, buffered with 0.165M MOPS at pH 7.0) for *A. fumigatus*]. Fifty μL of each inoculum were added to the appropriate wells of the 384-well microplates using a Thermo Scientific Multidrop Combi. The drug control Amphotericin B was included in each assay.

All organisms were read at either 530 nm using the Biotek Powerhouse XS Plate Reader or 544ex/590em (*C. albicans* and *A. fumigatus*) using the Polarstar Galaxy Plate Reader prior to and after incubation: *Candida spp.* at 35 °C for 24 hrs., *C. neoformans* at 35 °C for 70-74 hrs., and *A. fumigatus* at 35 °C for 46-50 hrs.
During the primary analysis, samples were tested in duplicate at one test concentration (40 μg/mL) and percent inhibitions were calculated relative to blank and growth controls. Samples showing at least 50% or greater inhibition in at least one test organism were selected for dose response (secondary) studies using either 384- or 96-well assays. During the secondary analysis, fractions were tested in duplicate at three test concentrations (5-fold dilutions). IC50s (concentrations that afford 50% inhibition relative to controls) were calculated using XLfit 4.2 software using fit model 201 to afford the concentration of the samples that inhibits the organism 50%. For isolated compounds, the minimum inhibitory concentration (MIC) is the lowest test concentration that prevents detectable growth (for *A. fumigatus*, no color change from blue to pink). Minimum fungicidal concentrations (MFC) were determined by removing 5 μL from each clear (or blue) well, transferring to fresh media and incubation as previously explained. The MFC is the lowest test concentration that kills the organism allowing no growth at all.

**Results**

There were a total of 13,787 fractions screened for % inhibition of various fungi. Out of these fractions, only 1,139 were confirmed to have antifungal activity using dose response (IC50). Eighteen of these confirmed active fractions were selected for LC-MS analysis based on the fact that little research had been conducted on the genus of these plant samples. Therefore, the extracts could potentially contain novel antifungal compounds.

The lead coded as 80689-c4, derived from the leaves and stems of *Hedera nepalensis* (Araliaceae), was active against *Candida albicans* with an IC50 (half maximal
inhibitory concentration) of 8.11 µg/mL and against *Cryptococcus neoformans* with an IC$_{50}$ of 9.00 µg/mL. This plant, which has the common name Nepal Ivy, was collected from the Missouri Botanical Garden in St. Louis, MO on September 19$^{th}$ 2005. *Hedera nepalensis* has been used medically as a diaphoretic and as a stimulant (Ummara et al. 2013). The UPLC-MS-ELSD-PDA profiles are shown in Figure 1. The ELSD detection process accurately determines the proportionate amount of individual compounds in a mixture even if they are not UV active. Looking at the photodiode array (PDA) chromatogram, which measures the UV absorption of the sample, significant UV activity does not appear around the retention time of 1.50 min where the majority of the compounds found in this fraction are detected.

The compound detected at the retention time 1.50 min showed an ion peak at 751.3 m/z [M+H]$^+$ in the (+)-ESIMS, indicating a molecular weight (MW) of 750 (Figure 1e). This molecular weight was confirmed with the ion peak at 749.6 m/z [M-H]$^-$ in the (-)-ESIMS, which also indicates a MW of 750 (Figure 1f). The compound was thus identified as Tauroside E with a MW of 750.96 m/z. This compound has been isolated from a plant of the *Hedera* genus and appears to have insignificant UV activity. Tauroside E has already been studied and found to have antifungal activity and is known to be produced in *Hedera taurica* (Shashkov et al. 1987). Therefore, it can be concluded that this compound is most likely the cause of 80689-c4’s antifungal activity. There is no further interest in the *Hedera nepalensis* plant as a novel antifungal drug source.

The lead coded 81069-c6, derived from the stems of *Guapira fragrans* (Nyctaginaceae), was active against *Cryptococcus neoformans* with an IC$_{50}$ of 11.72
(a) ELSD

(b) PDA

(c) (+)-ESI TIC

(d) (-)-ESI TIC

(e) (+)-ESIMS

(1.50 min)
**Figure 1:** UPLC-MS-ELSD-PDA analysis of lead 80689-c4. (a) ELSD chromatogram; (b) PDA chromatogram; (c) and (d) positive and negative ESIMS total-ion chromatograms (TIC), respectively; (e) and (f) positive and negative ESIMS at retention time 1.50 min.
11 μg/mL. This plant sample was collected at the Missouri Botanical Garden on March 16th 2006 and has the common name Four O’clock. Common uses for *Guapira fragrans* are generally unknown. The UPLC-MS-ELSD-PDA profiles are shown in Figure 2.

The compound detected at the retention time 1.48 min showed an ion peak at 284.8 m/z [M+H]^+ in the (+)-ESIMS, indicating a molecular weight of 284 m/z (Figure 2e). This MW was confirmed by the second peak in the (+)-ESIMS which gives a molecular weight of 569.0 m/z [2M+H]^+. This second peak is twice the MW of the first indicating that a dimer was formed. An ion peak showing a molecular weight of 282.9 m/z [M-H]^- can also be seen in the (--)-ESIMS at the same retention time (Figure 2f). There is a second peak at 567.1 m/z [2M-H]^- that again indicates a dimer was formed. A literature search for isolated compounds from the family Nyctaginaceae lead to the conclusion that the compound Wogonin was the one being detected. Wogonin has a molecular weight of 284.26 m/z, is UV active based on its conjugated system, and is known to have antifungal activity (Pal et al. 2003). Although little antifungal research has been conducted on the plant *Guapira fragrans*, this antifungal compound isolated from plants of the same family has a high probability of being found in this species as well. Therefore, isolation of the compound detected in this fraction of *Guapira fragrans* was deemed unnecessary.

The lead coded 79880-c6, derived from the plant *Oxytropis viscida* (Fabaceae), was active against *Cryptococcus neoformans* with an IC50 of 4.5 μg/mL. This plant sample was collected at the Missouri Botanical Garden on June 15th 2005 and has the common name Sticky Crazyweed. The use of this plant is not widely known. The UPLC-MS-ELSD-PDA profiles are shown in Figure 3.
Figure 2: UPLC-MS-ELSD-PDA analysis of lead 81069-c6. (a) ELSD chromatogram; (b) PDA chromatogram; (c) and (d) positive and negative ESIMS total-ion chromatograms (TIC), respectively; (e) and (f) positive and negative ESIMS at retention time 1.48 min.
(a) ELSD

(b) PDA

(c) (+)-ESI TIC

(d) (−)-ESI TIC

(e) (+)-ESIMS
(1.48 min)

[M^{2+40} + H]^+
Figure 3: UPLC-MS-ELSD-PDA analysis of lead 79880-c6. (a) ELSD chromatogram; (b) PDA chromatogram; (c) and (d) positive and negative ESIMS total-ion chromatograms (TIC), respectively; (e) and (f) positive and negative ESIMS at retention time 1.00 min.
The compound detected at the retention time 1.00 min showed an ion peak at 241.0 m/z [M+H]+ in the (+)-ESIMS, indicating a molecular weight of 240 m/z (Figure 3e). This MW was confirmed in the (−)-ESIMS with an ion peak of 238.9 m/z [M-H]−, which also indicates a MW of 240 m/z (Figure 3f). A literature search determined that there were no compounds isolated from this family or genus of plant that have a MW of 240 m/z. Thus, a scale-up bioassay-directed isolation was performed and confirmed the presence of an active compound with a MW of 240 m/z (Figure 3g). The isolated compound was determined to be trans-2',4'-dihydroxychalcone with an IC₅₀ of 1.06 μg/mL, a MIC of 5 μg/mL, and a MFC of 5 μg/mL against Cryptococcus neoformans.

**Conclusion**

Through the use of modern technological advances like high-throughput screening, larger amounts of natural product extracts can be screened at one time saving time and resources. These improvements have resulted in increased interest in natural products as a source for antifungal drug discovery. To determine which natural products have the highest probability of containing a novel antifungal compound, LC-MS data analysis, literature research, and biological data are all used to prioritize plant samples. This process promotes dereplication (minimizing re-discovery) and further preserves valuable resources and time.

The *Hedera nepalensis* fraction coded 80689-c4 was chosen for LC-MS data analysis because this plant sample produced active results during primary testing and no work on this plant had been found in literature pertaining to antifungal activity. The compound detected within the fraction had a molecular weight of around 750 m/z. Cross-
referencing this molecular weight with compounds isolated from the *Hedera* genus in literature, the compound Tauroside E was found. Tauroside E has been isolated from the plant *Hedera taurica* (Shashkov et al. 1987). A second literature search was performed on the biological activity of this compound which was determined to be antifungal against *Candida glabrata* (Woodward et al. 1998). However, no work could be found on the effects of Tauroside E on other fungi, such as *Candida albicans* or *Cryptococcus neoformans*. Therefore, this high-throughput testing and LC-MS analysis has led to the discovery of the antifungal activity of the compound Tauroside E against additional species of fungi. This compound was determined to be the cause of this fraction’s antifungal activity and there was no need to waste resources and time isolating the compound and performing further tests.

The *Guapira fragrans* sample had similar results with literature research leading to the Wogonin compound isolated from the same family, *Nyctaginaceae* (Pal et al. 2003). However, no evidence of compound isolation was found for the plant *Guapira fragrans* itself. Wogonin has been found to be antifungal against *Aspergillus niger*, *Penicillium frequentance*, *P. notatum*, and *Botrytis cinerea*. However, Wogonin has never before been confirmed to be antifungal against *Cryptococcus neoformans*.

The *Oxytropis viscida* fraction was found to have a compound of molecular weight around 240 m/z. The literature search determined that there was no compound of this molecular weight isolated from the same family or genus of *Oxytropis viscida*, warranting the isolation of the active fraction’s compound. The purified compound was then biologically tested and was found to be active against *Cryptococcus neoformans*. 
Using this high-throughput fractionation approach coupled with LC-MS data analysis, natural products can be efficiently prioritized for purification efforts. Novel antifungal compounds can be discovered with greater ease and with less required resources, time, and energy. This process also fuels dereplication efforts by preventing researchers from working on plant extracts or compounds that have already been extensively studied for antifungal activity.


