Distribution Agreement

In presenting this thesis as a partial fulfillment of the requirements for a degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis in whole or in part in all forms of media, now or hereafter now, including display on the World Wide Web. I understand that I may select some access restrictions as part of the online submission of this thesis. I retain all ownership rights to the copyright of the thesis. I also retain the right to use in future works (such as articles or books) all or part of this thesis.

Joan Shang April 13, 2017

Bringing century-old cures to the frontier of emerging antifungal resistance: A screen of natural products for anticandidal activity

by

Joan Shang

Dr. Cassandra Quave

Adviser

Biology

Dr. Cassandra Quave

Adviser

Dr. Tracy McGill

Committee Member

Dr. Meleah Hickman

Committee Member

2017

Bringing century-old cures to the frontier of emerging antifungal resistance: A screen of natural products for anticandidal activity

by

Joan Shang

Dr. Cassandra Quave

Adviser

An abstract of

a thesis submitted to the Faculty of Emory College of Arts and Sciences

of Emory University in partial fulfillment

of the requirements of the degree of

Bachelor of Science with Honors

Biology

2017

Abstract

Bringing century-old cures to the frontier of emerging antifungal resistance: A screen of natural products for anticandidal activity

By Joan Shang

Candida species rank as the seventh most common pathogen causing hospital-acquired infections and are the most common culprit of hospital-acquired bloodstream infections (Magill et al., 2014). *C. albicans* alone accounts for 90-100% of mucosal infections (Pfaller & Diekema, 2010). Although *C. albicans* is a commensal organism that normally inhabits the human gastrointestinal tract flora, it is an opportunistic fungal pathogen (Roemer & Krysan, 2014). The species is also responsible for approximately 50% of all candidiasis cases, which is associated with a crude mortality rate of 45-50% in critically ill patients, increased hospitalization length by over 14 days, and elevated medical costs by $6,000-29,000 (Gudlaugsson et al., 2003; Leroy et al., 2009; Morgan et al., 2005; Wisplinghoff et al., 2004). Patient populations at high risk for candidiasis include cancer and immunocompromised patients (Antinori, Milazzo, Sollima, Galli, & Corbellino, 2016).

There are only four classes of drugs that are used in the clinic to treat antifungal infections (azoles, echinocandins, polyenes, and pyrimidine analogs). Echinocandins and azoles are preferred by clinicians to treat *Candida* infections, however, their widespread use have contributed to resistance or decreased antifungal susceptibility by *Candida* strains (Antinori et al., 2016). With high rates of azole resistance and the emergence of echinocandin and multidrug resistance, there is a clear demand for new antifungal therapies (Antinori et al., 2016).

This study used an ethnobotanical approach to ultimately identify new chemical entities for antifungal drug development. By tapping into traditional herbal medicine that has persisted through centuries, we aimed to apply ancient wisdom to answer today's antimicrobial resistance crisis by screening and identifying novel antifungal compounds extracted from plants. A screening of over 300 natural products from the Quave Natural Product Library, which uniquely links each product to the traditional medical application and preparation, revealed 45 compounds that inhibit growth of *C. albicans* by at least 95% at a concentration of 16 μg/mL. Further analysis showed that five natural product extracts were from the leaves of the same plant species, *Schinus terebinthifolia.* Microbiological testing evaluated and demonstrated the growth inhibitory activity of the five extracts against both susceptible and drug-resistant *C. albicans* and non-*albicans* species. The demonstration of the bioactivity of *S. terebinthifolia* leaf compounds corroborates its use in traditional medicine to treat skin and soft tissue infections. These findings also identified the leaf of this plant as a promising source of antifungal molecules for drug development.

Bringing century-old cures to the frontier of emerging antifungal resistance: A screen of natural products for anticandidal activity

by

Joan Shang

Dr. Cassandra Quave

Adviser

A thesis submitted to the Faculty of Emory College of Arts and Sciences

of Emory University in partial fulfillment

of the requirements of the degree of

Bachelor of Science with Honors

Biology

2017

Acknowledgements

Dr. Cassandra Quave: Thank you for your mentorship, guidance, and support in helping me develop as a scientist and making this project possible. Your attentiveness and patience have made this Senior thesis experience one of my most formative memories as a science student. I am incredibly grateful for your encouragement and for serving as a role model.

Kate Nelson: Thank you for your patience and teaching. I greatly appreciate your energy while you trained me in lab techniques and data analyses that helped me bring this project to fruition.

Quave Lab: Thank you to the entire lab for supporting me in this research project.

Courtney Thomas: Thank you for sharing your experience with *Candida albicans* and providing feedback on my protocols.

Dr. Tracy McGill: Thank you for supporting me in my endeavors as a committee member and as a mentor. From answering my chemistry questions to helping me formulate a senior bucket list, you have helped me develop into a more well-rounded and balanced student.

Dr. Meleah Hickman: Thank you for your mentorship. I greatly appreciate your enthusiasm and willingness to share your *Candida albicans* expertise with me and for kindly providing resources to help me advance my project.

To my parents: Thank you for supporting me in my endeavors and providing me with unconditional love and support.

To my friends: Thank you for your continued support and memories over the past four years. From reading my drafts to driving me to the laboratory at midnight, you all have been such an integral part of this thesis experience and Emory journey.

Research support and funding sources: The National Institutes of Health (R01AT007052) provided support for this study.

Table of Contents

List of Tables and Figures

CHAPTER 1: INTRODUCTION

Candida albicans, candidemia, and the promise of plant-derived antifungals

Candida albicans is a commensal organism that typically occupies sites all over the human body in small populations and is also an opportunistic fungal pathogen (Pfaller & Diekema, 2010). Its overgrowth can confer pathogenicity and can cause skin and soft tissue infections, oral mucosal infections, and vulovaginal infections (CDC, 2015b). *C. albicans* can also cause candidemia, a common bloodstream infection and a major cause of morbidity and mortality in hospitalized patients (Horn et al., 2009; Leroy et al., 2009; Pfaller & Diekema, 2010). Most infections by *C. albicans* arise endogenously by the overgrowth of the fungus within a patient's own flora. Infection by exogenously transmitted *C. albicans* can also occur when a patient comes into contact with a hospital worker, another patient, or medical device carrying the fungal pathogen (Horn et al., 2009; Mean, Marchetti, & Calandra, 2008; Pappas, 2006; Pfaller & Diekema, 2010; Xu, Ramos, Vilgalys, & Mitchell, 2000). Individuals with weaker immune status are at the greatest risk for infection by *C. albicans* (Horn et al., 2009). For example, cancer patients undergoing chemotherapy and organ transplant patients receiving immunosuppressive drugs are particularly vulnerable to infection by *Candida* species (Pfaller & Diekema, 2010; Watamoto, Egusa, Sawase, & Yatani, 2015).

In the past two decades, the health care setting has witnessed an increased incidence of candidemia as part of a worrisome global trend towards the emergence of fungi as a significant human pathogen (Horn et al., 2009; Pfaller & Diekema, 2010). Between 2000 and 2005, the incidence of candidemia increased 52%, and the rate of candidemia-related hospitalizations increased to 14% in the United States (US) (Antinori et al., 2016; Zilberberg, Shorr, & Kollef,

2008). A large prospective study conducted in Paris, France reported that the incidence of candidemia in both the overall population and intensive care unit (ICU) patients increased from 2004 to 2009 (Lortholary et al., 2014). Similar upward trends for candidemia have also been reported in European studies conducted from 2000 to 2011 (Arendrup et al., 2013; Asmundsdottir, Erlendsdottir, & Gottfredsson, 2013). A more recent surveillance study on candidemia in Atlanta, GA and Boston, MA showed a decline in incidence of candidemia, but a significant increase in *Candida* isolates resistant to echincandin and multiple drugs from 2008-2013 (Cleveland et al., 2015; Vallabhaneni et al., 2015).

Candidemia has a tremendous clinical and economic impact. For an infected patient, the length of hospitalization increases by over 2 weeks and healthcare costs increase by $6,000-$29,000 (Morgan et al., 2005). *Candida* species are the most common cause of bloodstream infections acquired by patients in a hospital setting, accounting for 8-10% of all bloodstream infections (Magill et al., 2014; Morgan et al., 2005). This fungal pathogen is highly associated with device-related infections; they are the most common fungal species isolated from medical devices such as catheters and cardiovascular devices (Mukherjee & Chandra, 2004; Richards, Edwards, Culver, & Gaynes, 2000). The ability to form biofilms enables them to adhere to the surfaces of devices, which infect high-risk patients upon intravenous exposure (Mukherjee & Chandra, 2004).

Azoles, echinocandins, polyenes, and pyrimidine analogs are the four classes of drugs that are clinically used to treat *Candida* infections. Only the first three types are used to treat invasive candidiasis (Campoy & Adrio; Roemer & Krysan, 2014). The widespread use of azoles and echinocandins as first- and second-line therapy has majorly contributed to decreased antifungal susceptibility by *C. albicans* (Cleveland et al., 2015; Healey, Jimenez Ortigosa, Shor,

& Perlin, 2016; Kanafani & Perfect, 2008; Vallabhaneni et al., 2015). Increased resistance to azoles and echinocandins by *C. albicans* is particularly troubling given the limited number of remaining treatment options for antifungal infections (Campoy & Adrio). This may lead clinicians to resort to more toxic therapies that are not well tolerated by patients (Cosgrove & Carmeli, 2003; Kullberg & Arendrup, 2015).

The troubling emergence of antifungal resistant *C. albicans* echoes the broader threat of antimicrobial resistance. Since the advent of Alexander Fleming's discovery of penicillin, the threat of infectious diseases has been significantly reduced by breakthroughs in antimicrobials. However, overuse and misuse of antimicrobials have majorly contributed to the rising global health threats posed by antimicrobial resistance (Cosgrove & Carmeli, 2003; Kullberg & Arendrup, 2015). In the current era where existing antimicrobials have driven selection for resistant strains and new therapeutic developments face stagnation, the global health and economic issues posed by antimicrobial resistance loom over healthcare (Cosgrove & Carmeli, 2003; Savoia, 2012). The focused effort to contain antimicrobial resistance calls for the discovery of novel antimicrobials to combat resistance and increase susceptibility of resistant microbial strains to current treatments. Numerous drug discovery efforts in the pharmaceutical and scientific arena have turned to plants as a potent and largely untapped source of antimicrobial molecules in Western medicine (Savoia, 2012). Among the kingdoms of Fungi, Achaea, Bacteria, and Plantae from which natural drug products have been sourced from, plants are the most underutilized in drug discovery (Savoia, 2012).

A proposed definition of natural products is "any FDA-approved, unmodified natural material or compound, semisynthetic derivatives, or synthetic structures which were conceptually derived from a natural product" (Patridge, Gareiss, Kinch, & Hoyer, 2016). Natural

products and their derivatives have played significant roles in drug and therapeutic development, accounting for nearly 50% of all approved new molecular entities (NMEs) (Patridge et al., 2016). However, the increasing predominance of semisynthetic and synthetic plant derivatives as approved NMEs and the significant decline in unmodified products represented in NMEs warrants renewed efforts directed at natural products for therapeutic developments (Patridge et al., 2016). The representation of unmodified natural products in approved NMEs declined from 43% to 5.3% between 1940 and the present (Patridge et al., 2016). On the other hand, semisynthetic and synthetic plant derivatives have increased representation in NMEs from 14% to 28% (Patridge et al., 2016). With the increase and spread of antimicrobial resistance, it is more important than ever to replenish the drying drug discovery pipeline for unmodified products (Patridge et al., 2016). For each plant-derived NME discovered, drug discovery can be sustained with chemical diversity from the natural products and their derivatives (Patridge et al., 2016).

A comprehensive and systemic literature study revealed that the number of publications on anti-*C. albicans* natural products related to antifungal drug resistance increased from approximately 10 articles to over 120 articles from the time periods 1996-2005 and 2006-2015 (Zida, Bamba, Yacouba, Ouedraogo-Traore, & Guiguemde, 2017). The surge of interest in anti-*C. albicans* medicinal plants reflects the urgent search for novel antifungal therapeutics to combat the tremendous threat of antifungal resistance (Savoia, 2012; Zida et al., 2017). Anticandidal effects of numerous plant-derived compounds have already been reported. For example, *Enatia chlorantha* stem bark extracts, root extracts, and essential oils obtained from hydrodistillation of bark have been found to exert anticandidal effects via alkaloids (Olivier et al., 2015). Natural products that have been shown to exert anticandidal effects by inhibiting biofilm growth and formation include *Moringo oleifera* (Onsare & Arora, 2015).

Project aims and research question

This project uses an ethnobotanical approach to unite the ancient knowledge of plant-based antimicrobial agents honed by traditional medicine with the urgent demand in today's healthcare for novel antifungal agents against drug-resistant *C. albicans* strains and other non-*albicans* species. Discovery of a NME with antimicrobial activity that targets *C. albicans* may provide a promising candidate that could help replenish the drying pipeline of antifungal agents as either a primary treatment or adjunctive therapy. In the latter case, the NME could improve patient outcomes by increasing the susceptibility of pathogenic *C. albicans* to the effects of existing antifungals.

To begin establishing a series of plant-derived antifungal candidates to target *C. albicans*, over 300 extracts from plants that are utilized in traditional medicine for skin infections were screened from the Quave Natural Products Library (QNPL). The QNPL is composed of over 1000 herbal extracts from over 400 species of plants and fungi. QNPL is unique from other chemical libraries because it links herbal extracts with the unique ethnobotanical data on its preparation and use in traditional medicine. This library enables a targeted approach to determining anticandidal candidates among a large number of compounds. While natural products from the QNPL have been reported to exhibit antibacterial activity against *Propinibacterium acnes* and *Staphylococcus aureus,* the library has never been screened against fungal pathogens (Nelson, Lyles, Saitta, & Quave, 2016; Quave et al., 2015; Quave & Pieroni, 2015; Quave, Plano, & Bennett, 2011; Quave, Plano, Pantuso, & Bennett, 2008). Furthermore, this drug discovery initiative not only seeks to identify natural products that could be promising antifungal candidates of *C. albicans*, but also aims to determine whether active herbal extracts are effective against multiple *Candida* species, including *C. glabrata*, *C. parapsilosis, C.*

tropicalis, and *C. auris*. Determining the broad antifungal effects of active herbal extracts across an array of emerging *Candida* pathogens makes this project novel and innovative.

CHAPTER 2: LITERATURE REVIEW

Clinical Summary of *Candida albicans* and non-*albicans* strains

Candida species are normal inhabitants of the human body, living on the skin and mucous membranes of the mouth, throat, gastrointestinal tract, and genital areas in small populations without causing harm (CDC, 2015b). However, a weakened immune system, use of particular medicines, hormonal changes, or pH imbalance may cause these typically innocuous occupants to multiply. Fungal overgrowth often leads to infection in individuals with low immune status (CDC, 2015b). The genus *Candida* is comprised of over twenty yeast species that can infect humans. The majority of Candida fungal infections are caused by the species *C. albicans* (CDC, 2015a)

Infections caused by *Candida* species include candidiasis, which can be categorized into three types: oropharyngeal/esophageal, vulvovaginal/genital, and invasive (CDC, 2014, 2015a, 2015b). Table 2.1 summarizes the symptoms, relevant statistics, high-risk patient populations, and standard antifungal medication of the different *Candida* infections. Oropharyngeal candidiasis manifests when *Candida* species infect the mouth and throat, and esophageal candidiasis results when fungal overgrowth occurs in the esophagus (CDC, 2015b). Symptoms typically include plaques on oral mucous membranes, white patches on the tongue, redness, and soreness on infected areas (CDC, 2015b). Incidence of oropharyngeal and esophageal candidiasis in the general population is rare, but patient populations with weak immune systems are particularly vulnerable. The prevalence among babies less than one month old, HIV/AIDs

patients, and cancer patients are approximately 5-7%, 9-31%, and 20%, respectively (CDC, 2015b). Other vulnerable patient populations include the elderly and organ transplant patients (CDC, 2015b). The standard antifungal treatment for esophageal and oral candidiasis includes fluconazole and itraconazole. Severe esophageal candidiasis is treated with amphotericin B (CDC, 2015b).

Vulvovaginal and genital candidiasis are often referred to as "yeast infection" and develop due to yeast overgrowth in the vagina (CDC, 2014). Symptoms include burning, itching, and vaginal discharge. Infection occurs in nearly 75% of adult women at least once, but rarely in men. The frequency and severity of vulvovaginal and genital candidiasis increase in patients with weakened immune systems, diabetes, in pregnancy, and with the use of broad-spectrum antibiotics. The standard treatment is antifungal vaginal creams (CDC, 2014).

Invasive candidiasis develops when *Candida* species systemically spread to different foci in the body, leading to serious localized infections in other parts of the body, such as the heart, brain, eyes, bones, and blood (CDC, 2015a). Candidemia is the most common form of invasive candidiasis and commonly infects hospitalized patients seeking treatment for other medical conditions. Unlike candidiasis in the throat, mouth, and vagina, this infection of the bloodstream poses a more serious threat to the patient's health and, as a result, to public health as a whole. Candidemia is one of the most common hospital-acquired infections (HAI); it is usually acquired in the hospital setting and is associated with longer hospitalization and greater mortality (Cleveland et al., 2012; Leroy et al., 2009; Magill et al., 2014). An observational study in France reported a crude mortality rate of 45-50% for critical care patients with candidemia (Leroy et al., 2009). Another analyses of patients with candidemia reported a crude mortality rate of 35.2% in North America (Horn et al., 2009). Retrospective studies have reported that outcomes can

improve with early antifungal treatments, although the risk of mortality can be heightened to 40% even when patients with invasive candidiasis receive antifungal treatment (Kullberg & Arendrup, 2015; Pappas et al., 2016). The most common *Candida* species to cause invasive candidiasis is *C. albicans*, responsible for approximately one-third of cases in the US (CDC, 2015). *C. albicans* and four additional strains, *C. parapsilosis*, *C. krusei*, *C. glabrata*, and *C. tropicalis*, are responsible for approximately 95% of all invasive candidiasis (CDC, 2015). Virulence, epidemiology, and antifungal susceptibility varies between different *Candida* species, with *C. glabrata, C. albicans,* and *C. tropicalis* being more virulent than *C. parapsilosis* and *C. krusei* (Kullberg & Arendrup, 2015). The increased virulence of a species is correlated with greater mortality and morbidity among patients (Andes et al., 2012; Kullberg & Arendrup, 2015).

Unlike candidiasis in the throat, mouth, and vagina, which can arise in individuals with either intact or weakened immune function, invasive candidiasis almost exclusively affects patients with medical conditions or using medical treatments that lower immune function (Roemer & Krysan, 2014). Patient populations most vulnerable to invasive candidiasis are those who are immunocompromised or have weakened immune systems due to organ transplantation, HIV/AIDS, and cancer chemotherapy (CDC, 2015a; Kullberg & Arendrup, 2015). Other individuals at high risk for invasive candidiasis include those who have neutropenia, diabetes, kidney failure, as well as those who have either taken broad-spectrum antibiotics, have had an IV catheter, or have undergone surgery, especially gastrointestinal surgery (CDC, 2015a; Kullberg & Arendrup, 2015). Intensive care unit (ICU) patients are also at high risk of developing invasive candidiasis (Mean et al., 2008).

Lacking a characteristic array of clinical symptoms, invasive candidiasis is difficult to predict and diagnose early on (Antinori et al., 2016). Symptoms typically involve fever and chills, but can manifest in other ways when infection spreads to other body parts, such as the brain or kidney (Clancy & Nguyen, 2013). Since individuals typically develop invasive candidiasis when they are hospitalized for another condition, medical professionals face the challenge of pinpointing symptoms related to this particular *Candida* infection (Antinori et al., 2016). After invasive candidiasis diagnosis, the particular antifungal treatment administered depends heavily on factors such as the patient's immune status and age (CDC, 2015c). With only three drug classes available to treat invasive candidiasis, clinicians are limited to administering echinocandins (micafungin, anidulafungin, and caspofungin) and azoles to patients. Amphotericin B in the polyene drug class is not a preferred treatment as it is more toxic, and its side effects are not well-tolerated (Laniado-Laborin & Cabrales-Vargas, 2009).

Infection	Symptoms	Most Susceptible Individuals	Standard Antifungal Medication	Statistics
Oropharyngeal/Esophageal Candidiasis	-White patches/plaques on the tongue and other oral mucous membranes -Redness or soreness	-One month old or less babies -Elderly -Patients with weakened immune systems (organ transplant, cancer, HIV/AIDS	-Oral Candidiasis: clotrimazole troches, nystatin suspension, fluconazole, itraconazole -Esophageal candidiasis: fluconazole, itraconazole, amphotericin B	-5% to 7% of babies less than one month old -9 to 31% of AIDS/HIV patients -20% of cancer patients
Vulvovaginal/ Genital Candidiasis	-Itching -Burning -Vaginal discharge	-Women -Weakened immune systems -Diabetic patients -Recent antibiotic use	-Antifungal vaginal suppositories or creams	-Approximately 75% of all adult women are infected at least once in their life.
Invasive Candidiasis	-Fever -Chills -Other symptoms can develop if spread to other parts of the body.	-Exposed to hospital or healthcare setting -ICU patients -Weakened immune systems (HIV/AIDS, organ transplant, cancer) -Central venous catheter -Low neutrophil count -Medications such as broad-spectrum antibiotics -Kidney failure -Surgery patients	-Echinocandin (caspofungin, micafungin, or anidulafungin) -Fluconazole, amphotericin B	-Varies geographically. In Baltimore area, there are approximately 14 cases per 100,000 people in the Baltimore area. In the Atlanta area, there are approximately 10 cases per 100,000 people.

Table 2.1. Summary of infections caused by pathogenic *Candida* species.

Adapted from: CDC. (2014). Genital/vulvovaginal candidiasis Retrieved from https://www.cdc.gov/fungal/diseases/candidiasis/genital/index.html

CDC. (2015a, October 6, 2016). Candidiasis. Retrieved from https://www.cdc.gov/fungal/diseases/candidiasis/

CDC. (2015b). Oropharyngeal / Esophageal Candidiasis Retrieved from https://www.cdc.gov/fungal/diseases/candidiasis/
CDC. (2015c). Treatment for Invasive Candidiasis. Retrieved from https://www.cdc.gov/fungal/diseases/candidiasis/invasive/treatment.html

Health burden of invasive candidiasis

Approximately 2 million individuals develop HAIs annually in the US, and at any given time, 1 out of 25 hospitalized patients are affected (Guidos et al., 2011; Magill et al., 2014). The Healthy People 2020 has included the prevention of HAIs in high-priority objectives to decrease clinical complications. This could save $25 billion to $31.5 billion in medical costs (HealthyPeople, 2017). *Candida* species rank as the seventh-most reported causative pathogen for all healthcare-associated infections (HAI), accounting for 6.3% of all HAIs and 22% of hospital-acquired bloodstream infections (Magill et al., 2014). Moreover, high rates of mortality and morbidity are associated with invasive infection. According to one population-based candidemia surveillance study, mortality associated with candidemia is 19-24% (Morgan et al., 2005).

Every year, invasive candidiasis impacts over 250,000 people and causes over 50,000 deaths worldwide (Kullberg & Arendrup, 2015). With an estimated 46,000 annual cases in the US alone, invasive candidiasis poses an enormous health burden as the most common bloodstream HAI (Eggimann, Bille, & Marchetti, 2011). Within the intensive care unit (ICU) alone, invasive candidiasis accounts for approximately 5-10% of all ICU-acquired cases with the majority of these infections caused by *C. albicans* (Eggiman et al., 2011). A survey of over 1,000 ICUs in 75 countries showed that patients with microbial infections had twice the morality rate to that of uninfected ICU patients (Guidos et al., 2011; Vincent et al., 2009).

The most common form of invasive candidiasis is candidemia, and its incidence and distribution varies geographically and by patient population. Atlanta and Baltimore are designated locations for surveillance studies on candidemia incidence (Cleveland et al., 2015).

This most recent report indicated that the infection occurs in 14.4 per 100,000 individuals in the Baltimore area and 9.5 per 100,000 person-years in the Atlanta area (Cleveland et al., 2015).

Current treatment of *Candida* infections

Azoles, echinocandins, polyenes, and pyrimidine analogs are the four classes of antifungal drugs that can be used topically, intravenously, or orally (Campoy & Adrio). Only three structural classes of drugs are currently available to combat invasive fungal infections: echinocandins, azoles, and polyenes (Roemer & Krysan, 2014). In general, echinocandins are the preferred treatment for invasive candidiasis (Antinori et al., 2016). There are a sparse number of treatment options for fungal infections as compared to those for bacterial infections. The emergence of *Candida* strains resistant to these standard antifungal treatments has prompted an urgent demand for new therapeutics.

The most common antifungal drug class used in the clinic is azoles, which are cyclic organic molecules that are effective against a broad range of microbes. The azoles' antifungal activity relies on a specific interaction between the azole molecule and a fungal cytochrome P450. The cytochrome P450 enzyme, such as 14α-demethylase in the case of fluconazole, is inhibited by the azole, which results in growth and replication inhibition (Campoy & Adrio). Fluconazole is one of the most common drugs used to treated *C. albicans* infection. It is suspected that the prevalent use of fluconazole, as well as itraconazole, created strong selective pressure on susceptible *Candida* strains to develop azole resistance (Kanafani & Perfect, 2008).

Polyenes are amphiphilic macrocyclic organic molecules that bind with high affinity to lipid bilayers (Campoy & Adrio, 2016). They are a class of antifungals whose mechanism of action works by disruption of the cell membrane. Polyenes complex with ergosterol in the lipid bilayer, resulting in the formation of pores. This leads to cytoplasmic leakage and oxidative

damage (Campoy & Adrio, 2016). Amphotericin B is a standard antifungal treatment used for treating *Candida* infections (Campoy & Adrio, 2016).

Echinocandins are recommended as the first-line antifungal therapy and are the only antifungal drugs that have the capacity to target fungal cell walls (Campoy & Adrio). With a cyclic hexapeptide moiety and side chains at position R5, echinocandins are semisynthetic lipopeptides that act as noncompetitive inhibitors of the β-(1,3)-D-glucan synthase enzyme complex (Campoy & Adrio, 2016). Inhibiting the synthesis of this essential fungal cell wall component disrupts the cell wall structure and eventually leads to fungal cell death (Roemer & D.J. Krysan). Caspofungin, micafungin, and anidulafungin are the only three available antifungal echinocandins used in the clinical setting to target cell walls, and they have demonstrated antifungal activity against many *Candida* strains (Campoy & Adrio, 2016).

Pyrimidine analogs, such as fluctyosine, exert antifungal activity by interfering with pyrimidine metabolism, nucleic acid (RNA/DNA) synthesis, and protein synthesis (Campoy & Adrio, 2016). The fungal cytosine permease, a membrane transport protein that facilitates diffusion of particular molecules, transports the molecule into the fungal cell. When it is phosphorylated, the pyrimidine analog takes the place of a UTP when it is assembled into the RNA, which leads to protein synthesis inhibition. The converted form of the molecule can also be toxic to fungi by inhibiting thymidylate synthase, which inhibits DNA synthesis (Campoy & Adrio, 2016).

Health and economic impact of antimicrobial resistance

The United Kingdom government released a review that estimated that 10 million people will die every year by 2050 if the rising trend of antimicrobial resistance and stagnation of pharmaceutical development continues (O'Neil, 2016). Antimicrobial resistance can affect patient outcomes by "enhancing virulence, causing a delay in the administration of appropriate therapy, and limited available therapy" (Cosgrove & Carmeli, 2003). The ineffectiveness of antimicrobial treatments not only adversely impact patients with drug-resistant infections, but also to patients who undergo treatments that increase their risk of contracting such drug-resistant infections (Cosgrove & Carmeli, 2003. These treatments include chemotherapy for cancer patients, caesarean sections, and gut surgeries (O'Neil, 2016). For these reasons, antimicrobial resistance poses an enormous economic burden. The global economic impact will cost 100 trillion US dollars (O'Neil, 2016). In the US, alone, it costs the healthcare system $21 billion to $34 billion dollars annually (Guidos et al., 2011).

Mechanisms of antimicrobial resistance

There are two types antimicrobial resistance: Primary or intrinsic antimicrobial resistance refers to a microbe's ability to resist drug treatment prior to drug exposure, while secondary or acquired antimicrobial resistance arises when susceptible strains develop resistance after exposure to the drug (Kanafani & Perfect, 2008). Acquiring resistance against drugs is part of an evolutionary process, in which natural selection favors the microorganisms that can survive in the presence of the drug. The ability to evade toxic drug effects is often due to a change in gene expression (Kanafani & Perfect, 2008). There are many mechanisms of antifungal resistance at the molecular level that have led to the survival and proliferation of drug-resistant fungal strains by enabling them to evade or counteract the antifungal effects of drugs. Three of the most

common mechanisms of acquired resistance are: 1) altering drug targets 2) counteracting toxic effects of drugs by modifying the metabolism and 3) increasing drug efflux to decrease the effective drug concentration (Kanafani & Perfect, 2008).

As for *Candida* azole resistance, there are four main mechanisms by which acquired resistance may arise. It is important to note that the presence of multiple mechanisms in one particular resistant strain could confer additive effects. Azole-resistant *C. albicans* have been observed as to exhibit upregulation of two families of efflux transporter genes, *CDR* and *MDR* (Kanafani & Perfect, 2008). When CDR1 and CDR2, both of the ATP-binding cassette super family are upregulated, there is decreased cytosolic drug concentration in fungal cells. This phenomenon confers resistance to almost all azoles (Kanafani & Perfect, 2008). Greater expression of MDR1 of the major facilitator class confers resistance by also decreasing drug concentration via active efflux pumps, although it confers resistance specific to fluconazole (Kanafani & Perfect, 2008). In other *Candida* species, upregulation of additional transporter genes such as *PDH1, CdCDR1, CdMDR1,* and *CgCDR1* have been described (Kanafani & Perfect, 2008).

Azoles often exert antifungal effects by inhibiting the production of ergosterol, an essential component of the fungal cell membrane, which then leads to growth arrest (Kanafani & Perfect, 2008). *Candida* strains have developed two major mechanisms to evade the drug's effects: altering the drug target site and/or developing bypass pathways. *ERG11* mutation is one example of acquired resistance via altering the drug's target site, where the lanosterol 14 α-demethylase, a P450 enzyme, becomes a less favorable binding target. While the *ERG11* mutation confers azole resistance by target site alteration, the *ERG3* gene mutation confers antifungal resistance by giving rise to an alternative pathway for ergosterol biosynthesis

(Kanafani & Perfect, 2008). The bypass pathway produces 14α-methylfectosterol, which serves as a substitute for ergosterol. Since both azoles and polyenes deplete fungal cell membranes of ergosterol, *ERG3* mutation confers resistance to both of these classes of drugs (Kanafani & Perfect, 2008).

Polyenes constitute another major class of antifungals and exert their effects by insertion into the fungal cell membrane, associating with ergosterol, and forming porin channels. This leads to disruption of a functional membrane and impairment of cellular function (Kanafani & Perfect, 2008). The most commonly used polyene is amphotericin B. Resistant *Candida* strains commonly carry a *ERF3* mutation and use the previously-described bypass pathway to substitute ergosterol with other sterol products in the fungal membrane to maintain its functionality (Kanafani & Perfect, 2008).

So far, *FSK1* gene mutations have been described in echinocandin-resistant *Candida*. This gene codes for the β-1,3-D-glucan synthase complex. However the mechanism of echinocandin resistance remains elusive and requires further investigation (Kanafani & Perfect, 2008).

Drug Resistance Type	**Genes Altered**	**Mechanism of Drug Resistance**
Azole Resistance	Upregulation of *CDR1, CDR2, MDR1* (classes of transporter genes)	Decrease drug concentration via active efflux pumps
	ERG11	Alter target site - prevents azoles from binding to target enzyme involved in ergosterol synthesis of the fungal cell membrane
	ERG3	Bypass pathway that substitutes ergosterol with 14alpha-methylfecosterol to maintain cell membrane integrity
Polyenes Resistance	*ERG 3*	Bypass pathway that substitutes ergosterol with other sterol products to maintain cell membrane integrity
Echinocandin Resistance	*FSK1*	Exact mechanism remains elusive' Point mutation in beta-1,3-D-glucan synthase

Table 2.2. Mechanisms of Drug Resistance by *Candida* strains
Adapted from: Kanafani, Z. A., & Perfect, J. R. (2008). Resistance to Antifungal Agents: Mechanisms and Clinical Impact. Clinical Infectious Diseases, 46(1), 120-128. doi:10.1086/524071

Traditional medicine and plants: a source of novel antimicrobial agents

The search for new antimicrobial agents has led the pharmaceutical and scientific arenas to investigate plants as a potential source. Plants are rich reservoirs of antimicrobial agents as they have developed an arsenal of antimicrobial secondary metabolites to protect themselves

from the fungi, bacteria, and viruses that they are constantly exposed to in their environment (Savoia, 2012). In order to adapt to the environment, plants have developed secondary metabolites as part of its defense response. These metabolites can be categorized into two broad categories: 1) preformed compounds that were synthesized before pathogen exposure or 2) formed *de novo* in response to a pathogen (Lamothe et al., 2009; (Savoia, 2012). Secondary metabolites, such as antimicrobial compounds, are defined as plant products that are not involved in basic life functions such as growth and reproduction and are produced in low quantities (Bourgaud et al., 2001). However, these molecules play major roles in the plant's capacity to interact with the ecosystem and adapt to their environment.

Phytoanticipins are antimicrobial compounds that are produced by plants before the initial encounter with a pathogen (Lamonthe et al., 2009). They can be found on the surface of plants to combat pathogens. Other preformed compounds can also be found in different organelles, where they are stored until an infection triggers their release via a hydrolase (Lamothe et al., 2009). Examples of antimicrobial phytoanticipins molecules include saponin alpha-tomatine, produced by tomatoes (Lamothe et al., 2009). Phytoalexins are antimicrobial compounds that are produced by plants as part of its defense against pathogenic species. The detection of a pathogen induces transcriptional and/or translational activity by enzymes in the plant's biosynthetic pathway and leads to the production of phytoalexins, which are secreted to the site of infection (Lamonthe et al., 2009). It is worth noting that a molecule characterized as a phytoalexin in one plant species can be characterized as a phytoanticipin, as plant-derived antimicrobial molecules are shuttled into broad categories based on synthesis. Examples of antimicrobial phytoalexin molecules include nomilactone B from rice (Lamothe et al., 2009).

Given that secondary metabolites are involved in a broad range of biological activities, the use of plants in traditional medicine to combat ailments caused by fungal, viral, and bacterial species can be dated back many centuries (Lamothe et al., 2009). Today, there is a significant need for pharmaceutically useful secondary metabolites as alternatives to the current antimicrobial treatments. Utilizing the mostly unexploited plant kingdom for antifungal drug development is a promising approach to replenishing the drying pipeline of antimicrobial agents.

CHAPTER 3: MATERIALS AND METHODS

Source of natural product extracts

The plant and fungal materials from which the natural product was extracted were collected between 2006-2013 following the World Health Organization guidelines (WHO, 2003). Raw materials such as leaves, stems, and roots were separated, cut, dried, and ground into a fine powder using a Wiley mill (2 mm mesh size; Wiley Scientific). For two successive 72 hour periods, crude plant extracts were dissolved in either 95% ethanol or 100% methanol to create a ratio of 1 gram of plant material: 10 mL of organic solvent. After maceration, extracts were combined, concentrated, shell frozen, and then lyophilized. Extracts were suspended in DMSO or distilled water to create a stock concentration of 10 mg mL^{-1}. Stock concentrations were further diluted to 1 mg mL^{-1} for storage and use in screening assays. Figure 3.1 shows extraction of *S. terebinthifolia* leaves, which yields the crude extract (429) and its hexane, ethyl acetate, butanol, and aqueous partitions.

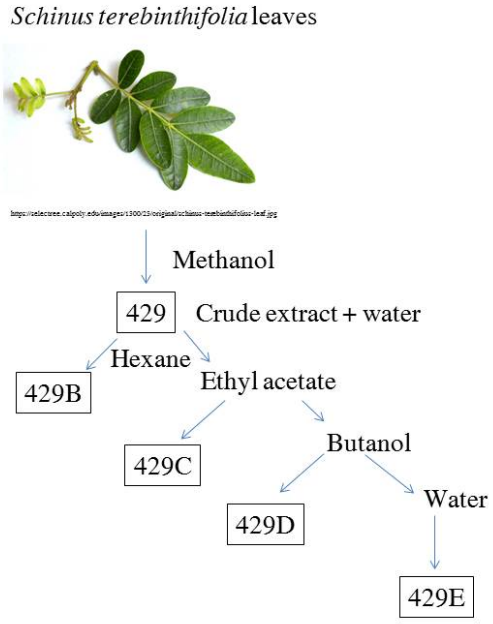

Figure 3.1. Extraction of *S. terebinthifolia* leaves.

Yeast maintenance

Frozen yeast cultures were inoculated on Sabouraud Dextrose Agar (SDA) media (Becton, Dickinson and Company) and incubated for 48 hours at 35°C. Restreaking from frozen yeast culture occurred weekly or biweekly to maintain a fresh culture on SDA media. To create overnight cultures, yeast strain was inoculated in 6 mL Difco™ YPD (Yeast Extract, Peptone, Dextrose) Broth (Becton, Dickinson and Company) media and grown overnight at 35°C. Four *C. albicans* isolates were used in the biological assays. EU52 (NR-29444) and EU54 (NR-29449) were obtained from BEI Resources. MH1 and MH12 clinical isolates were kindly provided by Dr. Meleah Hickman from Emory University. In addition, three *C. glabrata* strains were used to evaluate the growth inhibitory activity of the extracts. CDC0323 (AR-BANK#0323), and CDC0325 (AR-BANK#0325) were obtained from the CDC Antimicrobial Resistance Bank. The assay also used two *C. parapsilosis* isolates, CDC0337 (AR-BANK#0337) and CDC0342 (AR-BANK#0342), as well as one *C. tropicalis* isolate CDC345 (AR-BANK#0345). These strains were also obtained from the CDC Antimicrobial Resistance Bank. Strains were propagated from frozen culture and maintained by continuous passaging on SDA agar. Table 3.1 provides the strain information for the three *C. glabrata* strains, two *C. parapsolosis* strains, and one *C. tropicalis* strain used in the bioassays. Fluconazole-resistant *Candida* strains include one *C. albicans* isolate (MH12), one *C. parapsilosis* isolate (CDC0337), and one *C. tropicalis* isolate (CDC0345). Resistant strains to echinocandins (casopfungin, andiguldafungin, and micafungin) include one *C. glabrata* strain (CDC0323). The multidrug resistant *C. glabrata* strain (CDC0325) is resistant to both fluconazole and echinocandins (casopfungin, andiguldafungin, and micafungin)

Strain ID	Strain Information
MH1	*C. albicans* laboratory reference strain
MH12	*C. albicans* clinical strain, fluconazole resistant
EU52 (NR-29444)	*C. albicans* clinical strain, oral isolate
EU54 (NR-29449)	*C. albicans* clinical strain, vaginal isolate
CDC0323 (AR-BANK#0323)	*C. glabrata* clinical strain, known acquired resistance (FSK2 S663P), echinocandin (caspofungin, anidulgafungin, micafungin) resistant
CDC0325 (AR-BANK#0325)	*C. glabrata* clinical strain, known acquired resistance (FSK2 S663P), known acquired resistance (FSK2 S663P), fluconazole and echinocandin (caspofungin, anidulgafungin, micafungin) resistant
CDC0337 (AR-BANK#0335)	*C. parapsilosis* clinical strain, known acquired resistance (ERG11 SNP), fluconazole resistant
CDC0342 (AR-BANK#0342)	*C. parapsilosis* clinical strain, no resistance mechanism detected
CDC0345 (AR-Bank#0345)	*C. tropicalis* clinical strain, acquired resistance is unknown, fluconazole resistant

Table 3.1. *C. albicans* and non-*albicans* strain ID and strain information.

RPMI 1640 medium

RPMI 1640 powder (containing glutamine and phenol red, without bicarbonate, Sigma-Aldrich) was reconstituted in distilled H2O and buffered with 0.165 MOPS(3-[N-morpholino] propanesulfonic acid) to pH of 7. Prepared media was supplemented with 2% glucose with dextrose anhydrous (Fischer Scientific). Prepared medium was sterile filtered and stored at 4°C.

Initial antifungal susceptibility test of natural product extracts

To test antifungal susceptibility using broth microdilution testing, this study followed the Clinical and Laboratory Standards Institute (CSLI) M27-A3 guidelines and the European Committee for Antimicrobial Susceptibility Testing (EUCAST) with modifications (CLSI, 2008;

Rodriguez-Flores et al., 2014). Inoculum was prepared and adjusted to 0.5 McFarland turbidity standard with a spectrophotometer at an optical density of 530 nm. Suspension was then diluted with RPMI 1640 medium buffered with 0.165 mol/L MOPS to achieve a final cell density of 0.5-2.5x10^3 cells/mL. This final suspension was loaded in 96-well plates with QNPL extracts at an initial screening concentration of 16 µg/mL with negative controls (amphotericin B and fluconazoleat 64 µg/mL) and a positive control (DMSO). Other controls included untreated media and media blanks. The final volume in each well was 200 µL. Plates were incubated at 35°C, and growth reads were collected using a spectrophotometer (OD_{600}) at 24 hours and 48 hours. Percent inhibition and percent vehicle were then calculated with the following formula:

% Inhibition = 100 x 1-((experimental-positive control average)/(negative control average-positive control average)

% Vehicle = 100x ((experimental-positive control average)/(negative control average-positive control average)

Subsequent testing for minimum inhibition concentration of 50% and 90% of lead botanical candidates via serial-dilution MIC assays

Of the 45 extracts that exhibited over 95% inhibition (referred to as initial "hits"), five were further evaluated for the minimum concentration (MIC) necessary for 50 and 90 percent inhibition. Concentrations ranging from 0.125-16 ug/mL were evaluated. 429 and its partitions (B, C, D, and E) were pursued since they were extracted from *S. terebinthifolia* leaves. They were chosen for serial dilution, which also doubled as a screen against additional a*lbicans* and non-*albicans Candida* isolates. 429 is a crude extract of the leaves, 429B is the hexane partition,

429C is an ethyl acetate partition, 429D is a butanol partition, and 429E is an aqueous partition (Figure 3.1).

Determination of fungicidal or fungistatic effects of extracts on susceptible strains

To determine the *in vitro* minimal fungicidal concentration (MFC) of 429, 429B, 429C, 429D, and 429E, aliquots from each optically clear well were streaked onto SDA agar plates. These aliquots were selected from wells that had been incubated for 48 hours for the serial dilution MIC assays. Aliquots from wells with amphotericin B and fluconazole were also streaked onto SDA plates as controls. After 48 hours of incubation at 35°C, colonies were counted. The MFC was determined by the lowest concentration of test compound from the original plate where no recovery of cells was observed. An extract was characterized as fungistatic if more than 10 colonies were counted.

Statistical analysis

A two-tailed Student's t-test with unequal variance was calculated using Microsft Excel 2010 for all assays. *Candida* strains treated with extracts were compared to those treated by DMSO (vehicle control) for all statistical analyses. P-values less than 0.05 indicated statistical significance. The graphs presented in the result section use error bars to represent standard deviation.

CHAPTER 4: RESULTS

Extraction, Mass Spectrometry, and HPLC

Percent yield of 429 from initial dry plant leaf material was 16.9%. 429 was fractionated to produce 429B, 429C, 429D, and 429E.

Screening the QNPL against four *C. albicans* strains

This study evaluated over three hundred extracts from the QNPL for growth inhibitory activity against four *C. albicans* strains (MH1, MH12, EU52, and EU54) at 24 and 48 hours. The initial concentration of extracts in the screen was 16 μg/mL. MH1 is a *C. albicans* laboratory reference strain, MH12 is a fluconazole-resistant *C. albicans* clinical isolate, EU52 is a clinical oral isolate, and EU54 is a clinical vaginal isolate (Table 3.1). Table 4.1 summarizes the average percent inhibition and standard deviation of the 45 QNPL extracts that inhibited growth by at least 95% in at least one *C. albicans* strain when screened at 16 μg/mL. Six of the 45 extracts listed in Table 4.1 exhibited growth inhibitory activity against all four *C. albicans* strains (122, 164, 423, 429C, and 429D).

Linking the bioactive extracts in the QNPL with the plant source revealed that extracts of *S. terebinthifolia* leaves (429, 429B, 429C, 429D, and 429E) showed notable growth inhibitory activity against *C. albicans* strains (Table 4.1). Overall, 429, 429C, and 429D exhibited the broadest spectrum of growth inhibitory activity against the tested four *C. albicans* strains. 429D inhibited growth by 95% in all four strains at both time points. For at least one time read (24 or 48 hours), 429 and 429C inhibited growth by at least 95% of all four *C. albicans* strain. The growth inhibitory activity of 429B was observed in only one strain (EU52), while 95% inhibition by 429E was observed for MH12 at 24 hours and EU54 at 48 hours.

Extract No.	MH1 24 hours	MH1 48 hours	MH12 24 hours	MH12 48 hours	EU52 24 hours	EU52 48 hours	EU54 24 hours	EU54 48 hours
122	95.63 ± 1.6	96.98 ± 0.8	99.30 ± 0.11	99.54 ± 0.23	98.46 ± 0.32	98.05 ± 0.20	99.01 ± 0.77	99.166 ± 0.31
164	95.00 ± 0.8	96.65 ± 0.4	99.06 ± 0.19	99.37 ± 0.29	96.61 ± 0.51	-	97.24 ± 1.26	97.12 ± 1.84
423	95.12± 2.3	96.52 ± 1.8	99.44 ± 0.86	-	97.64 ± 0.624	-	97.56 ± 2.03	98.78 ± 0.35
429	94.46 ± 1.4	96.09 ± 1.1	99.63 ± 0.32	99.62 ± 0.17	97.88 ± 0.41	-	98.22 ± 0.51	99.21 ± 0.20
429C	97.23 ± 2.1	98.52 ± 1.1	99.63 ± 0.19	99.58 ± 0.17	96.62 ± 2.60	-	98.00 ± 1.76	99.17 ± 0.25
429D	96.10 ± 1.5	98.03 ± 0.9	99.43 ± 0	99.62 ± 0.24	96.15 ± 2.11	95.10 ± 1.93	96.89 ± 1.17	99.00 ± 0.20
490	99.83 ± 0.3	99.67 ± 3.4	-	-	96.44 ± 6.17	-	98.57 ± 2.47	97.67 ± 7.63
182	-	-	99.18 ± 0.29	98.61 ± 0.71	96.75 ± 0.41	95.48 ± 1.51	96.41 ± 1.96	96.89 ± 2.21
155	-	-	98.30 ± 0.50	96.42 ± 2.45	95.06 ± 1.12	-	-	-
187	-	-	98.18 ± 0.78	97.98 ± 2.03	97.22 ± 1.22	95.04 ± 2.65	97.52 ± 0.83	97.12 ± 1.84
430C	-	-	99.34 ± 0	99.46 ± 0.19	96.58 ± 1.87	96.93 ± 0.97	96.37 ± 1.47	98.31 ± 1.08
429B	-	-	-	-	-	98.02 ± 0.28	-	-
424	-	-	-	99.35 ± 0.17	97.62 ± 0.67	-	-	-
44	-	-	-	-	-	-	97.66 ± 1.90	98.72 ± 0.80
39	-	-	-	-	-	-	96.55 ± 1.67	98.27 ± 0.56
87	-	-	-	-	-	-	95.19 ± 2.79	98.04 ± 1.31
96	-	-	-	-	-	-	96.18 ± 3.36	-
108	-	-	97.65 ± 0.67	96.64 ± 0.63	-	-	98.77 ± 0.43	99.26 ± 0.28
114	-	-	98.29 ± 0.83	-	-	-	99.26 ± 0.74	99.52 ± 0.33
126	-	-	-	-	-	-	96.92 ± 1.30	95.35± 2.63
186	-	-	95.60 ± 2.33	-	-	-	95.31 ± 2.69	-
188	-	-	96.17 ± 4.56	-	-	-	95.172 ± 5.14	95.29 ± 6.01
191	-	-	-	-	-	-	96.69 ± 1.80	96.33 ± 1.57
195	-	-	-	-	-	-	97.66 ± 2.66	-
202	-	-	98.93 ± 0.47	95.76 ± 5.51	-	-	98.07 ± 0.96	-
223	-	-	-	-	-	-	97.23 ± 0.84	98.25 ± 0.76
224C	-	-	-	-	-	-	96.45 ± 1.71	97.82 ± 0.17

Extract No.	$MH1_{24\ hours}$	$MH1_{48\ hours}$	$MH12_{24\ hours}$	$MH12_{48\ hours}$	$EU52_{24\ hours}$	$EU52_{48\ hours}$	$EU54_{24\ hours}$	$EU54_{48\ hours}$
225	-	-	-	-	-	-	95.34 ± 2.40	-
229	-	-	-	-	-	-	95.78 ± 4.14	97.23 ± 2.41
252	-	-	96.88 ± 2.60	-	-	-	98.11 ± 1.07	99.31 ± 0.26
313	-	-	-	-	-	-	98.45 ± 0.51	98.61 ± 0.85
275	-	-	-	-	-	-	94.563 ± 0.69	95.74 ± 4.29
310	-	-	-	-	-	-	96.449 ± 2.87	98.84 ± 0.25
542	-	-	98.69 ± 0.66	98.52 ± 0.33	-	-	95.852 ± 2.14	98.34 ± 0.457
543	-	-	96.86 ± 3.75	97.98 ± 1.89	-	-	97.278 ±1.03	98.85 ± 0.41
495	-	-	-	-	-	-	97.148 ± 1.75	-
62	-	-	-	-	-	-	-	97.63 ± 0.77
132	-	-	-	-	-	-	-	98.46 ± 1.24
429E	-	-	99.20 ± 0.46	-	-	-	-	97.84 ± 0.38
487	-	-	-	-	-	-	-	96.28 ± 0.59
489	-	-	-	-	-	-	-	96.75 ± 2.81
491	-	-	-	-	-	-	-	97.23 ± 0.65
492	-	-	-	-	-	-	-	96.99 ± 2.88
220DF2	-	-	96.36 ± 1.26	96.95 ± 2.03	-	-	-	-

Table 4.1. Extracts from QNPL that inhibited growth by at least 95% at 16 μg/mL. Measured using OD_{600}.
Percent inhibition and standard deviation reported for each extract against MH1, MH12, EU52, and EU54 at 24 and 48 hours.

Growth inhibitory activity of *Schinus terebinthifolia* leaf extracts against *C. albicans*

Further testing was conducted in the five *S. terebinthifolia* leaf extracts (429, 429B, 429C, 429D, 429E) to determine MIC values (in μg/mL) against each of the four *C. albicans* strains. Table 4.2 summarizes the results of these experiments. The anti-*C. albicans* activity of 429, 429B, 429C, 429D, and 429E suggests that *S. terebinthifolia* leaves are promising sources of candidate antifungal therapeutics. The crude extract of the leaf (429) and ethyl acetate partition (429c) exhibited notable growth inhibitory effects for all four *C. albican*s isolates with MIC ranging from 0.125-16 μg/mL (Table 4.2

). 429D and 429E showed activity at against the four *C. albicans* isolates with MIC values ranging 0.125-16 μg/mL and 1-8 μg/mL , respectively (Table 4.2). Table 4.5 and Table 4.6 show a dose-response curve that represents the percent inhibition of *C. albicans* growth when treated with extracts and was measured using $OD_{600.}$ As extract concentrations increased, 429, 429C, and 429D antifungal activity against *C. albicans* also increased (Figure 4.1 and 4.2). 429B and 429E sometimes, but not always, exhibited this trend (Figure 4.1). P-values for extracts at each concentration are summarized in Table 4.3 for MH1 and MH12 and in Table 4.4 for EU52 and EU54.

	429	429B	429C	429D	429E
EU52					
MIC_{50} at 24 hrs	2	16	0.5	2	8
MIC_{90} at 24 hrs	4	-	2	4	16
MIC_{50} at 48 hrs	4	-	2	4	16
MIC_{90} at 48 hrs	8	-	4	8	16
EU54					
MIC_{50} at 24 hrs	1	1	1	1	8
MIC_{90} at 24 hrs	4	-	2	4	16
MIC_{50} at 48 hrs	2	-	2	4	16
MIC_{90} at 48 hrs	4	-	2	4	16
MH1					
MIC_{50} at 24 hrs	4	-	1	4	8
MIC_{90} at 24 hrs	4	-	2	8	16
MIC_{50} at 48 hrs	4	-	2	4	16
MIC_{90} at 48 hrs	4	1	4	8	16
MH12					
MIC_{50} at 24 hrs	1	1	0.25	1	4
MIC_{90} at 24 hrs	2	-	1	2	8
MIC_{50} at 48 hrs	2	-	0.25	2	4
MIC_{90} at 48 hrs	4	-	2	4	8

Table 4.2. Minimal inhibitory concentrations (MIC) in μg/mL of leaf extracts of *Schinus terebinthifolia* against *C. albicans* isolates. Measured using OD_{600}.

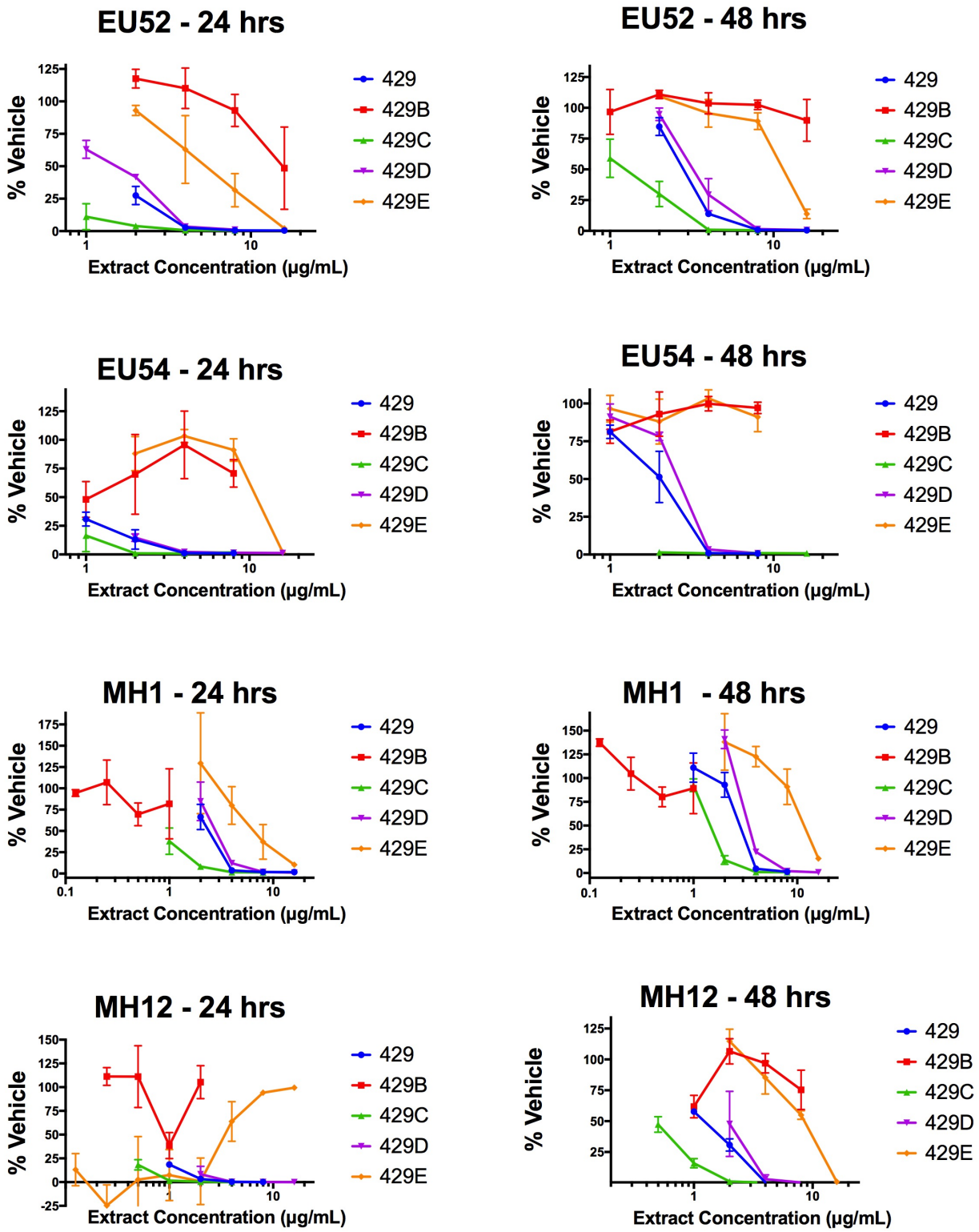

Figure 4.1. Dose-response curve of *C. albicans* strains (EU52, EU54, MH1, and MH12) treated with varying concentrations of extracts (429, 429B, 429C, 429D, 429E). Measured using OD_{600}.

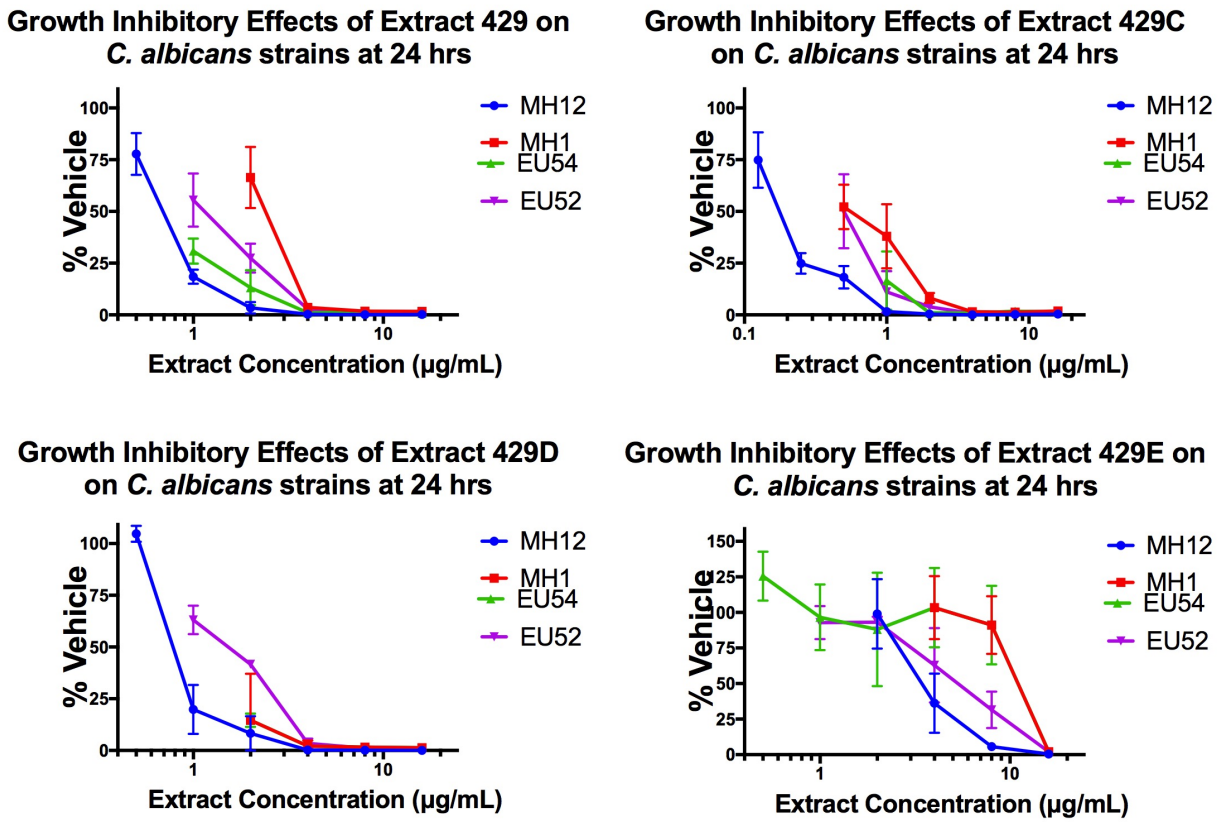

Figure 4.2. Dose-response curve of each extract against four *C. albicans* isolates. Measured using OD_{600}.

	MH1 -24 hours		MH1 - 48 hours		MH12- 24 hours		MH12 - 48 hours	
	µg/mL	p-value	µg/mL	p-value	µg/mL	p-value	µg/mL	p-value
429	16	p<0.05	8	p<0.05	8	p<0.05	8	P<0.01
	8	p<0.05	4	p<0.05	4	P<0.01	4	P<0.01
	4	p=0.06	2	p=0.57	2	p<0.05	2	p<0.05
	2	p<0.05	1	p<0.05	1	P=0.09	1	P=0.14
429B	1	P=0.63	1	P=0.66	2	P=0.81	8	P=0.11
	0.5	P=0.10	0.5	P=0.18	1	P=0.14	4	P=0.66
	0.25	p=0.73	0.25	P=0.70	0.5	P=0.80	2	P=0.63
	0.125	P=0.68	0.125	P=0.44	0.25	P=0.57	1	P=0.16
429C	8	p<0.05	8	p<0.05	4	P<0.01	4	P<0.01
	4	P=0.06	4	p<0.05	2	p<0.05	2	p<0.05
	2	P<0.01	2	P<0.01	1	P=0.07	1	p<0.05
	1	P=0.12	1	P=0.69	0.5	P=0.15	0.5	P=0.22
429D	16	p<0.05	16	p<0.05	16	P<0.01	16	P<0.01
	8	p<0.05	8	p<0.05	8	p<0.05	8	P<0.01
	4	P=0.07	4	P=0.05	4	P<0.01	4	P<0.01
	2	P=0.37	2	p<0.05	2	p<0.05	2	P=0.06
429E	16	p<0.05	16	p<0.05	16	P<0.01	16	P<0.01
	8	P=0.06	8	P=0.61	8	p<0.05	8	p<0.05
	4	P=0.53	4	P=0.36	4	p<0.05	4	P=0.19
	2	P=0.48	2	P=0.14	2	P=0.97	2	P=0.30

Table 4.3. Summary of p-values for varying concentrations of 429, 429B, 429C, 429D, and 429E when screened against MH1 and MH12.

	MH1 -24 hours		MH1 - 48 hours		MH12- 24 hours		MH12 - 48 hours	
	µg/mL	p-value	µg/mL	p-value	µg/mL	p-value	µg/mL	p-value
429	16	p<0.05	8	p<0.05	8	p<0.05	8	P<0.01
	8	p<0.05	4	p<0.05	4	P<0.01	4	P<0.01
	4	p=0.06	2	p=0.57	2	p<0.05	2	p<0.05
	2	p<0.05	1	p<0.05	1	P=0.09	1	P=0.14
429B	1	P=0.63	1	P=0.66	2	P=0.81	8	P=0.11
	0.5	P=0.10	0.5	P=0.18	1	P=0.14	4	P=0.66
	0.25	p=0.73	0.25	P=0.70	0.5	P=0.80	2	P=0.63
	0.125	P=0.68	0.125	P=0.44	0.25	P=0.57	1	P=0.16
429C	8	p<0.05	8	p<0.05	4	P<0.01	4	P<0.01
	4	P=0.06	4	p<0.05	2	p<0.05	2	p<0.05
	2	P<0.01	2	P<0.01	1	P=0.07	1	p<0.05
	1	P=0.12	1	P=0.69	0.5	P=0.15	0.5	P=0.22
429D	16	p<0.05	16	p<0.05	16	P<0.01	16	P<0.01

	MH1 -24 hours		MH1 - 48 hours		MH12- 24 hours		MH12 - 48 hours	
	8	p<0.05	8	p<0.05	8	p<0.05	8	P<0.01
	4	P=0.07	4	P=0.05	4	P<0.01	4	P<0.01
	2	P=0.37	2	p<0.05	2	p<0.05	2	P=0.06
429E	16	p<0.05	16	p<0.05	16	P<0.01	16	P<0.01
	8	P=0.06	8	P=0.61	8	p<0.05	8	p<0.05
	4	P=0.53	4	P=0.36	4	p<0.05	4	P=0.19
	2	P=0.48	2	P=0.14	2	P=0.97	2	P=0.30

Table 4.4. Summary of p-values for varying concentrations of 429, 429B, 429D, and 429E when screened against EU52 and EU54.

Growth inhibitory activity of *S. terebinthifolia* leaf extracts against *C.* non-*albicans*

To evaluate their potential for broad-spectrum antifungal activity, the extracts' growth inhibitory activity were also evaluated against non-*albicans* strains. Table 4.3 and Table 4.4 report MIC values (in μg/mL) of the five extracts against *Candida* non-*albicans* isolates. In addition to exhibiting good growth inhibitory effects for all four *C. albicans* strains, the crude extract of the leaf (429) and the ethyl acetate partition (429c) also showed good growth inhibitory effects for all non-*albicans Candida* isolates with MIC ranging from 0.125-8 μg/mL (Tables 4.5 and 4.6). 429E showed good antifungal activity against the two *C. glabrata* isolates (Table 4.5). Dose response assays showed that growth inhibition of *C. glabrata* isolates increased as concentrations increased (Figure 4.3).

429E also exhibited growth inhibitory effect against the two *C. parapsilosis* and one *C. tropicalis* isolates at 24 hours, 48 hours, or both time points (Table 4.6). At MIC values ranging from 0.125-16 μg/mL, 429D showed bioactivity against the two *C. glabrata,* two *C. parapsilosis* and one *C. tropicalis* isolates at 24 hours, 48 hours, or both time points (Table 4.5 and 4.6). 429B did not present good activity consistently against the different strains and time points (Table 4.2, 4.5, 4.6). The hexane extract did, however, exhibit good activity ranging against two *C. glabrata*

isolates at MIC ranging from 0.5-16 μg/mL at both 24 and 48 hours (Table 4.5). Further testing showed that these extracts inhibit growth via fungistatic effects.

Figure 4.6 shows a dose-response curve that represents the percent inhibition of *C. non-albicans* growth when treated with 429, 429C, and 429D extracts and was measured using $OD_{600.}$ As extract concentrations increased, 429, 429C, and 429D antifungal activity against *C. non-albicans* generally increased (Figure 4.4).

	429	**429B**	**429C**	**429D**	**429E**
CDC0325					
MIC_{50} at 24 hrs	0.5	4	0.25	0.5	0.5
MIC_{90} at 24 hrs	1	4	0.5	1	1
MIC_{50} at 48 hrs	2	16	0.5	2	2
MIC_{90} at 48 hrs	4	16	1	4	4
CDC0323					
MIC_{50} at 24 hrs	0.25	2	0.25	16	0.125
MIC_{90} at 24 hrs	2	2	4	-	16
MIC_{50} at 48 hrs	0.125	0.5	0.125	0.50	0.125
MIC_{90} at 48 hrs	0.25	2	0.125	0.125	2

Table 4.5. Minimal inhibitory concentrations (MIC) of leaf extracts of *S. terebinthifolia* against *C. glabrata* isolates. Measured using OD_{600}.

	429	429B	429C	429D	429E
CDC337					
MIC_{50} at 24 hrs	2	-	1	4	8
MIC_{90} at 24 hrs	4	-	2	4	8
MIC_{50} at 48 hrs	4	-	2	4	8
MIC_{90} at 48 hrs	4	-	4	8	16
CDC342					
MIC_{50} at 24 hrs	0.5	2	0.5	0.5	2
MIC_{90} at 24 hrs	8	-	4	8	16
MIC_{50} at 48 hrs	2	16	1	2	1
MIC_{90} at 48 hrs	16	-	8	-	-
CDC345					
MIC_{50} at 24 hrs	2	-	1	2	2
MIC_{90} at 24 hrs	2	-	2	4	8
MIC_{50} at 48 hrs	2	-	2	4	16
MIC_{90} at 48 hrs	4	-	2	8	16

Table 4.6. Minimal inhibitory concentrations (MIC) of leaf extracts of *S. terebinthifolia* against *C. parapsilosis and C. tropicalis* isolates. Measured using OD_{600}.

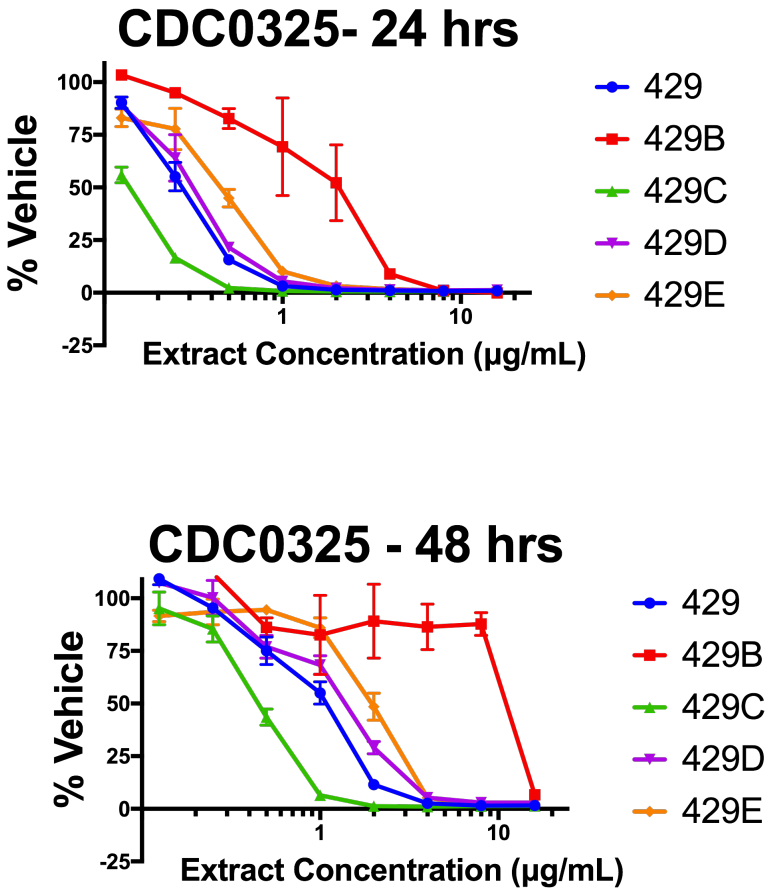

Figure 4.3. Dose-response curve of *C. glabrata* strain (CDC0325) treated with varying concentrations of extracts 429, 429B, 429C, 429D, and 429E (measured using OD_{600}

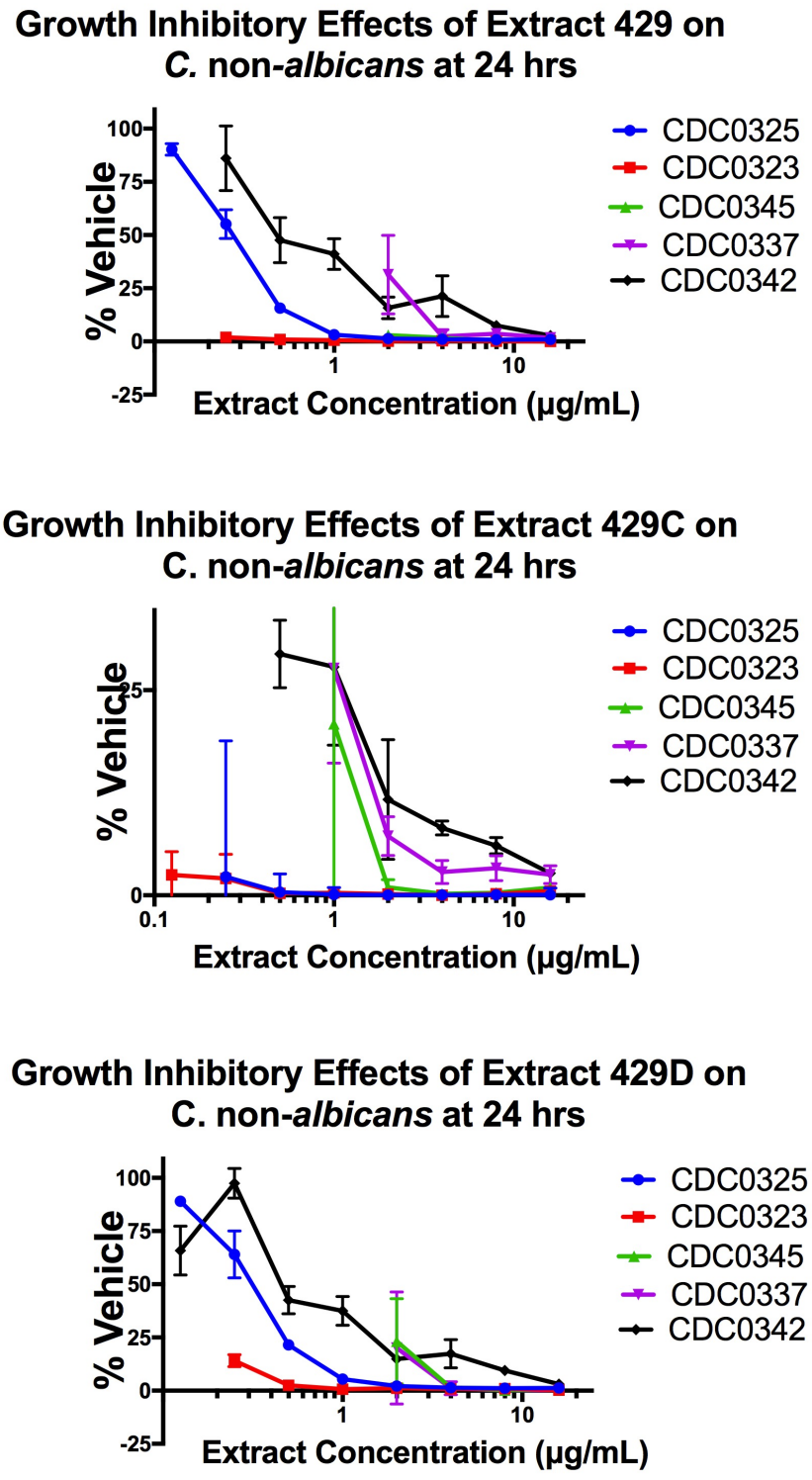

Figure 4.4. Dose-response curve of 429, 429C, and 429D against five *C*. non-*albicans* isolated (measured using OD_{600}).

CHAPTER 5: DISCUSSION

Fungistatic effects of 429, 429B, 429C, 429D, and 429E on *Candida* isolates

Experimental results support our hypothesis that there is at least one antifungal molecule present in the QNPL. Evaluation of growth inhibitory effects of over three hundred extracts led to the identification of five extracts from *S. terebinthifolia* leaves that can inhibit *Candida* growth through fungistatic effects. This suggests that these bioactive constituents are promising candidates that could be used in antifungal therapies by substituting current antifungal drugs or complementing effects of existing antifungal therapeutics. The results corroborate with previous reports of significant antifungal activity by this plant species against *C. albicans*. Aqueous and ethanolic extracts of the *S. terebinthifolia* have been shown to be active against *C. albicans* (Johann, Pizzolatti, Donnici, & Resende, 2007; Schmourlo, Mendonca-Filho, Alviano, & Costa, 2005). Reported MIC values of *S. terebinthifolia* (leaves) extract against *C. albicans* include 1.25 mg/mL, 0.80 mg/mL, and 60 μg/mL (Braga et al., 2007; El-Massry, El-Ghorab, Shaaban, & Shibamoto, 2009; Johann et al., 2007). Johann et al (2007) also reported antifungal activity of *S. terebinthifolia* (leaves) against *C. glabrata*, *C. tropicalis*, and *C. parapsilosis* with MIC values ranging from 30 to 250 μg/mL.

An initial screen of over 300 plant-derived compounds in the QNPL was conducted against four *C. albicans* strains in order to identify extracts that inhibited fungal growth by at least 95%. This screen revealed 45 active extracts that inhibited fungal growth. Using the ethnobotanical data linked to each extract in the QNPL, the goal was to trace the active extracts back to their respective plant species and part (stem, leaf, or bark). The plant species and part that showed the most potent and broadest antifungal activity across all four *C. albicans* were of interest and were pursued for screening against non-*albicans Candida* isolates. Of the initial 45

extracts, 5 (429, 429B, 429C, 429D, and 429E) were traced back to *S. terebinthifolia* leaves and pursued for further testing.

The five extracts were assayed against four *C. albicans*, three *C. glabrata,* two *C. parapsilosis*, and one *C. tropicalis* isolates to determine MIC values. The varied levels of bioactivity against different *C. albicans* and non-*albicans* strains by different extract partitions can be explained by the varying presence and concentrations of antimicrobial molecules in each partition. 429 and 429C inhibited growth of all four *C. albicans* strains, suggesting that the active antifungal constituents were present in the crude extract and in the ethyl acetate extract. 429D inhibited growth of all four *C. albicans* strains at all time points, suggesting that this partition may have concentrated the active antimicrobial molecules and maximized growth inhibitory effects against the fungal pathogen. 429B and 429E showed growth inhibitory effects against only one or two strains, respectively, at either 24 or 48 hours. This suggests that the hexane and aqueous partitions may have yielded a loss or lowered concentration of the antifungal molecules, which possibly diminishing growth inhibitory activity of bioactive constituents. It is reasonable that the growth inhibitory effects were not observed in 429B and 429E because the antimicrobial molecules were lost or too diluted for the growth inhibitory effects to be observed. It is likely that active antifungal constituents were lost in the partitioning process. It should be noted that 429B is the lipophilic partition of the leaves and contains high concentrations of waxes and fats (Muhs, 2015).

A proposed classification of the strength of plant-derived inhibitors was based on the following MIC in Table 5.1 (Braga et al., 2007). Experimental data summarized in Table 4.2, 4.3, and 4.4 indicate strong anti-candidal activity for extracts of *S. terebinthifolia* leaves, as the MIC value range for the most active extracts ranged from 0.125 to 16 μg/mL.

Strength of Plant-Derived Inhibitor	MIC Value Range (mg/mL)
Strong inhibitor	≥0.5
Moderate inhibitor	0.6-1.5
Weak inhibitor	>1.6

Table 5.1. Classifying the strength of plant-derived inhibitors by MIC value range in mg/mL.

Data source: Braga, F. G., Bouzada, M. L., Fabri, R. L., de, O. M. M., Moreira, F. O., Scio, E., & Coimbra, E. S. (2007). Antileishmanial and antifungal activity of plants used in traditional medicine in Brazil. *Journal of Ethnopharmacology, 111*(2), 396-402. doi:10.1016/j.jep.2006.12.006

Antifungal activity is likely due to the bioactivity of secondary metabolites such as tannins, phenols, flavones, xanthones, leucoanthocyanidins, flavonoids, saponins, triterpenes, and steroids in *S. terebinthifolia* leaves (Gomes, Procópio, Napoleão, Coelho, & Paiva, 2013; Johann et al., 2007). A study of the chemical compositions of *S. terebinthifolia* leaves from Egypt revealed that coumaric acid, caffeic acid, and syringic acid constituted the major phenolic compounds in ethanolic extracts (El-Massry et al., 2009). In addition, monoterpenes and sesquisterpenes are essential to the plant's chemical defense system. The terpene composition of *S. terebinthifolia* fresh and dried leaves are summarized in Table 5.2 (El-Massry et al., 2009; Muhs, 2015). Previous reports have also indicated that the aqueous and ethanolic extracts of *S. terebinthifolia* leaves are active against other pathogens such as *Escherchia coli*, *Staphyloccocus aureus,* and *Bacillus subtilis* (Johann et al., 2007).

	Fresh leaves	Dried Leaves
Monoterpenes	4.97	15.18
Sesquiterpenes	56.96	39.25
Oxygenated monoterpenes	34.37	8.13
Oxygenerated sesquiterpenes	3.32	36.07

Table 5.2. Terpene composition by percentage in *S. terebinthifolia* fresh and dried leaves

Data sources: El-Massry, K. F., El-Ghorab, A. H., Shaaban, H. A., & Shibamoto, T. (2009). Chemical compositions and antioxidant/antimicrobial activities of various samples prepared from *Schinus terebinthifolius* leaves cultivated in Egypt. *J Agric Food Chem, 57*(12), 5265-5270. doi:10.1021/jf900638c

Muhs, A. (2015). *Old cures for new wounds: Virulence inhibitors for Brazillian pepper*. (Bachelor of Sciences with Honors), Emory University, Atanta, GA.

Schinus terebinthifolia in traditional medicine

Schinus terebinthifolia Raddi (Anacardiaceae family), more commonly known as the Brazillian peppertree, has been heavily used in traditional medicine to treat skin infection. It is a native to Central and South America and also grows in North America (El-Massry et al., 2009). In 1898, *S. terebinthifolia* was first introduced to southern Florida and is now considered an invasive species (Morton, 1978). This plant was referenced in Brazil as early as 1648 in *Historia Naturalis Brasiliae* by Willem Piso, and its medicinal uses were subsequently described by 19th century European experts in natural history (Brandao et al., 2008; Muhs, 2015). Medicinal use of the plant's leaves, stalks, and flowers persist today to treat a range of conditions (Schmourlo et al., 2005). Ethnomedicinal uses include diabetes, hemorrhages, inflammation, sore throat, oral ulcers, diarrhea, and a range of respiratory problems (Braga et al., 2007). In Brazil, the leaves are

topically applied to treat skin wounds. Traditional healers administered the leaf through infusion to treat respiratory, urinary, and digestive tract-related infections (Gomes et al., 2013). Infusion of *S. terebinthifolia* leaves have also been used to treat oral candidiasis, and the essential oil can be topically applied to treat *Candida* infections (Gomes et al., 2013). In South Africa, the leaf is used in a tea to treat colds (El-Massry et al., 2009). A decoction is also used for depression, back pain, and hypertension (El-Massry et al., 2009).

Global distribution of *Candida* strains and the antimicrobial resistance crisis

Candida species attributed to the incidence of candidemia varies by geography (Eggimann et al., 2011). Figure 2.3 shows that while *C. albicans* remains the dominant causative agent of candidemia cases worldwide, there are epidemiological changes in the distribution of non-*albicans Candida* species causing candidemia. The frequency of *C. albicans* causing candidemia is declining in the U.S, mirroring global trends (Guinea, 2014); on the other hand, the frequency of non-*albicans Candida* species causing candidemia is increasing, such as *C. tropicalis* and *C. parapsilosis*, and *C. glabrata* (Guinea, 2014). This shift is very recent and has been occurred over just the past two decades (Pfaller & Diekema, 2010).

Population-based candidemia surveillance in Atlanta, GA and Baltimore, MD have showed that the dominant pathogen causative of candidemia, *C. albicans*, decreased in prevalence in this role while *C. glabrata* increased in prevalence (Lockhart et al., 2012). Figure 5.1 shows that the prevalence of *C. albicans* infections decreased from 43% to 34%, while *C. glabrata* infections increased from 28% to 31% in Atlanta, GA from 1998-2000 to 2008-2011 (Lockhart et al., 2012).

While the increase in candidemia cases caused by *Candida* non-*albicans* species has not been fully explained yet, it is hypothesized that increased fluconazole use, geography, and the

aging demographic may be major contributors (Healey et al., 2016). Currently, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* account for approximately 25%, 15%, and 10% of candidemia infections, respectively (Healey et al., 2016).

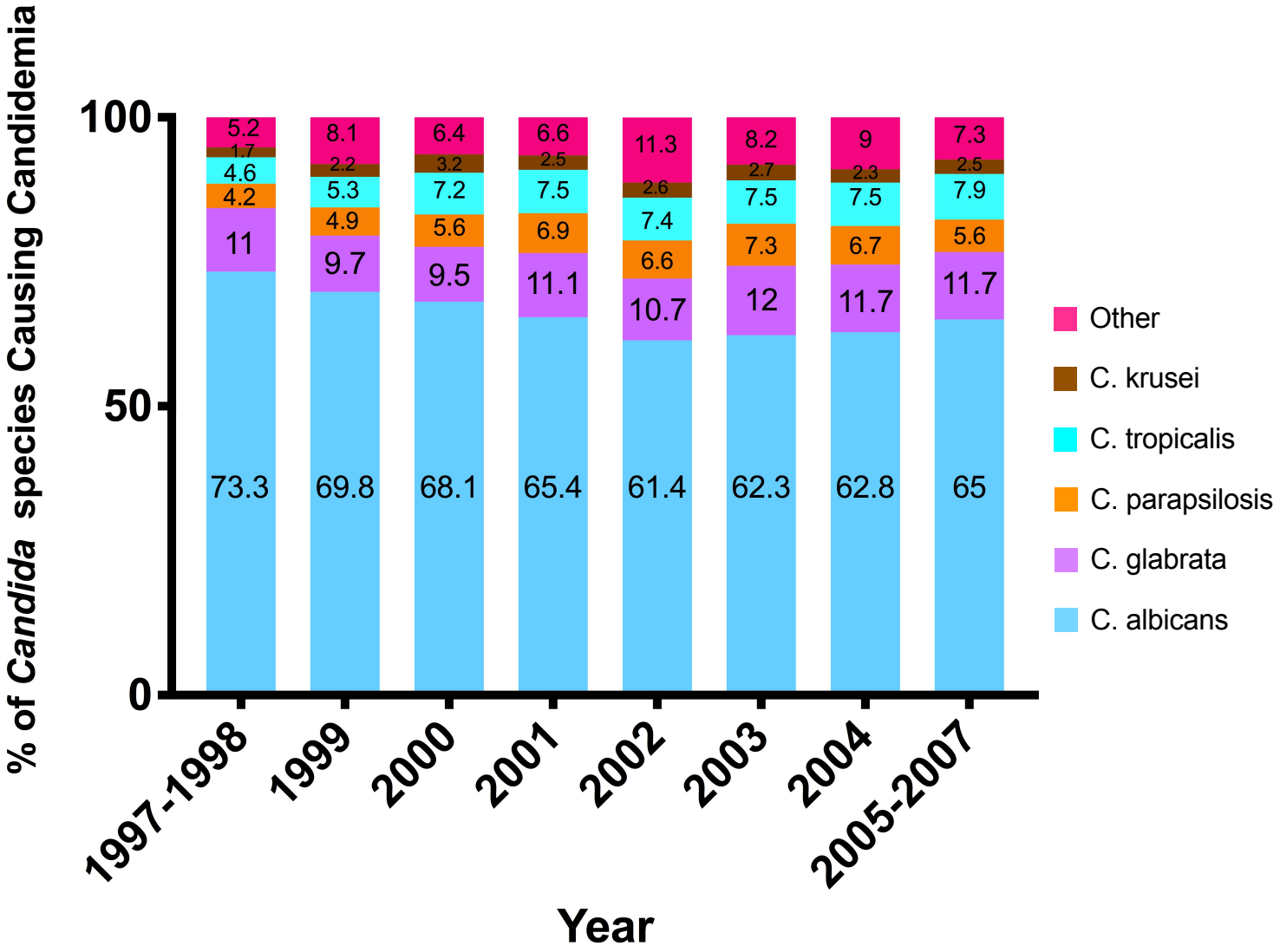

Figure 5.1. Change in invasive candidiasis-causing *Candida* species over time from 1997-2007 worldwide. Data from ARTEMIS DISK registry. Adapted from: Guinea, *Journal of Clinical Microbiology and Infection* 2014 20, 5-10DOI: (10.1111/1469-0691.12539)

Trends in antifungal resistance are depicted in Figure 5.2, which illustrates that both *C. albicans* and non-*albicans* species exhibit increased levels of drug resistance overall. The data shows that in 2012-13, approximately 7% of *Candida* isolates exhibited resistance to fluconazole, 4% to echinocandins, and 1% are multi-drug resistant. Both multidrug resistance and echinocandin-resistance have exhibited an upward trend since 2009 (CDC, 2015). The widespread use of antifungals, especially fluconazole, has driven selection for *Candida* strains that can more readily mutate and acquire resistance. This may account for the various resistant strains across *Candida* species. While less than 5% of *C. albicans* strains are fluconazole-resistant, approximately 30% of *C. glabrata* strains are resistant (Healey et al., 2016). Azole resistance is more likely to develop in *C. glabrata* partly because it can more readily develop mutations in the transcription factor PDR1. This mutation can cause an increased concentration of drug efflux pumps to transport toxic compounds out of the cell (Healey et al., 2016). The haploid state of *C. glabrata* has also been proposed as a contributor to frequent secondary resistance (Fidel, Vazquez, & Sobel, 1999). Due to the high prevalence of azole-resistant *C. glabrata* strains, echinocandins were introduced in the early 2000s as the first-line antifungal treatment (Vallabhaneni et al., 2015). The number of *C. glabrata* strains resistant to echinocandins has doubled from 4% to 8% from 2008 to 2014 (CDC, 2015). A study reported that FSK mutations, which reduces susceptibility or confers resistance to all echinocandins, was found in 14% *C. albicans* and 29% of *C. glabrata* isolates from patients with abdominal candidiasis (Alexander et al., 2013).

With high rates of resistance to both the first-line and second-line of antifungal treatment, *C. glabrata* poses a significant concern as further resistance is likely to continue evolving and spreading if the issue is not addressed (CDC, 2015). The greatest concern with regards to

changing trends in *Candida* species infectivity is the increased emergence of resistant strains, namely among *C. glabrata* (Kullberg & Arendrup, 2015).

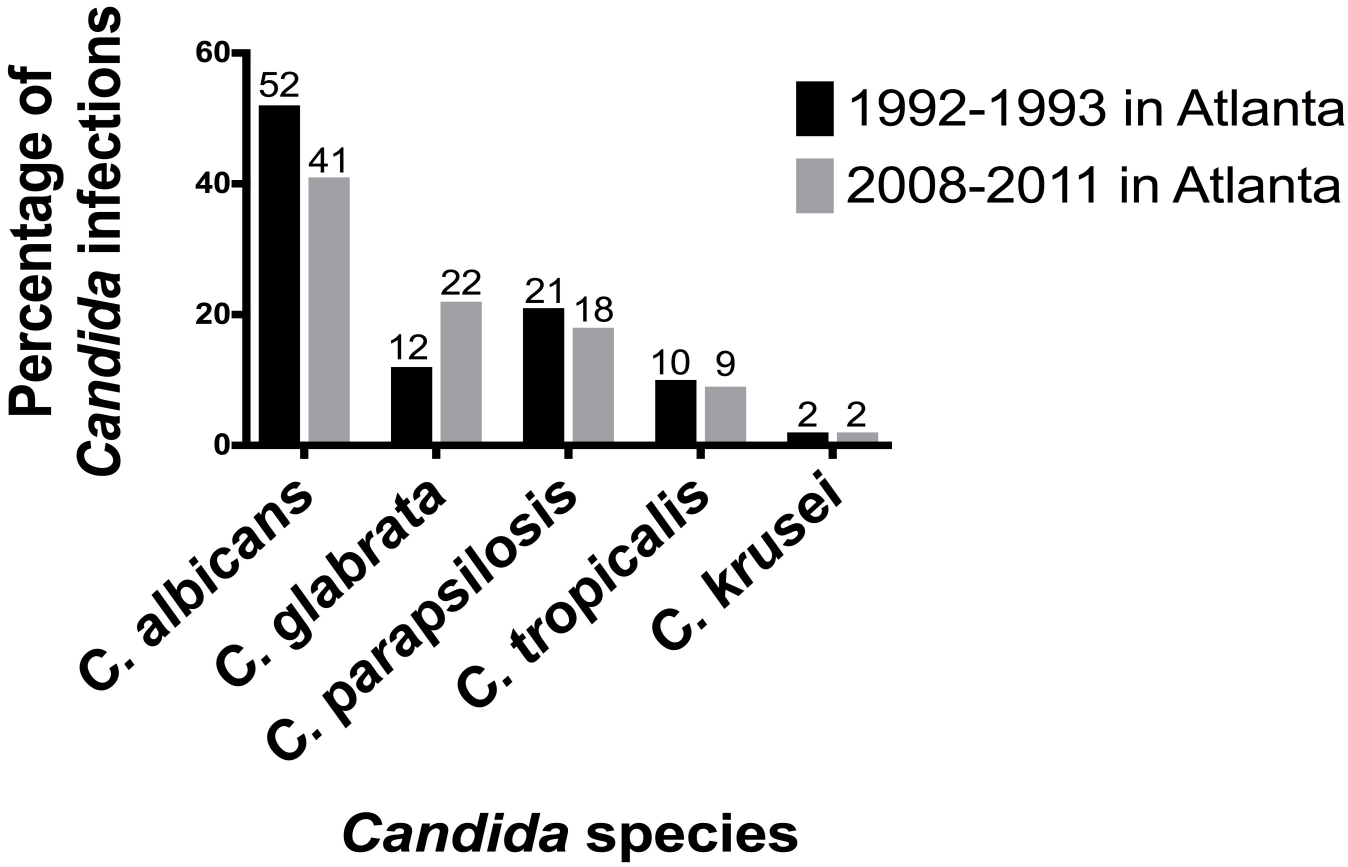

Figure 5.2. Species distribution changes in *Candida* bloodstream infections in Atlanta, GA between 1992-1993 and 2009-2011 in Atlanta, GA between 1998-2000 and 2008-2011 (n=1198). Adapted from: Lockhart et al. Journal of Clinical Microbiology. 2012; 50:3435-3442.

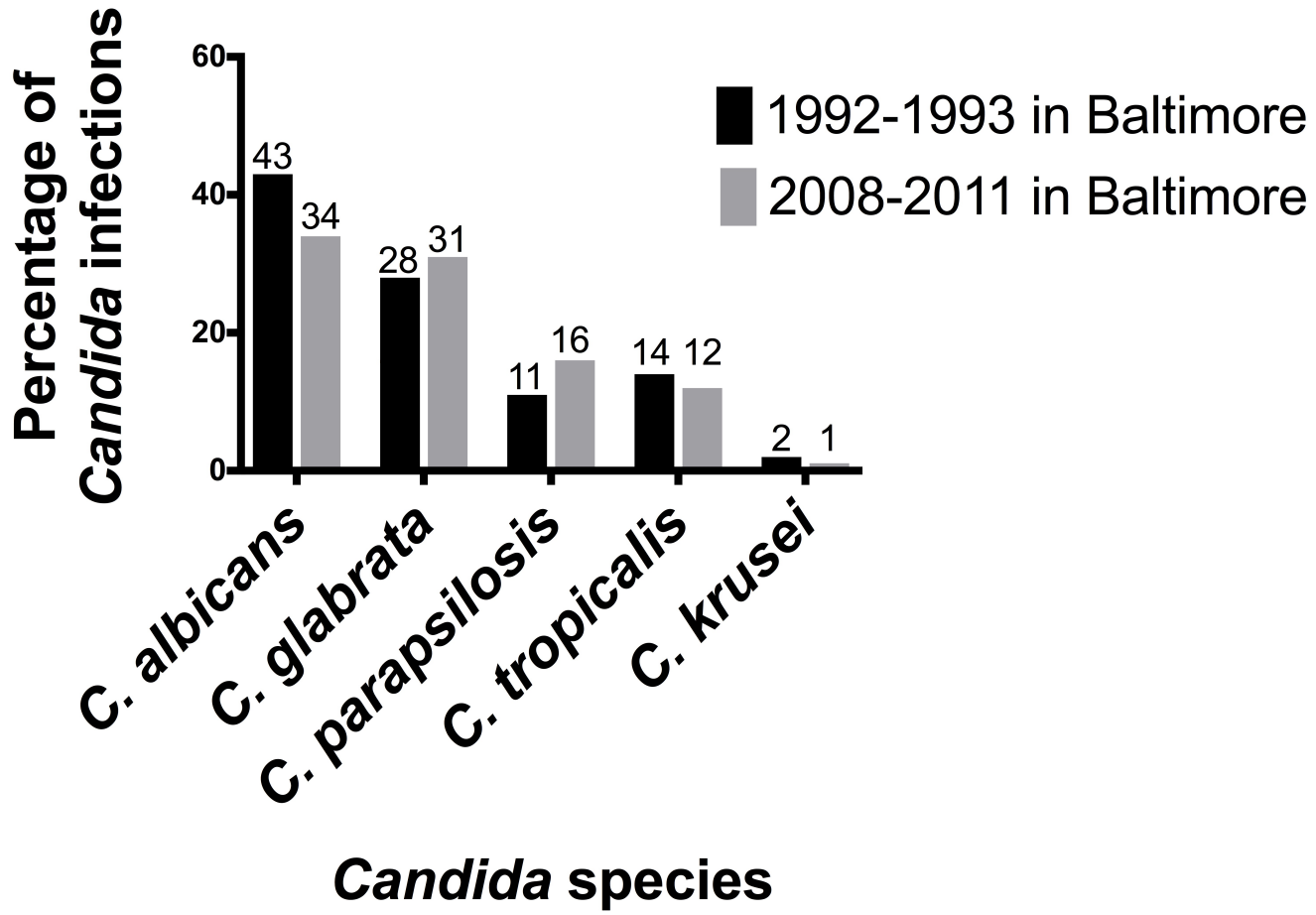

Figure 5.3. Species distribution changes in *Candida* bloodstream infections in Baltimore, MD between 1992-1993 and 2009-2011 in Baltimore, MD between 1998-2000 and 2008-2011 (n=1131). Adapted from: Lockhart et al. Journal of Clinical Microbiology. 2012; 50:3435-3442.

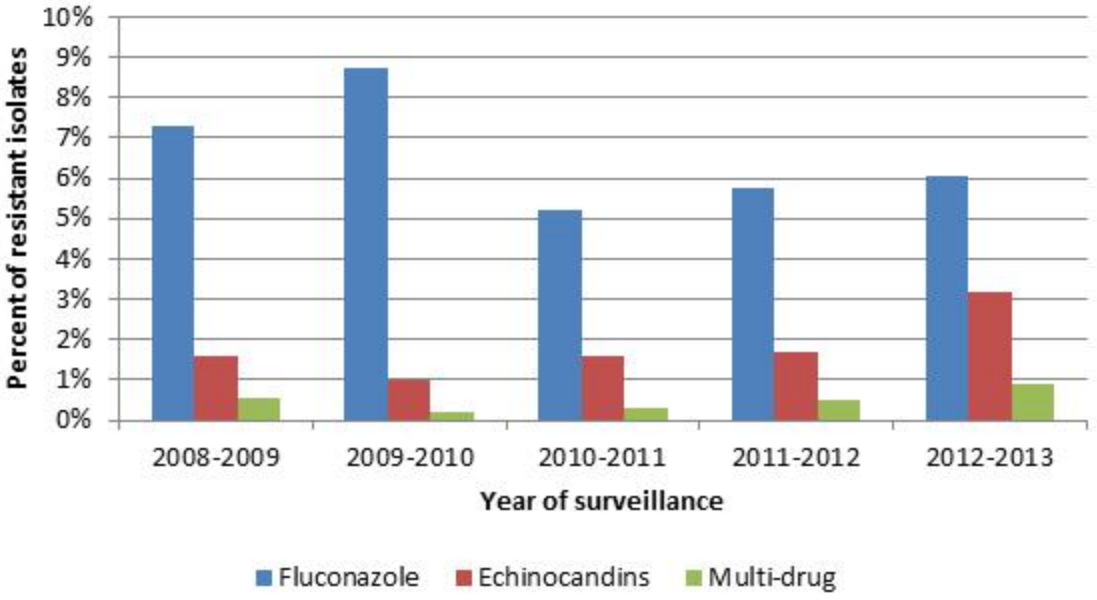

Figure 5.4. Change in the percentage of resistant *Candida* isolates from the bloodstream to fluconazole, echinocandins, and multiple drugs from 2008 to 2013. Source: Center for Disease and Control. (2017). *Invasive Candidiasis Statistics*.

Standard antifungal treatments against *C. albicans*, for example, have been used for a long time, which has provided sufficient time and exerted strong selective forces the evolution of resistant fungal species (O'Neil, 2016). For many types of microbial infections, doctors are running out of effective antibiotic treatments. The arsenal of antimicrobial treatments is already rapidly diminishing in the face of expanding range of drug-resistant infections. In addition, though drug-resistant infections have previously predominated the hospital setting and infected hospitalized patients, communities have begun noting its prevalence outside of the hospital (Guidos et al., 2011). For *Candida* species, these trends are particularly alarming as there are only four classes of antifungal treatments. The loss of effective antimicrobial treatments is concerning as antifungals and antibiotics are non-renewable sources. New antifungals and antibiotics have been steadily declining, as they are less profitable and less attractive for pharmaceutical companies to invest in than chronically prescribed drugs (Pierce & Lopez-Ribot,

2013). However, replenishing the treatment pipeline with novel antifungal agents is imperative to combat the global issue of emerging antifungal resistance.

Future Directions

The next steps include, but are not limited to, conducting experiments to lower standard deviations exhibited by some strains for particular extracts with modifications such as increasing the number of replicates from three to six. Cytotoxicity tests will be performed to determine if the extracts of *S. terebinthifolia* leaves are toxic to human cells in order to ensure the safety and efficacy of its potential therapeutic application. Future experiments will also evaluate the effectiveness of extracts aagainst other mechanisms that contribute to the virulence of *Candida* species, including biofilm formation and yeast-to-hyphae transition. Identification of specific active compounds in 429, 429B, 429C, 429D, and 429E through bioassay guided fractionation and flash chromatography will also be completed. To further substantiate the therapeutic potential of active antifungal constituents, we will utilize animal models to test the effectiveness of these extracts *in vivo* and to determine their safety and efficacy. In addition, only 5 of the 45 active compounds from the QNPL were pursued for further testing against *Candida* isolates. It would be worth repeating the experiments conducted in this project to determine the MIC values of the remaining active extracts against *albicans* and non-*albicans Candida* isolates.

Conclusion

Screening of the QNPL yielded 45 natural product extracts that inhibited four strains of *C. albicans* by at least 95% at a concentration of 16 μg/mL. This finding supports this study's hypothesis, which posited that the screening would yield at least one bioactive molecule against

C. albicans. The notable antifungal activity exhibited by extracts of *S. terebinthifolia* leaves against *C. albicans, C. tropicalis, C. glabrata,* and *C. parasiplosis* suggest that it is therapeutically useful and corroborates the medicinal use of the plant against *Candida* infections. By providing scientific validation of the use of *S. terebinthifolia* in traditional medicine, we demonstrate the efficacy of natural products that have been used for centuries in traditional medicine.

Heavy use of natural plants in medicinal remedies can be observed in India, Africa, Latin America, and Asia, while the US has left the plant kingdom largely unexploited in terms of therapeutics. Antifungal activity by natural plants against drug resistant *C. albicans* and non-*albicans* strains in the current study and others like it strongly suggests that natural plant products are promising sources for novel antifungal drug development. Given the paucity of antifungal therapeutics and the dismal success rate of standard drug discovery techniques, an interdisciplinary approach that blends biological and ethnobotanical knowledge may represent the most promising avenue for development of new therapeutics against *Candida* infections.

References

Alexander, B. D., Johnson, M. D., Pfeiffer, C. D., Jimenez-Ortigosa, C., Catania, J., Booker, R., . . . Pfaller, M. A. (2013). Increasing echinocandin resistance in Candida glabrata: clinical failure correlates with presence of FKS mutations and elevated minimum inhibitory concentrations. *Clinical Infectious Diseases* , *56*(12), 1724-1732. doi:10.1093/cid/cit136

Andes, D. R., Safdar, N., Baddley, J. W., Playford, G., Reboli, A. C., Rex, J. H., . . . Grp, M. S. (2012). Impact of Treatment Strategy on Outcomes in Patients with Candidemia and Other Forms of Invasive Candidiasis: A Patient-Level Quantitative Review of Randomized Trials. *Clinical Infectious Diseases, 54*(8), 1110-1122. doi:10.1093/cid/cis021

Antinori, S., Milazzo, L., Sollima, S., Galli, M., & Corbellino, M. (2016). Candidemia and invasive candidiasis in adults: A narrative review. *European Journal of Internal Medicine, 34*, 21-28. doi:10.1016/j.ejim.2016.06.029

Arendrup, M. C., Dzajic, E., Jensen, R. H., Johansen, H. K., Kjaeldgaard, P., Knudsen, J. D., . . . Schonheyder, H. C. (2013). Epidemiological changes with potential implication for antifungal prescription recommendations for fungaemia: data from a nationwide fungaemia surveillance programme. *Clinical Microbiology and Infection, 19*(8), E343-353. doi:10.1111/1469-0691.12212

Asmundsdottir, L. R., Erlendsdottir, H., & Gottfredsson, M. (2013). Nationwide study of candidemia, antifungal use, and antifungal drug resistance in Iceland, 2000 to 2011. *Journal of Clinical Microbiology, 51*(3), 841-848. doi:10.1128/JCM.02566-12

Braga, F. G., Bouzada, M. L., Fabri, R. L., de, O. M. M., Moreira, F. O., Scio, E., & Coimbra, E. S. (2007). Antileishmanial and antifungal activity of plants used in traditional medicine in Brazil. *Journal of Ethnopharmacology, 111*(2), 396-402. doi:10.1016/j.jep.2006.12.006

Brandao, M. G. L., Zanetti, N. N. S., Oliveira, P., Grael, C. F. F., Santos, A. C. P., & Monte-Mor, R. L. M. (2008). Brazilian medicinal plants described by 19th century European naturalists and in the Official Pharmacopoeia. *Journal of Ethnopharmacology, 120*(2), 141-148. doi:10.1016/j.jep.2008.08.004

Campoy, S., & Adrio, J. L. Antifungals. *Biochemical Pharmacology*. doi:http://dx.doi.org/10.1016/j.bcp.2016.11.019

CDC. (2014). Genital/vulvovaginal candidiasis Retrieved from https://www.cdc.gov/fungal/diseases/candidiasis/genital/index.html

CDC. (2015a, October 6, 2016). Candidiasis. Retrieved from https://www.cdc.gov/fungal/diseases/candidiasis/

CDC. (2015b). Oropharyngeal / Esophageal Candidiasis Retrieved from https://www.cdc.gov/fungal/diseases/candidiasis/

CDC. (2015c). Treatment for Invasive Candidiasis. Retrieved from https://www.cdc.gov/fungal/diseases/candidiasis/invasive/treatment.html

Clancy, C. J., & Nguyen, M. H. (2013). Finding the "missing 50%" of invasive candidiasis: how nonculture diagnostics will improve understanding of disease spectrum and transform

patient care. *Clinical Microbiology and Infection, 56*(9), 1284-1292. doi:10.1093/cid/cit006

Cleveland, A. A., Farley, M. M., Harrison, L. H., Stein, B., Hollick, R., Lockhart, S. R., . . . Chiller, T. M. (2012). Changes in Incidence and Antifungal Drug Resistance in Candidemia: Results From Population-Based Laboratory Surveillance in Atlanta and Baltimore, 2008-2011. *Clinical Infectious Diseases, 55*(10), 1352-1361. doi:10.1093/cid/cis697

Cleveland, A. A., Harrison, L. H., Farley, M. M., Hollick, R., Stein, B., Chiller, T. M., . . . Park, B. J. (2015). Declining incidence of candidemia and the shifting epidemiology of Candida resistance in two US metropolitan areas, 2008-2013: results from population-based surveillance. *PLoS One, 10*(3), e0120452. doi:10.1371/journal.pone.0120452

Cosgrove, S. E., & Carmeli, Y. (2003). The impact of antimicrobial resistance on health and economic outcomes. *Clinical Infectious Diseases, 36*(11), 1433-1437. Retrieved from <Go to ISI>://WOS:000183126500013

D.K., O., S.F., V. V., & A.N., M. (2015). Annickia affinis and A. chlorantha (Enantia chlorantha) – A review of two closely related medicinal plants from tropical Africa. *Journal of Ethnopharmacology, 176*, 438-462.

Eggimann, P., Bille, J., & Marchetti, O. (2011). Diagnosis of invasive candidiasis in the ICU. *Annals of Intensive Care, 1*(1), 1.

El-Massry, K. F., El-Ghorab, A. H., Shaaban, H. A., & Shibamoto, T. (2009). Chemical compositions and antioxidant/antimicrobial activities of various samples prepared from Schinus terebinthifolius leaves cultivated in Egypt. *Journal of Agricultural and Food Chemistry, 57*(12), 5265-5270. doi:10.1021/jf900638c

Fidel, P. L., Jr., Vazquez, J. A., & Sobel, J. D. (1999). Candida glabrata: review of epidemiology, pathogenesis, and clinical disease with comparison to C. albicans. *Clinical Microbiology Reviews, 12*(1), 80-96. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9880475

Gomes, F., Procópio, T., Napoleão, T., Coelho, L., & Paiva, P. (2013). Antimicrobial lectin from Schinus terebinthifolius leaf. *Journal of Applied Microbiology, 114*(3), 672-679.

Gudlaugsson, O., Gillespie, S., Lee, K., Vande Berg, J., Hu, J., Messer, S., . . . Diekema, D. (2003). Attributable mortality of nosocomial candidemia, revisited. *Clinical Infectious Diseases, 37*(9), 1172-1177. doi:10.1086/378745

Guidos, R. J., Spellberg, B., Blaser, M., Boucher, H. W., Bradley, J. S., Eisenstein, B. I., . . . Idsa. (2011). Combating Antimicrobial Resistance: Policy Recommendations to Save Lives. *Clinical Infectious Diseases, 52*, S397-S428. doi:10.1093/cid/cir153

Healey, K. R., Jimenez Ortigosa, C., Shor, E., & Perlin, D. S. (2016). Genetic Drivers of Multidrug Resistance in Candida glabrata. *Frontiers in Microbiology, 7*, 1995. doi:10.3389/fmicb.2016.01995

HealthyPeople. (2017). Healthcare-Associated Infections. Retrieved from https://www.healthypeople.gov/2020/topics-objectives/topic/healthcare-associated-infections

Horn, D. L., Neofytos, D., Anaissie, E. J., Fishman, J. A., Steinbach, W. J., Olyaei, A. J., . . . Webster, K. M. (2009). Epidemiology and Outcomes of Candidemia in 2019 Patients: Data from the Prospective Antifungal Therapy Alliance Registry. *Clinical Infectious Diseases, 48*(12), 1695-1703. doi:10.1086/599039

Institute, C. a. L. S. (2008). Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeast: Approved Standard-Third Edition. (Vol. M27-A3
). Wayne, PA: Clinical and Laboratory Standards Institute.

Johann, S., Pizzolatti, M. G., Donnici, C. L., & Resende, M. A. d. (2007). Antifungal properties of plants used in Brazilian traditional medicine against clinically relevant fungal pathogens. *Brazilian Journal of Microbiology, 38*(4), 632-637.

Kanafani, Z. A., & Perfect, J. R. (2008). Resistance to Antifungal Agents: Mechanisms and Clinical Impact. *Clinical Infectious Diseases, 46*(1), 120-128. doi:10.1086/524071

Kullberg, B. J., & Arendrup, M. C. (2015). Invasive Candidiasis. *N Engl J Med, 373*(15), 1445-1456. doi:10.1056/NEJMra1315399

Laniado-Laborin, R., & Cabrales-Vargas, M. N. (2009). Amphotericin B: side effects and toxicity. *Revista Iberoamericana de Micología, 26*(4), 223-227. doi:10.1016/j.riam.2009.06.003

Leroy, O., Gangneux, J. P., Montravers, P., Mira, J. P., Gouin, F., Sollet, J. P., . . . AmarCand Study, G. (2009). Epidemiology, management, and risk factors for death of invasive Candida infections in critical care: a multicenter, prospective, observational study in France (2005-2006). *Critical Care Medicine, 37*(5), 1612-1618. doi:10.1097/CCM.0b013e31819efac0

Lockhart, S. R., Iqbal, N., Cleveland, A. A., Farley, M. M., Harrison, L. H., Bolden, C. B., . . . Chiller, T. (2012). Species identification and antifungal susceptibility testing of Candida bloodstream isolates from population-based surveillance studies in two U.S. cities from 2008 to 2011. *Journal of Clinical Microbiology, 50*(11), 3435-3442. doi:10.1128/JCM.01283-12

Lortholary, O., Renaudat, C., Sitbon, K., Madec, Y., Denoeud-Ndam, L., Wolff, M., . . . French Mycosis Study, G. (2014). Worrisome trends in incidence and mortality of candidemia in intensive care units (Paris area, 2002-2010). *Intensive Care Med, 40*(9), 1303-1312. doi:10.1007/s00134-014-3408-3

Magill, S. S., Edwards, J. R., Bamberg, W., Beldavs, Z. G., Dumyati, G., Kainer, M. A., . . . Healthcare, E. I. P. (2014). Multistate Point- Prevalence Survey of Health Care-Associated Infections. *New England Journal of Medicine, 370*(13), 1198-1208. doi:10.1056/NEJMoa1306801

Mean, M., Marchetti, O., & Calandra, T. (2008). Bench-to-bedside review: Candida infections in the intensive care unit. *Critical Care, 12*(1), 204. doi:10.1186/cc6212

Morgan, J., Meltzer, M. I., Plikaytis, B. D., Sofair, A. N., Huie-White, S., Wilcox, S., . . . Teutsch, S. M. (2005). Excess mortality, hospital stay, and cost due to candidemia: A case-control study using data from population-based candidemia surveillance. *Infection Control and Hospital Epidemiology, 26*(6), 540-547. doi:Doi 10.1086/502581

Morton, J. F. (1978). Brazilian Pepper - Its Impact on People, Animals and the Environment. *Economic Botany, 32*(4), 353-359. doi:Doi 10.1007/Bf02907927

Muhs, A. (2015). *Old cures for new wounds: Virulence inhibitors for Brazillian pepper*. (Bachelor of Sciences with Honors), Emory University, Atanta, GA.

Mukherjee, P. K., & Chandra, J. (2004). Candida biofilm resistance. *Drug Resistance Updates, 7*(4-5), 301-309. doi:10.1016/j.drup.2004.09.002

Nelson, K., Lyles, J., Saitta, A., & Quave, C. L. (2016). Discovery of plants and fungi with antibacterial activity against Propionibacterium acnes. *Journal of Investigative Dermatology, 136*(5), S90-S90. Retrieved from <Go to ISI>://WOS:000380028800509

O'Neil, J. (2016). *Tackling drug-resistant infections globally: final report and recommendations*. The review on antimicrobial resistance.

Onsare, J. G., & Arora, D. S. (2015). Antibiofilm potential of flavonoids extracted from Moringa oleifera seed coat against Staphylococcus aureus, Pseudomonas aeruginosa and Candida albicans. *Journal of Applied Microbiology, 118*(2), 313-325. doi:10.1111/jam.12701

Pappas, P. G. (2006). Invasive candidiasis. *Infectious Disease Clinics of North America, 20*(3), 485-506. doi:10.1016/j.idc.2006.07.004

Pappas, P. G., Kauffman, C. A., Andes, D. R., Clancy, C. J., Marr, K. A., Ostrosky-Zeichner, L., . . . Sobel, J. D. (2016). Clinical Practice Guideline for the Management of Candidiasis: 2016 Update by the Infectious Diseases Society of America. *Clinical Infectious Diseases, 62*(4), e1-50. doi:10.1093/cid/civ933

Patridge, E., Gareiss, P., Kinch, M. S., & Hoyer, D. (2016). An analysis of FDA-approved drugs: natural products and their derivatives. *Drug Discovery Today, 21*(2), 204-207. doi:10.1016/j.drudis.2015.01.009

Pfaller, M. A., & Diekema, D. J. (2010). Epidemiology of invasive mycoses in North America. *Critical Reviews in Microbiology, 36*(1), 1-53. doi:10.3109/10408410903241444

Pierce, C. G., & Lopez-Ribot, J. L. (2013). Candidiasis drug discovery and development: new approaches targeting virulence for discovering and identifying new drugs. *Expert Opinion on Drug Discovery, 8*(9), 1117-1126. doi:10.1517/17460441.2013.807245

Quave, C. L., Lyles, J. T., Kavanaugh, J. S., Nelson, K., Parlet, C. P., Crosby, H. A., . . . Horswill, A. R. (2015). Castanea sativa (European Chestnut) Leaf Extracts Rich in Ursene and Oleanene Derivatives Block Staphylococcus aureus Virulence and Pathogenesis without Detectable Resistance. *PLoS One, 10*(8), e0136486. doi:10.1371/journal.pone.0136486

Quave, C. L., & Pieroni, A. (2015). A reservoir of ethnobotanical knowledge informs resilient food security and health strategies in the Balkans. *Nature Plants, 1*, 14021. doi:10.1038/nplants.2014.21

Quave, C. L., Plano, L. R., & Bennett, B. C. (2011). Quorum sensing inhibitors of Staphylococcus aureus from Italian medicinal plants. *Planta Medica, 77*(2), 188-195. doi:10.1055/s-0030-1250145

Quave, C. L., Plano, L. R., Pantuso, T., & Bennett, B. C. (2008). Effects of extracts from Italian medicinal plants on planktonic growth, biofilm formation and adherence of methicillin-resistant Staphylococcus aureus. *Journal of Ethnopharmacology, 118*(3), 418-428. doi:10.1016/j.jep.2008.05.005

Richards, M. J., Edwards, J. R., Culver, D. H., & Gaynes, R. P. (2000). Nosocomial infections in combined medical-surgical intensive care units in the United States. *Infection Control & Hospital Epidemiology, 21*(8), 510-515. doi:10.1086/501795

Roemer, T., & Krysan, D. J. (2014). Antifungal Drug Development: Challenges, Unmet Clinical Needs, and New Approaches. *Cold Spring Harbor Perspectives in Medicine, 4*(5). doi:10.1101/cshperspect.a019703

Savoia, D. (2012). Plant-derived antimicrobial compounds: alternatives to antibiotics. *Future Microbiology, 7*(8), 979-990. doi:10.2217/Fmb.12.68

Schmourlo, G., Mendonca-Filho, R. R., Alviano, C. S., & Costa, S. S. (2005). Screening of antifungal agents using ethanol precipitation and bioautography of medicinal and food plants. *Journal of Ethnopharmacology, 96*(3), 563-568. doi:10.1016/j.jep.2004.10.007

Vallabhaneni, S., Cleveland, A. A., Farley, M. M., Harrison, L. H., Schaffner, W., Beldavs, Z. G., . . . Smith, R. M. (2015). Epidemiology and Risk Factors for Echinocandin Nonsusceptible Candida glabrata Bloodstream Infections: Data From a Large Multisite Population-Based Candidemia Surveillance Program, 2008-2014. *Open Forum Infectious Diseases, 2*(4), ofv163. doi:10.1093/ofid/ofv163

Vincent, J. L., Rello, J., Marshall, J., Silva, E., Anzueto, A., Martin, C. D., . . . Investigators, E. I. G. o. (2009). International study of the prevalence and outcomes of infection in intensive care units. *JAMA, 302*(21), 2323-2329. doi:10.1001/jama.2009.1754

Watamoto, T., Egusa, H., Sawase, T., & Yatani, H. (2015). Screening of Pharmacologically Active Small Molecule Compounds Identifies Antifungal Agents Against Candida Biofilms. *Frontiers in Microbiology, 6*, 1453. doi:10.3389/fmicb.2015.01453

WHO. (2003). *WHO guidelines on good agricultural and collection practices (GACP) for medicinal plants*.

Wisplinghoff, H., Bischoff, T., Tallent, S. M., Seifert, H., Wenzel, R. P., & Edmond, M. B. (2004). Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clinical Infectious Diseases, 39*(3), 309-317. doi:10.1086/421946

Xu, J., Ramos, A. R., Vilgalys, R., & Mitchell, T. G. (2000). Clonal and spontaneous origins of fluconazole resistance in Candida albicans. *Journal of Clinical Microbiology, 38*(3), 1214-1220. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10699025

Zida, A., Bamba, S., Yacouba, A., Ouedraogo-Traore, R., & Guiguemde, R. T. (2017). Anti-Candida albicans natural products, sources of new antifungal drugs: A review. *Journal de Mycologie Médicale, 27*(1), 1-19. doi:10.1016/j.mycmed.2016.10.002

Zilberberg, M. D., Shorr, A. F., & Kollef, M. H. (2008). Secular trends in candidemia-related hospitalization in the United States, 2000-2005. *Infection Control and Hospital Epidemiology, 29*(10), 978-980. doi:10.1086/591033