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.

Stephanie Pintas April 1, 2018

Antifungal Activity of Botanical Extracts against *Malassezia furfur* Implicated in Common Skin Conditions

By

Stephanie Pintas

Dr. Cassandra Quave

Adviser

Department of Dermatology

Dr. Cassandra Quave

Adviser

Dr. Meleah Hickman

Committee Member

Dr. Sarah Chisolm

Committee Member

2018

Antifungal Activity of Botanical Extracts against *Malassezia furfur* Implicated in Common Skin Conditions

by

Stephanie Pintas

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 Arts with Honors

Human Health Department

2018

Abstract

Antifungal Activity of Botanical Extracts against *Malassezia furfur* Implicated in Common Skin Conditions

By Stephanie Pintas

Malassezia furfur is a commensal lipophilic fungus found on the surface of the skin that is implicated in the pathogenesis of several prevalent skin conditions, including Atopic Dermatitis (AD), Seborrheic Dermatitis (SD), and Tinea Versicolor (TV). With antifungal resistance becoming an increasing concern, it is important to identify new therapeutic options. While several studies have demonstrated anti-*Malassezia* activity with botanical extracts, the concentration tested is often high (>500 μg/mL) and not evaluated for mammalian cytotoxicity.

The purpose of this study was to screen for potential anti-*Malassezia* activity of botanical extracts using a unique chemical library, the Quave Natural Products Library (QNPL), based on plants used in the traditional treatment of infectious and inflammatory skin disease. Initial screen of the QNPL (~1000 extracts) was conducted at 16 μg/mL, but failed to demonstrate anti-*Malassezia* activity. A second screen of the library was conducted at 128 μg/mL by broth microtiter plate dilution method in triplicate.

The screen revealed potential activity for crude extracts 31, 55, 66, 637, and 1251 and pure compounds 448, 449, and 451. Further analysis via two-fold serial dilution in quadruplicate demonstrated 48-hour MIC_{90} at 128 μg/mL for extract 1251, *Allium amethystinum*, and 16 μg/mL for pure compound 451 as compared to 2 μg/mL for Ketoconazole control. Minimum fungicidal concentration (MFC) was determined by agar plate test. MFC was 256 μg/mL for both pure compound 451 and extract 1251. We also conducted two-fold serial dilution on extracts 158 and 237, ethanolic and methanolic crude extracts of *Allium cepa* (onion) in the same genus as *A. amethystinum*, but failed to observe any growth inhibition at either 24 or 48-hour optical density reads.

To our knowledge, this is the first report of antifungal activity of *Allium amethystinum*. Mammalian cytotoxicity as determined by testing of human keratinocytes (HaCaTs) via LDH assay revealed that extract 1251 did not achieve an IC_{50} in HaCaTs at 512 μg/mL, twice the concentration that exhibited fungicidal activity. This provides promising evidence for potential topical treatments that include *Allium amethystinum*.

Chemical inhibition of *Malassezia furfur* at lesional skin sites could support reduced allergenic responses and inflammation, improving symptoms of disease and decreasing risk of fungal resistance from current topical treatments. Future efforts to isolate the active compound(s) of extract 1251 via iterative bioassay guided fractionation are warranted.

Antifungal Activity of Botanical Extracts against *Malassezia furfur* Implicated in Common Skin Conditions

by

Stephanie Pintas

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 Arts with Honors

Human Health Department

2018

Acknowledgements

The idea for my thesis all began because of my own struggles with atopic dermatitis. I grew increasingly frustrated with the inefficacy of topical steroids at relieving my symptoms long-term. I started doing personal research online and found several “natural” remedies such as tea tree oil and apple cider vinegar. However, I wanted to put these ideas to the test and see if I could discover botanical extracts that truly exhibited antimicrobial effects in the lab. My hope is that this research will provide insights and information for future clinical testing and topical natural products that may help individuals who also suffer from chronic skin conditions.

I would like to express my sincere appreciation and gratitude to Dr. Quave, for supporting my research, guiding me through this immense process, and pushing me to learn more about botanical medicine than I could ever imagine. Without her guidance and expertise, this project would not have been possible, and I am so appreciative that she was willing to accept me into her lab to pursue this project. I would also like to thank my thesis committee members, Dr. Chisolm, for providing knowledge on the skin conditions I researched and Dr. Hickman for her interest and expertise in fungal microbes.

I would like to especially thank Kate Nelson for teaching me the microbiology techniques necessary for my experiments and Dr. Lyles for his expertise in phytochemistry. A huge thank you to Dr. Freeman for providing her support and endless tips for creating a thorough thesis and presentation.

I am thankful to my parents, especially my mother who sparked my interest in this topic because of its clinical relevance in dermatology. I would also like to thank my father, grandparents, and brothers for supporting and encouraging me throughout the process.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1 Introduction

1.1 Skin Microbiome

The skin is the body's largest organ and is colonized by various microbes, including bacteria, fungi, and viruses, most of which contribute to a symbiotic relationship (Grice and Segre, 2011). The relationship with commensal organisms prevents and protects our skin from more harmful and invasive organisms in two central ways. First, resident commensal organisms prevent harmful microbes from colonizing, and potentially invading, through competition for resources (Naik et al., 2012). Secondly, the commensal bacteria and fungi on our skin, such as *Staphylococcus epidermidis*, may activate T cells in response to cousin pathogenic microorganisms such as *Staphylococcus aureus* (Naik et al., 2012).

The primary goal of the skin is to provide a physical barrier from invasive organisms and toxic substances. To accomplish this goal, the skin remains slightly acidic around a pH of 5 to inhibit growth of pathogenic microorganisms such as *Staphylococcus aureus* (*S. aureus*), which thrive at a higher pH (Eberlein-Konig et al., 2000; Valero et al., 2009). Besides low pH, free fatty acids are another important component of the skin that protect against pathogenic microbes. Sebum triglycerides are broken down into free fatty acids by lipases secreted from commensal bacteria such as *Propionibacterium acnes* (Nguyen et al., 2015). These fatty acids are then incorporated into the cell membrane of microbes, increasing fluidity and disrupting membrane integrity (Nguyen et al., 2015). In *S. aureus*, lauric acid, capric acid, myristic acid, and linoleic acid exhibit antibacterial activity by causing protein leakage and disrupting nutrient uptake (Nguyen et al., 2015). Lastly, antimicrobial peptides (APs) produced by our innate immune

system play an essential role in targeting invasive organisms – their role in protecting the skin and implication with *Malassezia* spp. is discussed in further detail below.

Disruptions in the skin microbiome leading to colonization of pathogenic microorganisms and skin disorders can be endogenous or exogenous in nature. Endogenous or internal disruptions include age, sex, diet, genetic variations that determine an individual's microbial community, or the immune system, which alters the microbiome based on prior infectious exposures and/or inflammation (Grice and Segre, 2011). Adults have greater microbial diversity than adolescents and the elderly, and males have increased colonization of *Propionibacterium* and *Corynebacterium* species (Ying et al., 2015). Males also have higher colonization of *Malassezia* spp. in sebum-rich areas due to higher androgen production that creates oilier skin and a richer growth environment (Dessinioti and Katsambas, 2013). Exogenous factors include excessive handwashing which removes both beneficial and harmful microorganisms from the surface, location and climate, hygiene, and antibiotic usage (Grice and Segre, 2011). Atopic dermatitis (AD), for example, is found in higher rates in developed, urban areas with increased hygienic practices (Williams and Gallo, 2015), while tinea versicolor (TV) is more common in humid climates (Levin, 2009; Williams and Gallo, 2015).

Pathogenicity of *Malassezia* in Chronic Skin Disorders

Malassezia is a commensal fungus found on the skin of healthy individuals; however, it takes on a pathogenic role in several skin disorders including Atopic dermatitis (AD), Seborrheic dermatitis (SD), and Tinea Versicolor (TV). *Malassezia* is the most common fungal genus on the skin and may constitute as much as 80% of the total skin fungal population (Gao et al., 2010). *Malassezia* species are more commonly found in sebaceous areas because all species except for

M. pachydermatis, a primarily zoonotic species, are lipophilic and require exogenous lipids for survival (Sugita et al., 2012).

Individuals with AD, SD, and TV show improvement in symptoms such as pruritus, erythema, skin discoloration, and scaling at the site of skin lesions concurrent with decreased *Malassezia* colonization after topical or oral antifungal treatment (Back et al., 1995; Rad et al., 2014; Stratigos et al., 1988). These results provide evidence for the involvement of *Malassezia* spp. in the pathogenesis of skin disorders. A study using real-time PCR found that patients with severe AD had 2-5 times more *Malassezia* colonization than patients with mild to moderate AD and healthy individuals (Kaga et al., 2009). Research on SD has not found an increase in *Malassezia* colonization throughout the entire body, however there is a positive correlation between fungal density at lesional skin sites and increased severity of symptoms (Heng et al., 1990). Further, reduction of *Malassezia* spp. on the scalp using topical antifungal therapy improves symptoms of scaling, pruritus, and erythema in SD patients (Gunduz et al., 2005; Stratigos et al., 1988). Presentation of TV is directly linked to colonization of *Malassezia* yeasts, specifically increased colonization of *M. globosa*, *M. furfur*, or *M. sympodialis* depending on the geographic region (Gupta et al., 2002; White et al., 2014). Pathogenesis in TV occurs when *Malassezia* yeast transform into their mycelial (hyphal) form and penetrate the stratum corneum, and the exact mechanism of action is discussed in further detail below (Gupta et al., 2002; White et al., 2014).

Interactions between *Malassezia* and the Immune System

Our innate immune system is essential in recognizing and destroying pathogenic microorganisms on the surface of the skin. Commensal and pathogenic microorganisms contain

microorganism-associated molecular patterns (MAMPs) – patterns that are unalterable and essential for survival of microbes – such as mannan and zymosan in fungal cell walls and peptidoglycan found in gram-positive bacteria (Mills, 2011). In healthy individuals, keratinocytes – the most common skin cell type – contain extracellular receptors called pattern recognition receptors (PRRs) (Mills, 2011)**.** There are several families of PRRs, including toll-like receptors (TLRs), cytosolic NOD-like receptors, C-type lectin receptors, and RIG-I-like receptors that detect conserved patterns from bacteria, fungi, viruses, and parasites (Mills, 2011). TLR1, TLR2, and TLR4 are extracellular receptors that bind to molecular agonists in fungi, such as zymosan for TLR1 and TLR2, and mannan for TLR4 (Baroni et al., 2006; Mills, 2011). Activation of TLRs via binding to MAMPs causes a downstream cascade of various immune responses, including transcription of genes encoding for inflammatory cytokines that activate T cells and production of antimicrobial peptides (APs) of the β-defensin family that are released to the surface of the skin (Baroni et al., 2006). APs create ion channels that disrupt the phospholipid bilayer of pathogenic microbes, causing leakage of ions and leading to cell death (Dessinioti and Katsambas, 2013). There are three β-defensins: human β-defensin 1 (HBD-1), HBD-2, and HBD-3 (Baroni et al., 2006). Baroni et al. showed that *M. furfur* isolated from psoriatic scalp lesions up-regulates the expression of TLR-2, HBD-2, HBD-3, and IL-8 mRNA in keratinocytes, providing evidence for a pro-inflammatory response in certain skin conditions. Cathelicidins are another family of APs involved in fungal defense. Cathelicidin LL-37 inhibited growth of *M. furfur* isolated from patients with tinea versicolor (Lopez-Garcia et al., 2006). Further, a modified synthetic AP, P5, showed antifungal activity against *M. furfur* in normal human keratinocytes demonstrating the role of our innate immune system in preventing pathogenesis (Ryu et al., 2011).

While *Malassezia* species are a commensal organism, they can take on a more pathogenic role, especially in individuals with impaired skin barrier function. Individuals with AD, for example, have low levels of AP production and therefore do not destroy pathogenic microorganisms as well as healthy individuals (Grice and Segre, 2011). Further, in patients with AD, *Malassezia* species can easily penetrate beneath the surface of the skin, causing *Malassezia*-specific IgE production, and bind to TLR-2 on keratinocytes and dendritic cells leading to increased cytokine production (Baroni et al., 2006; Sugita et al., 2012). These mechanisms, coupled with decreased AP production may contribute to inflammation and perpetuate symptoms of chronic inflammatory skin diseases.

Malassezia **and Extracellular Lipases**

Several other biochemical mechanisms are involved in the pathogenesis of *Malassezia* spp., ensuring survival of the fungal cells and preventing attack by the host immune system while worsening symptoms of inflammatory skin conditions. All *Malassezia* spp., except *M. pachydermatis* most frequently found in animals, require lipid supplementation to grow (Brunke and Hube, 2006; Harada et al., 2015). This lipid requirement is due to the inability of *Malassezia* spp. to produce myristic acid, a precursor of long-chain fatty acids (Brunke and Hube, 2006). *Malassezia* spp. preferentially colonize sebaceous areas such as the face, neck, and scalp to take advantage of the rich source of triglycerides present in those areas (Grice and Segre, 2011). *M. furfur* and *M. globosa* contain genes (*MfLIP1* and *LIP1*, respectively) that encode for extracellular lipases (Brunke and Hube, 2006; Harada et al., 2015). These lipases break down phospholipids and triglycerides into free fatty acid metabolites like oleic and arachidonic acid, stimulating an inflammatory response at the surface of the skin. (Grice and Segre, 2011; Harada

et al., 2015; Plotkin et al., 1998). In SD, these free fatty acids act as scalp irritants by causing scaling (Gupta et al., 2004b; Harada et al., 2015). Further, arachidonic acid-derived mediators of inflammation were detected at high levels in atopic skin lesions as compared to healthy controls, suggesting that free fatty acids may also perpetuate cutaneous inflammation in AD (Ruzicka et al., 1986).

Encoding for extracellular lipases is also an important function of *Malassezia* defense. Extracellular lipases incorporate fatty acids into the membrane of *Malassezia* spp., creating a hydrophobic cell wall that can more readily adhere to human cells and create a biofilm (Angiolella et al., 2018). Biofilm production is an important part of *Malassezia* pathogenesis, further promoting virulence factors such as antifungal resistance and release of proinflammatory cytokines, including IL-1α, IL-6, and IL-8 in *M. furfur*, and IL-6 in *M. globosa* (Akaza et al., 2012). In a previous study comparing hydrophobic and hydrophilic cell walls in *C. albicans*, hydrophobic cell walls were less sensitive to phagocytosis (Hazen et al., 2000). This evidence suggests that cell-surface hydrophobicity helps *Malassezia* spp. establish a biofilm and promote virulence factors, while also protecting the yeasts from phagocytosis.

Study Aims

The purpose of this study is to identify potential botanical extracts that exhibit fungistatic and fungicidal activity against *Malassezia* spp. without compromising human skin cells. Resistance to antifungal treatments is a growing issue, especially in immunosuppressed patients (Balkis et al., 2012; Dessinioti and Katsambas, 2013; Loeffler and Stevens, 2003; Schwartz et al., 2006; Vandeputte et al., 2012). Further, certain antifungals such as oral ketoconazole have severe side effects such as hepatotoxicity (García Rodríguez et al., 1999). Lastly, cost-

effectiveness and risk of recurrence are both pertinent issues that need to be addressed in the clinical management of these chronic skin conditions (Gupta et al., 2004a; Shemer et al., 1999). Therefore, it is beneficial to develop treatments with botanical extracts that can be used as both prophylaxis and treatment, with decreased risk of recurrence, treatment resistance, and side effects.

1.2 Characteristics of *Malassezia* spp.

Malassezia fungi are part of the Basidiomycota phylum and are superficial commensals of the skin found in more sebaceous areas such as the head, face, and neck (Saunders et al., 2012). The fungal cells were initially identified from skin scrapings in 1846 by Carl Ferdinand Eichstedt (Gupta et al., 2002). In 1874, cells of the fungi from SD lesions were described by Louis-Charles Malassez, a French histologist who contributed to the genus name (Gupta et al., 2002; Harada et al., 2015). The yeasts were first cultured in 1913 and named *Pityrosporum ovale*, more commonly referred to as *Malassezia furfur* today (Gupta et al., 2002). In 1951, *P. orbiculare* (synonym: *M. furfur*) was isolated from non-lesional human skin providing the first evidence that *Malassezia* yeasts compose the skin microbiome (Gupta et al., 2002).

There are nine species found in humans, and five that are most commonly found on healthy skin including *M. globosa*, *M. restricta*, *M. furfur*, *M. dermatis* and *M. sympodialis* (Harada et al., 2015; Saunders et al., 2012). *Malassezia* spp. have a small genome size, with *M. globosa* containing around 4,300 genes, likely due to their adaptation of only thriving on the skin of warm-blooded vertebrates (Saunders et al., 2012). These microorganisms can present as ovoid, cylindrical, spherical or elongated in morphology (Levin, 2009; Sugita et al., 2012). *Malassezia* spp. are dimorphic, presenting on the skin as both branching, interconnected hypha and distinct yeast cells (Grice and Segre, 2011). *Malassezia* spp. are believed to play a role in

seborrheic dermatitis, atopic dermatitis, *Malassezia* folliculitis, ptiryasis (tinea) versicolor, and psoriasis (Harada et al., 2015; Levin, 2009).

One study which conducted real-time PCR analysis detected *M. restricta* and *M. globosa* in 100% of samples from AD patients (Sugita et al., 2012). *M. sympodialis* was found in 61.9% and *M. furfur* in 28.6% of samples (Harada et al., 2015; Sugita et al., 2012). Further, as severity of AD increases, the ratio of *M. restricta* to *M. globosa* decreases; *M. globosa* is detected at much higher levels in patients with severe AD, pointing to its potential pathogenesis in worsening AD symptoms (Sugita et al., 2012). Research on seborrheic dermatitis found *M. restricta* in 64-74.2% and *M. globosa* in 41-93.5% of samples from patients, although *M. sympodialis* and *M. furfur* are also believed to play a role (Dessinioti and Katsambas, 2013; Harada et al., 2015; Levin, 2009). *M. globosa, M. restricta* and *M. sympodialis* seem to be the most prevalent yeasts involved in TV, although *M. furfur* and *M. dermatis* also play a role, especially in warmer climates (Harada et al., 2015; White et al., 2014). Levin et al. (2009) reported *M. globosa* in 77-90% of TV isolates in Spain, 55% in Japan, and 25% in Canada. *M. sympodialis* was found in 32-41% of TV isolates in Spain, 9% in Japan, and 59% in Canada (Levin, 2009). *M. furfur* was isolated in 11% of TV samples from patients in Canada (Levin, 2009). It is important to note that colonization of *Malassezia* spp. varies based on geographical region as well as sampling site on the body (Glatz et al., 2015). Further, when determining the distribution of *Malassezia* spp. in samples, different media results in better growth of certain species and may alter determinations (Glatz et al., 2015).

1.3 Atopic Dermatitis

Atopic dermatitis (AD) is a common skin disorder that is estimated to affect about 15-30% of children and up to 10% of adults (Glatz et al., 2015). AD is more commonly referred to as eczema and it typically begins in early childhood (2017a). Symptoms include erythema and intensely itchy and dry, flaky skin (2017a). The exact cause is not known, but the etiology is believed to be multifactorial. AD is part of the "atopic triad" and individuals with a personal or family history of asthma or allergic rhinitis, also known as seasonal allergies, have an increased chance of development (2017c).

There may be certain gene variations in individuals who develop AD that decreases the skin's ability to provide protection from bacteria, fungi, and allergens (2017a). In individuals with AD, genetic mutations have been found in filaggrin (FLG), a gene that involves tight junctions and the formation of the skin barrier (Williams and Gallo, 2015). This gene variation may contribute to the impaired skin barrier function, increased water loss, and increased surface pH seen in individuals with AD (Glatz et al., 2015). Mutations have also been found in T helper cell cytokines IL-4, IL-10, and IL-13 (Williams and Gallo, 2015). Mutations in these cytokines reduce the production of antimicrobial peptides (APs) which target pathogenic microorganisms and are important components of the skin's innate immune system as discussed previously. Additionally, genome-wide scans have shown multiple chromosome loci where gene expression has been altered (Williams and Gallo, 2015). AD is also more common in urban areas and developed countries, which some researchers believe may be due to excessive hygiene which disrupts the skin microbiome by reducing beneficial organisms that normally colonize the surface of the skin (Williams and Gallo, 2015). In the last 30 years alone, rates of AD have

doubled or tripled in industrialized countries, providing evidence of an environmental trigger for skin microbiome fluctuations (Hanifin and Reed, 2007; Ricci et al., 2010).

Although AD is not lethal, it leads to enormous healthcare and financial burdens for families due to treatment costs and occupational disabilities. It is estimated that the cost of caring for a child with moderate to severe AD is more expensive than caring for a child with asthma (Hanifin and Reed, 2007). Direct costs in the US are estimated to be as much as $4 billion per year due to work losses (Hanifin and Reed, 2007). In individuals with moderate to severe AD the constant scratching and rubbing increases risk of secondary infection, such as *S. aureus* colonization, because of open wounds (Huang et al., 2009). Most individuals who develop AD in early childhood will continue to struggle with eczema for the rest of their lives (2017a). In certain individuals, AD symptoms will improve in adulthood, but most will continue to experience flare-ups and symptoms throughout their life (2017a). Stress, fungal or bacterial infection, hormone fluctuations, and weather variations are all factors that can trigger an AD flare-up (2017c). In addition, individuals with AD have increased risk for depression, anxiety, and suicidal ideation as compared to healthy adults (Thyssen et al., 2017).

There are multiple treatments available, but most medication is expensive and presents with side effects, decreasing patient compliance (Filip et al., 2010). Individuals with mild to moderate AD may be prescribed topical steroids, while those with more severe AD may try oral corticosteroids like prednisone (2017a). Corticosteroid treatment cannot be used long term, however, due to skin thinning, hypopigmentation, acne, and risk for secondary infections (2017a; Paller et al., 2001). Other first line treatments include topical calcineurin inhibitors (TCIs), such as the topical treatments tacrolimus and pimecrolimus, and bleach baths for individuals with secondary bacterial infection (Gupta et al., 2004a; Huang et al., 2009).

Individuals with AD are thought to have a skin microbiome more susceptible to pathogenic bacteria like *Staphylococcus aureus* (Grice and Segre, 2011). For this reason, topical antibiotics such as cloxacillin and cephalexin seem to decrease symptoms of AD (Goh et al., 1997), however long-term use is not recommended because of the possibility for resistance and worsening infections in the future (2017a). One study explored the antibacterial effects of virgin coconut oil against *S. aureus* colonization in adult AD patients (Verallo-Rowell et al., 2008). Patients applied virgin coconut oil twice daily for four weeks. At follow-up 19/20 (95%) of individuals had complete clearance of SA colonies at the site of treatment. Coconut oil is just one example of a botanical treatment that exhibits antibacterial and antifungal effects to decrease inflammation and colonization implicated in AD (Verallo-Rowell et al., 2008). Understanding the underlying mechanisms of AD and developing novel treatments is a highly important and relevant issue today.

1.4 The Role of *Malassezia* species in Atopic Dermatitis

Although the role of *Staphylococcus aureus* is well-known for its implications in AD, less research has been conducted on the involvement of *Malassezia* species. Individuals with AD respond well to topical antifungal therapy at lesional sites, showing decreased colonization of *Malassezia* spp. and improved symptom severity such as pruritus, erythema, and flaking (Sugita et al., 2012). This evidence suggests that *Malassezia* spp. take on a pathogenic role, likely through interaction with antigen presenting immune cells such as Langerhans cells (Sugita et al., 2012). Interestingly, studies have shown that *Malassezia* spp. cause a more allergenic response with the increase in pH found in AD. Mala s5-13 are common *Malassezia* allergens that are very similar to their human homologs (Saunders et al., 2012). Mala s5-13 activate T cells, but also

cross-react with human enzymes, likely worsening skin inflammation (Glatz et al., 2015; Saunders et al., 2012). Due to decreased skin barrier function in AD patients, *Malassezia* cells penetrate beneath the surface and are recognized by TLRs on keratinocytes and dendritic cells (Sugita et al., 2012). This causes the release of pro-inflammatory cytokines that elicit a T cell response and production of *Malassezia*-specific IgE antibodies (Sugita et al., 2012). These IgE-specific antibodies may also trigger skin inflammation through mast cells (Glatz et al., 2015). A previous study showed that *M. sympodialis* activated mast cells to release leukotrienes and increase IL-6 production (Saunders et al., 2012). It is also believed that *Malassezia* spp. can release nanovesicles that carry *Malassezia* allergens, inducing more inflammatory cytokine responses and contributing to the inflammation and itching found in AD (Saunders et al., 2012).

According to Glatz et al. (2015), standardized kits (ImmunoCAP® m227) that measure serum IgE have found *Malassezia*-specific IgE in 5-27% of children and 29-65% of adult AD patients. Sensitization rates are likely lower in children because they have less sebum production than adults and therefore provide a worse environment for *Malassezia* growth (Glatz et al., 2015). Further, a recent study found a positive correlation between AD severity and sensitization to *Malassezia* spp.-specific IgE in adults. Sensitization rates were also worse in individuals presenting with head and neck types of AD (Glatz et al., 2015). This data provides evidence for the pathogenesis of *Malassezia* spp. by penetrating beneath the stratum corneum and initiating a multifaceted immune response.

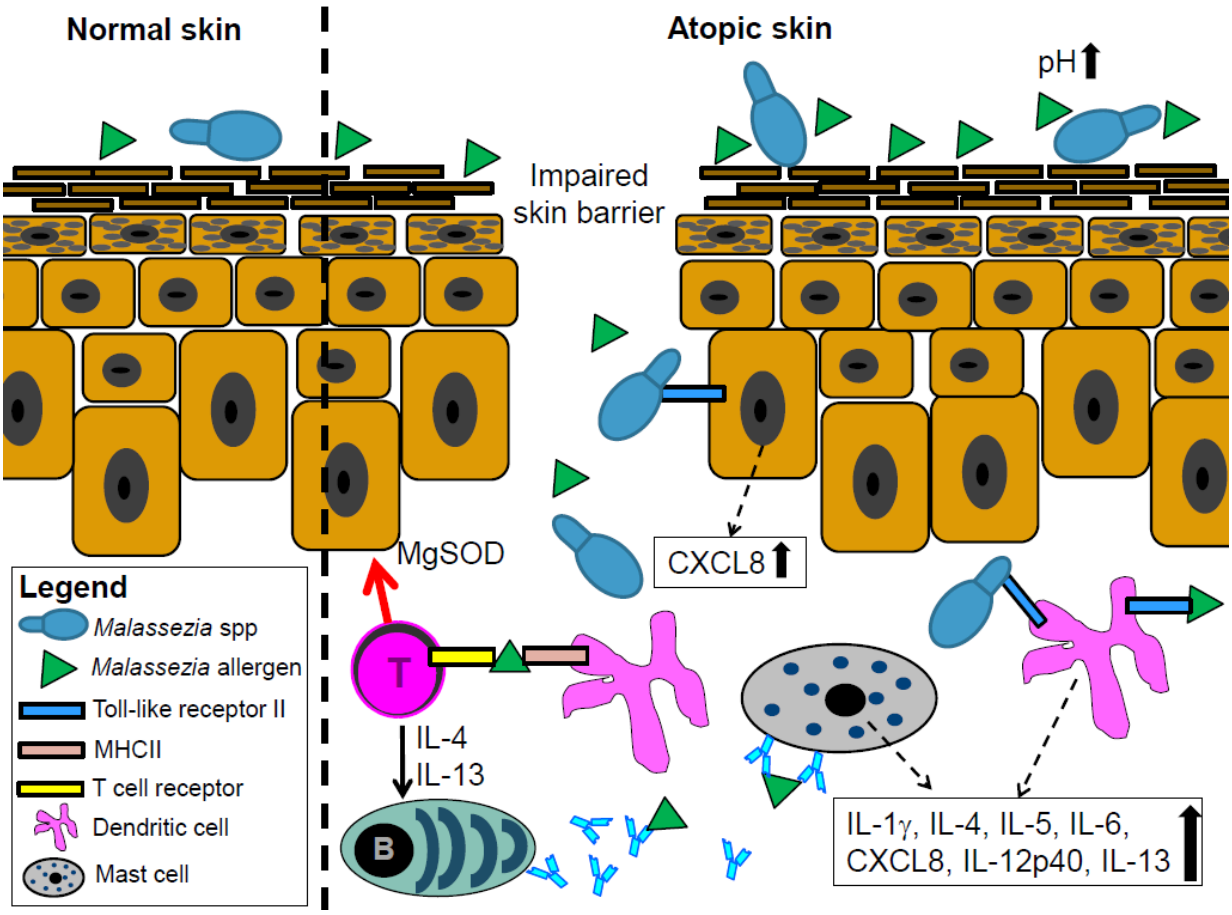


Figure 1. Skin Microbiome Differences between Healthy and Atopic Dermatitis Patients. Proposed mechanisms by which *Malassezia* spp. contributes to skin inflammation in Atopic Dermatitis. In normal skin, tight junctions prevent *Malassezia* spp. and allergens from penetrating the stratum corneum. In atopic skin, however, MAMPs of *Malassezia* spp. – specifically zymosan composing the fungal cell wall – acts as an agonist for TLR-2 on keratinocytes and dendritic cells, increasing inflammatory cytokine production. *Malassezia* allergens also bind to mast cells, causing IgE antibody production and histamine release to worsen inflammation at the surface of the skin and symptoms of atopic dermatitis. Of note, the increased pH seen in atopic skin provides a more favorable environment for increased *Malassezia* allergen production. Image permission obtained under the terms and conditions of Creative Commons Attribution License (Glatz et al., 2015).

The exact species isolated from AD patients depends on geographic region, ethnicity, and culture method, but evidence points to *M. globosa*, *M. restricta*, *M. furfur*, and *M. sympodialis* being most commonly implicated. A study in Japan looked at both lesional and non-lesional skin in 46 patients. The researchers found *M. globosa* in 14%, *M. furfur* in 21%, and *M. sympodialis* in 7% of lesional skin as compared to *M. globosa* in 33% and *M. furfur* in 11% of non-lesional skin (Nakabayashi et al., 2000). However, a study conducted in Canada found the principal species to be *M. sympodialis*, which was detected in 51.3% of atopic skin lesion samples obtained from various anatomic sites. *M. globosa* was isolated from 17.9%, *M. furfur* from 10.3%, and *M. restricta* from 7.7% of samples (Gupta et al., 2001). These two studies point to the variance of isolated species based on regional differences and culture techniques. Therefore in 2001 a new technique was developed to analyze *Malassezia* DNA directly using real-time PCR instead of culture-dependent methods (Sugita et al., 2001). 32 patients with AD in Japan had samples collected from skin lesions on the scalp, back, and neck. *M. globosa* and *M. restricta* were found in 90% of samples from all AD patients independent of the lesion site; *M. furfur* and *M. sympodialis* were detected in approximately 40% of patients (Sugita et al., 2001). As comparison, *Malassezia* DNA was only detected in 78% of samples obtained from healthy patients without skin disease; of the positive samples, *M. globosa*, *M. restricta*, and *M. sympodialis* were detected in 44-61% of samples and *M. furfur* was found in 11% of samples. Further, AD patients had greater diversity of *Malassezia* spp. as compared to healthy controls (2.7 species detected in each AD individual vs. 1.8 species per healthy control). This data provides evidence of higher colonization of *Malassezia* species at lesional skin sites in AD, contributing to worsening symptoms of disease.

The role of oral antifungal treatment in managing AD symptoms has been explored in various studies. In one study, 36 patients were treated with oral ketoconazole (KTZ) and 39 AD patients treated with placebo (Lintu et al., 2001). Itching ($p<0.005$), extent of dermatitis (area percentage, $p<0.01$), erythema, and dryness ($p<0.05$) improved significantly in the KTZ group as compared to placebo. Further, there was a significant decrease in positive cultures of *M. furfur* from 60% to 31% in the KTZ group as compared to the placebo group (64% to 56%). Bäck et al. (1995) detected IgE antibodies in 44% of AD patients, but did not detect any antibodies in controls. Therefore, patients positive for IgE antibodies were given 200 mg KTZ daily for 2 months and then tapered to 200 mg twice weekly for 3 more months. Clinical scores of AD improved, along with a reduction in *Malassezia*-specific IgE. Another study specifically looked at patients with head and neck AD, randomly assigning 18 patients to 200 mg itraconazole (ITZ) daily and 17 patients to 400 mg ITZ daily for 2 weeks. There was a significant improvement in SCORAD of the head and neck area at days 7 and 14 for patients taking both doses of ITZ ($p<0.05$). While these results show promising efficacy of oral antifungals in improving AD symptoms, antifungal resistance is a growing issue and prolonged antifungal treatment may not be an effective option for patients (Balkis et al., 2012).

1.5 Seborrheic Dermatitis

Seborrheic dermatitis (SD) is a common skin condition affecting sebaceous (oily) areas of the body such as the scalp, face, retro-auricular area (behind the ear), upper chest, and axillae (Borda and Wikramanayake, 2015). The disorder is characterized by erythematous, scaly, and oily patches of the scalp, face, and upper chest (Schwartz et al., 2006). Dandruff and SD are considered the same dermatological condition, but affect different sebaceous parts of the body (Borda and Wikramanayake, 2015). Dandruff is a milder form of SD only found on the scalp and is estimated to affect up to 50% of the population (Gupta et al., 2004b). SD, on the other hand, is found on the scalp, face, and trunk and is estimated to affect 1-3% of the general adult population (Dessinioti and Katsambas, 2013). The causes of SD are multifactorial, including fluctuation in hormone levels, genetics, fungal infection, and neurogenic factors (Schwartz et al., 2006).

SD is prominent in three age periods: infancy, puberty, and adulthood from 30-60 years of age, concurrent with increased sebaceous gland activity (Borda and Wikramanayake, 2015). In infancy, SD appears in the first three months of life when sebaceous glands are activated by maternal androgens (Borda and Wikramanayake, 2015). It mainly affects the scalp, face, and diaper area with cases appearing in about 40% of infants (Borda and Wikramanayake, 2015). The disease reappears during puberty when sebaceous glands are activated again and cause increased sebum production. It is prevalent in about 10% of adolescents and mainly affects the scalp, face, chest, and axillae (Borda and Wikramanayake, 2015; Schwartz et al., 2006). Both dandruff and SD are more common in men than women, likely due to increased androgen production and subsequent sebum secretion, which stays elevated between 30-60 years of age (Borda and Wikramanayake, 2015; Dessinioti and Katsambas, 2013). Hormone fluctuation may

also explain why SD ceases in childhood, but reappears in adolescence when sebum production increases (Schwartz et al., 2006). However, sebum production is not the only cause of SD, as individuals with normal sebum production have been diagnosed with SD, while some individuals with excessive sebum production do not present with SD (Borda and Wikramanayake, 2015).

Malassezia yeasts contribute to the pathogenesis of SD due to their preference for lipid-rich areas of the body (Borda and Wikramanayake, 2015; Dessinioti and Katsambas, 2013; Schwartz et al., 2006). The increase in sebum production during the first few months of birth and again during adolescence creates a favorable environment for *Malassezia* growth (Gupta et al., 2004b). Gupta et al. (2001) conducted a study with 28 SD patients in Canada and isolated *M. sympodialis* (37.5%), *M. globosa* (45%), *M. furfur* (7.5%), and *M. slooffiae* (10%) from skin lesions. Another study that looked at 98 Japanese patients with SD isolated *M. furfur* (21%), *M. globosa* (21%), and *M. sympodialis* (6%) from lesional skin samples (Nakabayashi et al., 2000). On the other hand, researchers used PCR technique on 60 samples from Iranian SD patients and found frequencies of *M. globosa* (61.6%), *M. furfur* (23.3%), *M. sympodialis* (8.4%), and *M. restricta* (6.7%) (Didehdar et al., 2014). This evidence suggests that *M. globosa*, *M. sympodialis*, and *M. furfur* are most commonly implicated in SD skin lesions.

Researchers have observed an association between *Malassezia* density, specifically *M. furfur*, and increased SD severity (Heng et al., 1990; Levin, 2009). As mentioned previously, *Malassezia* yeasts exhibit extracellular lipase activity, breaking down triglycerides on the skin into oleic and arachidonic fatty acids (Borda and Wikramanayake, 2015; Plotkin et al., 1998). Oleic and arachidonic acid disrupt skin barrier function through desquamation (scaling) of skin cells, triggering an inflammatory response that worsens itching and erythema in SD (Borda and Wikramanayake, 2015; Dessinioti and Katsambas, 2013). Further, arachidonic acid metabolites,

such as 12-hydroxyeicosatetraenoic acid and leukotrienes are major mediators of skin inflammation (Plotkin et al., 1998) In addition to increased *Malassezia* density at lesional sites and inflammatory responses caused by extracellular lipases, SD may be driven by disrupted skin barrier function (Warner et al., 2001). Transmission electron microscopy of scale samples revealed an altered stratum corneum, including distorted corneocyte shape and intercellular *Malassezia* yeasts, which may further induce an inflammatory response (Warner et al., 2001).

One study observed induced inflammatory genes in both dandruff involved and uninvolved skin pointing to other causal factors that make certain individuals more susceptible, such as the neurogenic theory and immunomodulatory theory (Borda and Wikramanayake, 2015; Dessinioti and Katsambas, 2013). SD is associated with Parkinson's disease and other neurological disorders including epilepsy and depression (Borda and Wikramanayake, 2015). Symptoms may also be exacerbated by emotional stress (Borda and Wikramanayake, 2015). Further, SD is more common in immunocompromised individuals such as HIV/AIDs patients and organ transplant patients (Borda and Wikramanayake, 2015). Overall, these theories suggest that the pathogenesis of SD is complex and multifactorial.

Seborrheic dermatitis and dandruff have vast financial consequences. It is estimated that about $300 million dollars is spent annually on over-the-counter products to treat scalp itching and flaking (Borda and Wikramanayake, 2015). SD office visits cost about $60 million alone and more than $100 million is spent on prescription drugs each year (Borda and Wikramanayake, 2015). In addition, symptoms of SD such as scalp scaling decrease patient's self-esteem and about $51 million is spent annually on lost work days (Borda and Wikramanayake, 2015). The economic and emotional burden of SD and dandruff are immense and developing effective antifungal therapies for long-term maintenance of symptoms is pertinent.

There are various treatment options for patient with seborrheic dermatitis. SD patients respond well to antifungal treatment including topical antifungals like ketoconazole and antifungal shampoos containing selenium sulfide (Borda and Wikramanayake, 2015). Oral antifungals are rarely prescribed due to the potential for hepatotoxicity (García Rodríguez et al., 1999; Levin, 2009). Antifungal use shows improvement of symptoms and decreased number of *Malassezia* yeasts at lesional skin sites (Levin, 2009; Stratigos et al., 1988). Corticosteroids and topical calcineurin inhibitors (TCIs) are also recommended for short-term use and are effective at resolving symptoms (Borda and Wikramanayake, 2015). TCIs are preferred due to their potent fungicidal and anti-inflammatory effects without the risk of skin thinning found in corticosteroid use (Schwartz et al., 2006). Meshkinpour et al. (2003) prescribed 0.1% tacrolimus ointment to SD patients for four weeks. At the end of the treatment regimen, 61% of patients had 100% clearance of symptoms and the remaining patients had 70-99% clearance (Meshkinpour et al., 2003). Discontinuing treatment, however, is associated with recurrence of symptoms pointing to the need for effective long-term therapies without adverse effects (Borda and Wikramanayake, 2015).

Other treatments include the use of keratolytics, such as zinc pyrithione shampoos and salicylic acid, which act as peeling agents to soften and shed the outer layer of the skin (Schwartz et al., 2006). There has also been limited research on alternative medical therapies with botanicals. One study prescribed 5% tea tree oil (*Melaleuca alternifolia*) and exhibited 41% improvement in symptom severity score as compared to 11% in the placebo group (Satchell et al., 2002). Further botanical medicine research results are discussed in table 2. In the face of increasing antifungal resistance, continued research is needed on potential botanical therapies for long-term use.

1.6 Tinea Versicolor

Tinea versicolor (TV) is a skin infection caused by *Malassezia* yeasts that affects the stratum corneum. There are three distinct forms of *Malassezia* yeasts that present in all layers of the stratum corneum including conidia, budding yeasts, and mycelia (Gupta et al., 2002). Typically, *Malassezia* is a commensal yeast that inhabits the skin microbiome without pathogenesis (Gupta et al., 2002). However, under certain endogenous and exogenous conditions such as increased antibiotic use, humid climates, and poor hygiene, *Malassezia* spp. convert to the mycelial phase, causing inflammation and hypopigmentation (Gupta et al., 2002). In the mycelial phase, yeasts are located between keratinocytes; invasion of these skin cells creates a clear area around *Malassezia* spp. to promote pathogenesis (Gupta et al., 2002).

TV is estimated to affect as many as 60% of individuals in tropical and humid areas (Gupta et al., 2002; Levin, 2009). The exact cause of TV is not known, however individuals who are immunocompromised or frequently use antibiotics and/or corticosteroids are more prone to infection (Gupta et al., 2002). Other endogenous risk factors include malnutrition, birth control use, and genetic factors (Gupta et al., 2002). Exogenous risk factors for TV include living in a humid environment, sweating, and infrequent washing (Levin, 2009). It is most prevalent in adolescents when sebum production is highest (Gupta et al., 2002).

Typically, TV will appear on the upper trunk, shoulders, and back (Gupta et al., 2002). Unlike AD, it is not commonly found on the face and neck (Levin, 2009). The lesions are circular with hypopigmentation or hyperpigmentation, as well as flaking around the border (Gupta et al., 2002). *Malassezia* yeasts produce azaleic acid which inhibits tyrosinase, an important enzyme in melanin synthesis, thus contributing to the hypopigmentation of TV skin lesions (Levin, 2009). *Malassezia* yeasts also produce pityriacitrin, a compound that absorbs UV

light and results in discoloration and lightening of skin (Levin, 2009). Further, Malassezin is a compound formed by *Malassezia* yeasts that acts as an agonist in melanocytes (skin cells that produce melanin) to stimulate apoptosis and decrease melanin production in affected sites (Levin, 2009). Besides the discolored lesions, patients are usually asymptomatic, although some experience itching (Gupta et al., 2002).

The exact *Malassezia* species involved varies depending on region, but one study isolated samples from 22 patients with TV and found *M. globosa* at a frequency of 55%, and all other strains at a frequency below 10%, pointing to *M. globosa* as being the probable pathogenic agent in TV (Nakabayashi et al., 2000). Other research found that *M. globosa* affects 77-90% of patients in Spain, 55% in Japan, and 25% in Canada (Levin, 2009). Prohic et al. found *M. globosa* (62%) the most common species to affect patients with TV. Finally, through real-time PCR of samples from Japanese patients, Harada et al. (2015) found *M. globosa* and *M. restricta* in 93.9% of samples, *M. sympodialis* in 34.6%, and *M. furfur* in 10.2% of TV samples.

Wood's light is a diagnostic tool that uses filtered UV light at 365 nm to identify TV (Gupta et al., 2002). Lesions will appear bright yellow indicating the mycelial form of the yeast (Gupta et al., 2002). However, Wood's light is only effective in about 1/3 of patients that have TV. This is because *M. furfur* is the only species that forms the indole compounds that fluoresce under Wood's light (Gupta et al., 2002). Overall, this provides strong evidence that while *M. globosa* and *M. restricta* may be the most frequent species, *M. furfur* is also a contributor to TV (Gupta et al., 2002).

Current treatments for TV include Ketoconazole shampoos and lotions, 2.5% selenium sulfide shampoos and lotions, and the oral antifungals ketoconazole or fluconazole. Ketoconazole and fluconazole exhibit a fungistatic effect, disrupting formation of the fungal cell

membranes (Gupta et al., 2002). Relapse is common, especially in immunocompromised individuals so patients may use oral ketoconazole or itraconazole if the infection persists (Levin, 2009). Other topical antifungals include tolciclate, terbinafine, and selenium sulfide. Tolciclate blocks sterol biosynthesis in fungal cells, weakening the plasma membrane (Gupta et al., 2002). Terbinafine disrupts squalene epoxidation, leading to a toxic buildup of squalene (Balkis et al., 2012). Antifungal therapy is usually effective within a week— the cells will appear wrinkled under a microscope, indicating lysis in the cytoplasm (Gupta et al., 2002). Other dermatological treatments include keratolytic agents, such as propylene glycol and salicylic acid that cause peeling of the skin (Gupta et al., 2002). TV infection has an estimated 60-80% rate of recurrence with current topical therapies demanding the need for novel botanical treatments that exhibit potent antifungal activity (Borelli et al., 1991).

Chapter 2 Background

2.1 Antifungal Resistance and Toxicity: The Need for New Solutions

Antifungal resistance is a growing issue, especially among immunosuppressed individuals such as HIV-infected individuals and cancer patients (Balkis et al., 2012). There are only five different categories of antifungals, three of which are effective against *Malassezia* spp. (Balkis et al., 2012). The first category includes azoles, which function by inhibiting the synthesis of ergosterol. There are two main types of azoles: imidazoles, which include ketoconazole and miconazole, and triazoles, which includes fluconazole, itraconazole, voriconazole, posaconazole, and ravuconazole (Balkis et al., 2012).

Ergosterol is the main sterol found in yeast and is essential for building the fungal cell membrane (Balkis et al., 2012). Specifically, azoles inhibit lanosterol 14α-demethylase, an enzyme involved in the conversion of lanosterol to ergosterol (Balkis et al., 2012). A reduction of ergosterol levels alters the cell membrane and reduces function; it also leads to a build-up of sterol precursors, which cause growth arrest and are toxic to fungal cells (Balkis et al., 2012).

ERG11 is the name of the gene that encodes 14α-demethylase (Balkis et al., 2012). Modification of *ERG11* by merely a single point mutation leads to the inability of azoles to bind its target (Balkis et al., 2012). Further, overexpression of *ERG11* is associated with increased resistance to fluconazole, leading to cross-resistance in KTZ and ITZ, but not amphotericin B (Balkis et al., 2012; Loeffler and Stevens, 2003). This is likely because a mutation alters ergosterol synthesis and reduces intracellular concentrations of target enzymes (Balkis et al., 2012). Therefore, it is likely that prolonged exposure to one azole can lead to cross-resistance to other azoles and decreased efficacy in future infections (Vandeputte et al., 2012). Further, modification of *ERG11* is also implicated in overexpression of drug efflux pumps, which also contribute to decreased effectiveness (Balkis et al., 2012). Therefore, future research should also explore the role of efflux-pump inhibitors coupled with antifungal treatments.

Human sterol synthesis is also affected by azole administration, specifically ketoconazole, leading to decreased synthesis of cholesterol (Balkis et al., 2012). KTZ also inhibits transport of cholesterol, which affects plasma membrane formation and may play a role in its oral toxicity (Balkis et al., 2012). One clinical trial observed significantly increased acute liver injury in patients prescribed oral KTZ (García Rodríguez et al., 1999).

The second category of antifungal drugs includes allylamines, such as the widely prescribed terbinafine for fungal nail infections (Balkis et al., 2012). Allylamines work by

inhibiting the synthesis of ergosterol in a similar manner to azoles, by preventing the conversion of squalene to lanosterol (Balkis et al., 2012). This build-up of squalene is toxic to fungal cells and leads to death (Balkis et al., 2012).

The third category includes polyenes, such as amphotericin B (AmB) (Balkis et al., 2012). Polyene bind to sterols in the fungal membrane such as ergosterol and cholesterol, creating pores through which cytoplasmic materials can leak (Balkis et al., 2012). This increased permeability causes protons and cations to flow out of the cell, leading to cell death (Balkis et al., 2012). AmB should be cautiously utilized because it has a narrow therapeutic index— there is a close line between efficacy and toxicity and if too high of a dose is prescribed, it can lead to kidney toxicity (Balkis et al., 2012). Resistance to polyene antifungals is not common, although this has not been specifically studied in *Malassezia* spp. (Balkis et al., 2012). Polyene resistance that has been observed is likely due to change in lipid composition through decreased sterol content which decreases the binding ability of AmB (Balkis et al., 2012; Loeffler and Stevens, 2003). With decreased binding of AmB, the antifungal is less effective at degrading the plasma membrane (Loeffler and Stevens, 2003). Other components may be an increase in β-1,3 glucans in the fungal cell wall which increases stability and prevents large molecules such as Amphotericin B from entering (Loeffler and Stevens, 2003).

The fourth category includes candins which prevent the formation of the fungal cell wall by inhibiting β-1,3-glucan synthesis, a main structural component (Balkis et al., 2012). Candin resistance includes fungal factors that increase β-1,3-glucan levels to increase stability and prevent large molecules from entering the cell (Loeffler and Stevens, 2003). The last category includes flucytosine which functions by inhibiting macromolecular synthesis (Balkis et al., 2012).

Unfortunately, there is mixed evidence on antifungal resistance in *Malassezia* species. This is due to the lack of accepted susceptibility testing guidelines under the Clinical Laboratory Standards Institute (CLSI), unlike other yeasts like *Candida* spp. (Rincón et al., 2006). Growing *Malassezia* spp. in different broths can affect growth (Rincón et al., 2006). Further, susceptibility breakpoints have not been established for *Malassezia* yeasts (Rincón et al., 2006). MICs below the susceptibility breakpoint exhibit antifungal activity, while any MIC above the breakpoint exhibits resistance (Rincón et al., 2006). Studies on candidal infections demonstrate that MIC $\geq$ 4 μg/mL for Voriconazole and $\geq$ 1 μg/mL for itraconazole indicate a poor therapeutic response in patients (Rincón et al., 2006).

One study did not find ketoconazole or itraconazole resistance in *M. furfur*, *M. globosa*, or *M. restricta* tested (Sugita et al., 2005). Further, it was observed that treatment with tacrolimus and oral ketoconazole together produced a synergistic effect that increased antifungal activity and relief of symptoms for AD patients (Sugita et al., 2005). On the other hand, Rincon et al. (2006) found that in vitro testing of *M. globosa* showed MIC greater than 8 μg/mL for voriconazole, itraconazole, and ketoconazole as compared to MIC $\leq$ 0.5 μg/mL for *M. furfur* and $\leq$ 2.0 μg/mL for *M. restricta*. Gupta et al. (2000) found resistance to terbinafine in *M. globosa*, with two strains exhibiting a MIC of 8 μg/mL and one strain with a MIC of 16 μg/mL as compared to two *M. globosa* strains with MIC equal to 0.06 μg/mL. Similarly, two strains of *M. furfur* were resistant to terbinafine with a MIC of 32 μg/mL and seven strains with a MIC of 16 μg/mL as compared to three strains with MIC $\leq$ 0.03 μg/mL (Gupta et al., 2000). Another study found 28.2% of *M. furfur* isolates and 8.3% of *M. globosa* isolates had a fluconazole MIC $\geq$ 8 μg/mL, suggesting that fluconazole may not be a good treatment option for *Malassezia* fungal infection (Rojas et al., 2014).

Malassezia pachydermatis, a species more commonly found in animals, has also demonstrated azole resistance. One study in canines found higher MICs for ketoconazole (KTZ) and itraconazole (ITZ) in the canines with AD compared to controls (Watanabe et al., 2014). A case study also discovered fluconazole and flucytosine-resistant *M. pachydermatis* in a pre-term neonate (Al-Sweih et al., 2014).

Although susceptibility breakpoints have not been established, these studies raise concern for increasing resistance in *Malassezia* yeasts. Therefore, the present research seeks to discover new antimycotic treatments from plant extracts that retains the acidic environment of the skin and decreases presence of *Malassezia* spp. for long-term resolution of symptoms.

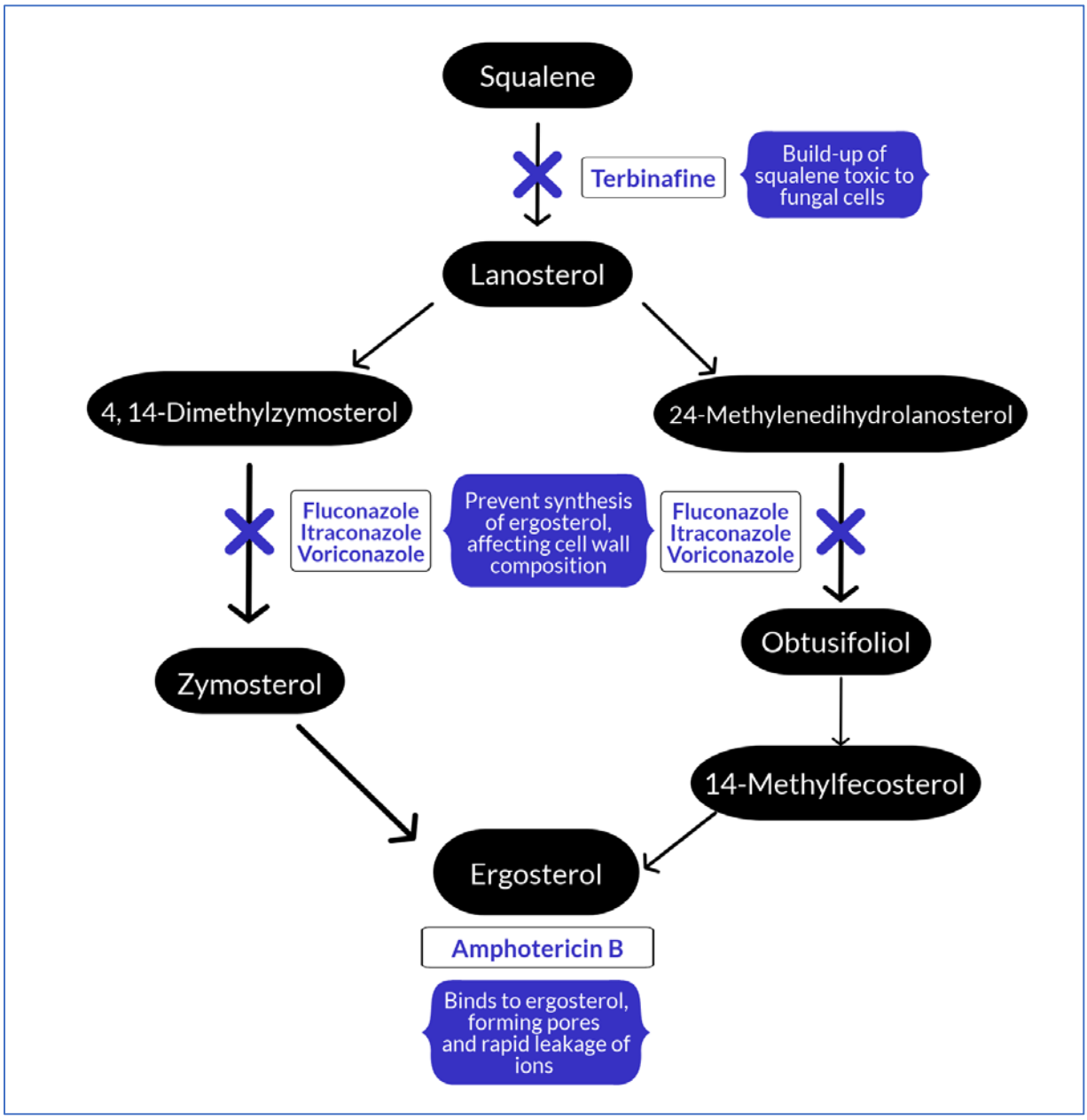


Figure 2. Mechanisms of Antifungal Activity against *Malassezia* Species. There are three classes of antifungals active against *Malassezia* species. Terbinafine, an allylamine, blocks the conversion of squalene to lanosterol leading to toxic build-up of squalene. Azole antifungals work further down in the pathway to prevent the creation of ergosterol, weakening the fungal cell wall. Amphotericin B, a polyene, binds directly to the cell membrane, forming holes where ions can leak out and lead to cell death (Balkis et al., 2012; Ghannoum and Rice, 1999).

2.2 Implications of Other Treatments

As stated previously, corticosteroid use is not recommended for long-term use due to risk of skin thinning, adrenal gland suppression, and secondary infections (Ashcroft et al., 2005; Paller et al., 2001). Due to these side effects, patient compliance and adherence to steroid treatment is poor, providing further evidence for the need of effective long-term treatment (Ashcroft et al., 2005). More recently, calcineurin inhibitors have been utilized in AD and SD because they have potent anti-*Malassezia* activity (Sugita et al., 2012). Calcineurin inhibitors were initially used systemically to prevent rejection of organs after transplant surgery (Arellano et al., 2007). Calcineurin inhibitors block the pathway that leads to transcription of genes encoding for inflammatory cytokines that worsen symptoms of AD and SD (Hanifin et al., 2001).

Tacrolimus may be an effective treatment for both children and adults who struggle with either AD or SD. One study tested the effects of tacrolimus treatment in pediatric patients aged 2-15 years with AD (Paller et al., 2001). Patients were either prescribed a vehicle ointment, 0.03% tacrolimus ointment, or 0.1% tacrolimus treatment. Both the 0.03% and 0.1% tacrolimus groups had significant improvement of symptoms after 12 weeks of treatment, including reduced swelling, scaling, pruritus (itchiness), and erythema (redness). A similar studied was conducted in adult patients with AD (Hanifin et al., 2001). Patients were either prescribed vehicle, 0.03% tacrolimus, or 0.01% tacrolimus twice daily for 12 weeks. A 90% or better improvement was observed for 27.5% of patients in the 0.03% group and for 36.8% of patients in the 0.1% tacrolimus group, compared to only 6.6% of controls. Lastly, another study conducted an open-label trial using 0.1% tacrolimus ointment in 18 adult patients with SD, 11 men and 7 women for

4 weeks (Meshkinpour et al., 2003). 61% of patients showed complete clearance of symptoms at the end of the trial, while the remaining 39% showed 70-99% clearance.

Tacrolimus seems to be an effective treatment for reduction of symptoms in individuals with both SD and AD. Some individuals may experience skin irritation or burning during initiation of treatment, but these symptoms usually reside after one week (Schneeweiss et al., 2009). However, TCIs are often only prescribed for intermittent use and discontinuation is associated with a return of symptoms, indicating the need for other long-term treatments such as botanical remedies for sustained relief (Arellano et al., 2007). Further, there has been concern over TCI safety. Studies found an increased risk of lymphoma and nonmelanoma skin cancer in transplant patients that use tacrolimus systemically (Arellano et al., 2007; Hui et al., 2009; Margolis et al., 2007). This caused the FDA to require a "black box" warning on labels in 2006 because of possible association between topical calcineurin inhibitor (TCI) use and cancer (Arellano et al., 2007; Margolis et al., 2007). Over the last few years, several studies have also exhibited an increased risk of lymphoma with topical calcineurin inhibitor use, so other treatments with decreased side effects are needed (Hui et al., 2009; Schneeweiss et al., 2009).

One study assessed a cohort of individuals with dermatitis using TCIs and risk of lymphoma (Schneeweiss et al., 2009). The study obtained a cohort of individuals from a health insurance claims database. The study assessed individuals using pimecrolimus, tacrolimus, and topical steroid use based on pharmacy dispensing records. They had two control groups: patients with AD who were non-users, and patients who did not have a dermatitis diagnosis and were also non-users.

The study determined that pimecrolimus users did not have a difference in rate of lymphoma compared to untreated dermatitis patients. However, the risk ratio was 1.79,

suggesting an approximately 79% increased rate of lymphoma among pimecrolimus users. Further, pimecrolimus users were 2.89 times more likely to develop lymphoma compared to the general population (RR = 2.89). Tacrolimus users were 5.28 times more likely to develop Hodgkin's Lymphoma (HL) and 1.35 times more likely to develop non-Hodgkin's Lymphoma (NHL) compared to untreated dermatitis patients and had a 97% increased rate of "any lymphoma" compared to non-users. The study also demonstrated an adjusted odds ratio of 2.98 for AD patients who used a cumulative amount of 61-100 grams of pimecrolimus compared to topical steroids and 4.17 times for >100 grams. This means that AD patients who used a total amount of 61-100 grams of pimecrolimus were 2.98 times more likely to develop lymphoma compared to topical steroid users. This is a statistically significant difference and suggests that TCI use may increases risk over topical steroid use.

Another study conducted a retrospective cohort of individuals with AD and TCI use and found an increased risk for T-cell lymphoma (Hui et al., 2009). The study obtained its data from Kaiser Permanente in California and assessed 953,064 subjects with atopic dermatitis or eczema. After chart review and adjustment for confounding variables, tacrolimus users were 3.13 times more likely to develop T-cell lymphoma compared to non-users (P-value: 0.005). There was an 86% increased development in pimecrolimus patients, but this was not statistically significant (P-value= 0.204). Limitations to this study include the fact that the sample size was only obtained from health records in California. Additionally, the follow-up time was only 2.4 years for tacrolimus and 1.9 years for pimecrolimus. Strengths include the large sample size and stringent chart review.

Overall, TCI use may increase risk for different types of lymphoma. Although Arellano et al. (2007) did not find an association, the study did not differentiate between AD individuals

who developed HL, T-cell NHL, and B-cell NHL as done in the other two studies that did show an association (Hui et al., 2009; Schneeweiss et al., 2009). Longer term studies need to be conducted to assess the risk of TCI use in addition to the importance of dosing and length of treatment.

2.3 Current Research on Botanical Extracts

As stated previously, antifungal resistance is a growing problem. Although there is limited evidence on *Malassezia* spp. specifically, turning to botanical extracts is a relevant field of study. There is evidence that botanical extract treatments may provide powerful antifungal activity, without the side effects associated with current treatments (Simonetti et al., 2017). Through human keratinocyte (HaCaT) LDH cytotoxicity testing, studies have shown the availability of botanical extracts that demonstrate antifungal activity and preserve the integrity of keratinocytes (Biabiany et al., 2012). It should be noted, however, that *natural* does not mean better or safer. Antifungal resistance is still a possibility with botanical extracts, which is why it will continue to be important to establish stringent dose recommendations based on clinical trials. The current study only focused on botanical extracts that showed growth inhibition greater than 90% to decrease risk of resistance. It has been established that some *Malassezia* spp. including *M. pachydermatis* and *M. furfur* exhibit biofilm formation (Angiolella et al., 2018; Figueredo et al., 2013; Sardi et al., 2014; Simonetti et al., 2015). Biofilm inhibition is an important field of study because it focuses on inhibiting secondary defenses such as microbial cell-cell communication (Figueredo et al., 2013). It also targets sessile cells which deplete nutrients from the host while worsening symptoms and increasing risk of recurrence (Figueredo et al., 2013). Therefore, future studies should focus on botanical extracts with antibiofilm activity to knock down the secondary defenses of *Malassezia* spp., decreasing attachment to the host and

promotion of virulence factors without promoting resistance (Figueredo et al., 2013; Iatta et al., 2014).

2.4 *In Vitro* Research against *Malassezia* species

Plants produce both primary and secondary metabolites (Bourgaud et al., 2001). Primary metabolites are implicated in growth and reproduction and are therefore essential to survival (Bourgaud et al., 2001). Secondary metabolites, on the other hand, are not essential for survival, but help plants thrive in their ecosystem (Bourgaud et al., 2001). Common examples of secondary metabolites include alkaloids, terpenoids, and phenolics (Bourgaud et al., 2001). These compounds are beneficial to the plant in several ways. For example, flavonoids are phenolic compounds responsible for the yellow pigment in many plants, thereby attracting pollinators and increasing chances of reproduction (Bourgaud et al., 2001). In humans, flavonoids play a potent antioxidant role, decreasing free radicals in the body, and promoting anti-inflammatory, antiallergenic, and vasodilatory properties (Pietta, 2000). Secondary metabolites also protect plants from pathogens via the production of phytoalexins, exhibiting antimicrobial, antimycotic, and antiviral properties (Bourgaud et al., 2001). Plants and their secondary metabolites contain therapeutic properties that are important against many human skin diseases, including seborrheic dermatitis, atopic dermatitis, and tinea versicolor. The following table (Table 1) includes plant extracts that were previously tested against *Malassezia* spp. implicated in the common skin disorders AD, SD, and TV.

Table 1. Botanical Extracts Tested In Vitro against *Malassezia* species

Family	Botanical Name	Plant part	Species tested	Methods	Results	Source
Amaryllidaceae	*Allium sativum*	Cloves	*M. furfur*	Agar well diffusion method	Microwave dried garlic powder MIC_{100} 268.33µg/mL and aqueous garlic extract 249.6µg/mL	(Gitanjali et al., 2014)
	Allium sativum	Cloves	*M. furfur*	Agar well diffusion with extract at 100 mg/mL	IZ 15.5±0.86 mm as compared to Selsun shampoo control IZ of 30.0±0.05 mm	(Kaur et al., 2016)
	Allium cepa	Bulbs	*M. furfur*	Broth microdilution with aqueous extract	MIC_{50} at 2mg/mL and MIC_{90} at 8mg/mL as compared to KTZ MIC_{90} at 2 µg/mL	(Shams-Ghahfarokhi et al., 2006)
	Allium sativum	Bulbs	*M. furfur*	Broth microdilution with aqueous extract	MIC_{50} at 31.2 µg/mL and MIC_{90} at 125 µg/mL as compared to KTZ MIC_{90} at 2 µg/mL	(Shams-Ghahfarokhi et al., 2006)
Anacardiaceae	*Semecarpus anacardium*	Leaves	*M. furfur*	Broth microdilution with methanolic extract	MIC and MFC 400 mg/mL	(Pednekar and Raman, 2013b)
Apiaceae	*Anethum graveolens*	Seed	*M. furfur*	Agar well diffusion with aqueous extract at 4 mg/mL	IZ 60 mm for aqueous extract as compared to IZ of 40 mm for KTZ control MIC_{100} determined to be 6 mg/mL for extract	(Mahmoud et al., 2015)
Aquifoliaceae	*Ilex paraguariensis*	Leaves	*M. furfur*	Agar well diffusion with extract at 1000 mg/mL	IZ 18.5±2mm at 1000 mg/mL as compared to 22.9±2.6 mm for KTZ control at 5.12 µg/mL	(Filip et al., 2010)
Asparagaceae	*Asparagus racemosus*	Roots	*M. furfur* *M. globosa*	Agar disk diffusion and broth microdilution	MIC 25 mg/mL for methanolic extract MIC of saponin-enriched extract 0.20 mg/mL for *M. furfur* and 0.40 mg/mL for *M. globosa*	(Onlom et al., 2014)
Asteraceae	*Pluchea carolinensis*	Leaves	*Malassezia* spp. from clinical isolate	Agar well diffusion	MIC_{100} of 400 µg/mL for EtOH/water extract as compared to 16 µg/mL for Fluconazole control HaCaT IC_{50} 400 µg/mL	(Biabiany et al., 2012)

	Artemisia abrotanum	-	*M. furfur* *M. slooffiae* *M. globosa* *M. sympodialis*	Agar dilution method	MIC 2 mg/mL for ethanol extract in all *Malassezia* spp. tested *M. furfur* and *M. sympodialis*: MIC 0.6 mg/mL for oil extract *M. slooffiae*: MIC 0.3mg/mL for oil extract *M. globosa*: MIC 1.3 mg/mL for oil extract	(Brodin et al., 2007)
	Eclipta alba	Whole plant	*M. furfur*	Agar disk diffusion of ethanolic extract at 250 µg/mL and 500 µg/mL	250 µg/mL: 12.1±0.34 mm 500 µg/mL: 13.9±0.32 mm as compared to clotrimazole IZ of 16.1 mm at 25 µg/mL	(Regupathi and Chitra, 2015)
	Dittrichia viscosa	-	*M. furfur*	Agar disk diffusion	IZ 10.66 ± 1.54 mm for ethanolic extract at 50 mg/mL. IZ 9.0 ±1.73 mm for methanolic extract at 50 mg/mL and 8.0 ± 1.0 mm at 10 mg/mL	(Rhimi et al., 2017)
Berberidaceae	*Mahonia aquifolium*	Stem, bark	*Malassezia* spp. from clinical isolates	Agar dilution method	MIC ranged from $\leq$50 µg/mL to $\geq$1000 µg/mL depending on isolate	(Vollekova et al., 2001)
Burseraceae	*Bursera simaruba*	Bark	*Malassezia* spp. from clinical isolate	Agar well diffusion method	MIC_{100} of 500 µg/mL for EtOH extract as compared to 16 µg/mL for Fluconazole control HaCaT IC_{50} 1.5µg/mL	(Biabiany et al., 2012)
Cladoniaceae	*Cladia aggreagata*	Lichen material	*M. furfur* *M. restricta* *M. globosa* *M. sympodialis*	Broth microdilution with ethanolic extract	*M. furfur*: 2720 µg/mL as compared to 34 µg/mL for FLC control *M. globosa* 630 µg/mL as compared to 6 µg/mL for FLC *M. sympodialis*: 1280 µg/mL as compared to 51 µg/mL for FLC *M. restricta*: no activity in extract	(Pandey et al., 2013)

Combretaceae	*Terminalia bellerica*	Fruit coat	*M. furfur*	Agar disk diffusion	IZ of 23 mm at 22.5 mg/mL for water extract and 24 mm at 30 mg/mL for ethanol extract as compared to IZ of 23 mm for zinc pyrithione control at 50 µg/mL	(Balakrishnan et al., 2011)
	Terminalia chebula	Fruit coat	*M. furfur*	Agar disk diffusion	IZ of 24 mm at 22.5 mg/mL for water extract and 18 mm at 30 mg/mL for ethanol extract as compared to IZ of 23 mm for zinc pyrithione control at 50 µg/mL	(Balakrishnan et al., 2011)
Compositae	*Vernonia cinerea*	Leaves	*M. furfur*	Agar disk diffusion: extract at 10 mg/mL	IZ of 19 mm for ethyl acetate extract as compared to IZ of 21 mm for antidandruff shampoo control	(Dhanalakshmi et al., 2013)
Ericaceae	*Chimaphila umbellata*	-	*M. globosa* *M. restricta*	Broth microdilution	Chimaphilin compound extracted from *C. umbellata* exhibited MIC_{80} of 0.39 mg/mL for *M. globosa* and 0.55 mg/mL for *M. restricta*	(Galvan et al., 2008)
Euphorbiaceae	*Emblica officinalis*	Fruit coat	*M. furfur*	Agar disk diffusion	IZ of 18 mm at 22.5 mg/mL for water extract and 30 mg/mL for ethanol extract as compared to IZ of 23 mm for zinc pyrithione control at 50 µg/mL	(Balakrishnan et al., 2011)
Fabaceae	*Acacia concinna*	Pods	*M. furfur* *M. globosa* *M. restricta* *M. obtusa* *M. slooffiae* *M. sympodialis*	Agar disk diffusion at 256 µg/mL and broth microdilution	*M. furfur*: 22.9 mm *M. globosa*: 24.0 mm *M. obtusa* 23.3 mm *M. restricta*: 21.6 mm *M. slooffiae* 22.3 mm *M. sympodialis* 23.0 mm as compared to Itraconazole control tested at 16 µg/mL IZ 22.3 mm MIC: *M. globosa* 32 µg/mL	(Sibi et al., 2014)

Hypericaceae	*Hypericum perforatum*	Roots	*M. furfur*	Broth microdilution with methanolic extract	MIC_{50} 16 µg/mL and MIC_{90} 32µg/mL.	(Simonetti et al., 2015)
Lamiaceae	*Ocimum kilimandscharicum*	Leaves	*M. furfur*	Serial microplate dilution with essential oil	MIC 128 µg/mL as compared to KTZ MIC 16 µg/mL	(Pooja et al., 2013)
	Origanum dictamnus	Dried flowering herbs	*M. furfur* *M. restricta* *M. globosa*	Broth microdilution with aqueous extract	*M. restricta* and *M. furfur*: 32.8 mg/mL *M. globosa*: 64.5 mg/mL	(Varsani et al., 2017)
Lauraceae	*Cinnamomum verum*	Bark	*M. furfur* *M. globosa* *M. sympodialis*	Agar well diffusion and agar dilution with methanolic extract	*M. furfur*: IZ of CV extract 25 mm at 10 µg/mL as compared to KTZ control 30 mm IZ *M. globosa*: IZ of extract 24 mm as compared to KTZ 40 mm IZ *M. sympodialis* 10 mm IZ as compared to 35 mm IZ of KTZ; MIC_{100} for *M. furfur* is 1.1 mg/mL, *M. globosa* is 0.75 mg/mL, *M. sympodialis* is 1.5 mg/mL	(Mariappan et al., 2013)
	Cinnamomum verum	Bark	*M. furfur*	Serial microplate dilution with essential oil	MIC 32 µg/mL as compared to KTZ MIC 16 µg/mL	(Pooja et al., 2013)
Lythraceae	*Lawsonia inermis*	Leaves	*M. furfur* *M. globosa* *M. restricta* *M. obtusa* *M. slooffiae* *M. sympodialis*	Agar disk diffusion at 256 µg/mL and broth microdilution	MIC 64 µg/mL against *M. globosa*	(Sibi et al., 2014)
	Lawsonia inermis	Leaves	*M. furfur*	Agar disk diffusion and broth microdilution	Methanol extract: IZ 13.8 mm at 25mg/mL and 10.63 mm at 12.5 mg/mL as compared to IZ of 16.06 mm for KTZ at 0.125 mg/mL MIC: 0.781 mg/mL for methanolic extract	(Sreelatha et al., 2015)

Malvaceae	*Hibiscus rosa-sinensis*	Flower	*M. furfur* *M. globosa* *M. restricta* *M. obtusa* *M. slooffiae* *M. sympodialis*	Agar disk diffusion at 256 µg/mL and broth microdilution	*M. furfur*: 20.5 mm *M. globosa*: 21.1 mm *M. obtusa* 21.7 mm *M. restricta*: 19.6 mm *M. slooffiae* 20.8 mm *M. sympodialis* 22.0 mm as compared to Itraconazole control tested at 16 µg/mL IZ 22.3 mm MIC: *M. sympodialis* 64 µg/mL	(Sibi et al., 2014)
Meliaceae	*Azadirachta indica*	Leaves	*M. furfur* *M. globosa* *M. restricta* *M. obtusa* *M. slooffiae* *M. sympodialis*	Agar disk diffusion at 256 µg/mL and broth microdilution	MIC *M. sympodialis* 64 µg/mL	(Sibi et al., 2014)
Myrtaceae	*Syzygium aromaticum*	Buds	*M. furfur*	Agar disk diffusion with extract at 100 mg/mL	IZ 20.0±0.53 mm as compared to Selsun shampoo control IZ of 30.0±0.05 mm	(Kaur et al., 2016)
	Melaleuca alternifolia	Essential oil (tea tree)	*M. furfur* (clinical isolates)	Agar dilution method	Growth inhibition of all strains at concentrations of 556.2-4,450 µg/mL with a geometric mean of 1,261.6 µg/mL 12/15 (80%) of dandruff isolated inhibited at 1,112 µg/mL 100% of SD and TV isolates inhibited at 2,225 µg/mL.	(Nenoff et al., 1996)
	Melaleuca leucadendron	Leaves	*M. furfur*	Serial microplate dilution with essential oil	MIC 64 µg/mL as compared to KTZ MIC 16 µg/mL	(Pooja et al., 2013)
	Melaleuca alternifolia	Leaves	*M. furfur*	Serial microplate dilution with essential oil	MIC 32 µg/mL as compared to KTZ MIC 16 µg/mL	(Pooja et al., 2013)

	Syzygium aromaticum	Flower buds	*M. furfur*	Agar disk diffusion and broth microdilution	Hexane extract: IZ 18.36 mm at 25mg/mL and 13.9 mm at 12.5 mg/mL as compared to IZ of 16.06 mm for KTZ at 0.125 mg/mL MIC: 0.391 mg/mL for hexanolic extract	(Sreelatha et al., 2015)
Nelumbonaceae	*Nelumbo nucifera*	Seeds extracted with hexane into oil	*M. furfur*	Agar disk diffusion	IZ of 17.33±3.06 mm 20.67± 3.21 mm, 24.67± 3.51 mm at 25, 50, and 75 µg/mL as compared to IZ of 20.67±1.15 mm for fluconazole control	(Arumugam and Dhailappan, 2012)
Oleaceae	*Nyctanthes arbor-tristis*	Leaves	*M. furfur*	Agar disk diffusion with extract at 25 mg/mL	IZ 8.33 ± 0.57 mm for methanol extract and 7.73 ± 0.46 mm for hexane extract MIC 25 mg/mL and MYC 50 mg/mL.	(Lalitha et al., 2016)
	Nyctanthes arbor-tristis	Leaves	*M. furfur* *M. restricta* *M. globosa* *M. sympodialis*	Broth microdilution with ethanolic extract	MIC: *M. furfur* 1.22 µg/mL *M. restricta* 1.47 µg/mL *M. globosa* 1.05 µg/mL *M. sympodialis* 1.35 µg/mL MFC: 3.12 µg/mL for all strains	(Mishra et al., 2016)
Parmeliaceae	*Usnea barbata*	Lichen material	*M. furfur*	Agar dilution with aqueous extract	MIC 100 at 10 µg/mL	(Weckesser et al., 2007)
Phyllanthaceae	*Phyllanthus emblica*	Fruits	*M. furfur* *M. globosa* *M. restricta* *M. obtusa* *M. slooffiae* *M. sympodialis*	Agar disk diffusion at 256 µg/mL and broth microdilution	*M. furfur*: 23.7 mm *M. globosa*: 22.2 mm *M. obtusa* 20.1 mm *M. restricta*: 22.6 mm *M. slooffiae* 19.3 mm *M. sympodialis* 22.8 mm as compared to Itraconazole control tested at 16 µg/mL IZ 22.3 mm MIC: *M. furfur* 32 µg/mL	(Sibi et al., 2014)

Pinaceae	*Pinus densiflora*	Needles	*M. furfur*	Agar disk diffusion method with extract at 100 mg/mL	IZ of 1.1 mm for water extract, 1.2 for ethyl acetate, 1.3 mm for n-butyl alcohol extract, and 1.9 mm for n-hexane extract	(Choi et al., 2016)
Piperaceae	*Piper nigrum*	Seeds	*M. furfur*	Agar disk diffusion with extract at 100 mg/mL	IZ 14.5±1.3 mm as compared to Selsun shampoo control IZ of 30.0±0.05 mm	(Kaur et al., 2016)
Posidoniaceae	*Posidonia oceanica*	Rhizomes	*M. furfur*	Agar disk diffusion	Aqueous extract had no activity IZ of 6-12 mm for chloroform extract at concentration of 50 mg/mL	(Bernard and Pesando, 1989)
Primulaceae	*Embelia ribes*	Fruit	*M. furfur*	Broth microdilution	65% growth inhibition of EA extract and 77% inhibition of PE extract at 500 µg/mL	(Sivasankar et al., 2017)
Punicaceae	*Punica granatum*	Fruit rind	*M. furfur*	Agar disk diffusion with extract at 25 mg/mL	At 25 mg/mL IZ 7.33 ± 0.57 mm of methanolic extract MIC and MYC 25 mg/mL	(Lalitha et al., 2016)
Rutaceae	*Citrus aurantifolia*	Peel	*M. furfur*	Agar disk diffusion with oil extracts at 2 mg/mL	IZ 26 mm for methanolic extract at 2 mg/mL as compared to control Itraconazole IZ of 15 mm at 2 mg/mL	(Lee and Lee, 2010)
	Murraya koenigii	Leaves	*M. furfur* *M. globosa* *M. restricta* *M. obtusa* *M. slooffiae* *M. sympodialis*	Agar disk diffusion at 256 µg/mL and broth microdilution	*M. furfur*: 23.7 mm *M. globosa*: 22.2 mm *M. obtusa* 20.1 mm *M. restricta*: 22.6 mm *M. slooffiae* 19.3 mm *M. sympodialis* 22.8 mm as compared to Itraconazole control tested at 16 µg/mL IZ 22.3 mm MIC: *M. furfur* 32 µg/mL	(Sibi et al., 2014)
Schisandraceae	*Illicium verum*	Fruits	*M. furfur*	Agar disk diffusion with extract at 25 mg/mL	IZ 7.66 ± 0.57 mm of hexane extract. MIC 25 mg/mL and MYC 50 mg/mL	(Lalitha et al., 2016)

Verbenaceae	*Lantana camara*	Leaves	*M. furfur*	Agar well diffusion	No IZ for aqueous extract IZ 13 mm for ethanol extract at 30 mg/mL as compared to IZ of 23 mm for zinc pyrithione control at 50 µg/mL	(Balakrishnan et al., 2011)
	Lippia nodiflora	Whole plant	*M. furfur*	Agar disk diffusion of ethanolic extract at 250 µg/mL and 500 µg/mL	250 µg/mL: 13.7±0.54 mm 500 µg/mL: 15.4±0.55 mm as compared to clotrimazole IZ of 16.1 mm at 25 µg/mL	(Regupathi and Chitra, 2015)
Vitaceae	*Ampelocissus latifolia*	Leaves	*M. furfur*	Broth microdilution with methanolic extract	MIC 200 mg/mL and MFC 800 mg/mL	(Pednekar and Raman, 2013a)
	Vitis vinifera	Seeds	*M. furfur*	Broth microdilution method against three different *M. furfur* strains (geometric mean provided) based on 6 different grape varieties	MP 2012 A (AEtOH/H2O, 7:3 v/v): 64 µg/mL MP 2012 B (EtOH): 256 µg/mL MP 2012 C (MeOH) 102 µg/mL ITA 2012 A (AEtOH/H2O, 7:3 v/v): 81 µg/mL ITA 2012 B (EtOH): 256 µg/mL ITA 2012 C (MeOH) 203 µg/mL as compared to FLZ MIC_{100} 55 µg/mL	(Simonetti et al., 2017)
Zingiberaceae	*Curcuma xanthorrhiza*	Xanthorrhizol isolated from ethyl acetate fraction of methanol extract	*M. furfur*	Broth microdilution	MIC: 1.25 µg/mL MFC: 5 µg/mL as compared to MIC 0.625 µg/mL and MFC 5 µg/mL of Zinc pyrithione control	(Rukayadi and Hwang, 2007)

IZ – inhibition zone (in millimeters); MIC – minimum inhibitory concentration; MFC/MYC – minimum fungicidal concentration; MeOH – methanol, EtOH – ethanol, H_2O – water, AEtOH – ethyl acetate; FLC – fluconazole, KTZ – ketoconazole; EA – ethyl acetate, PE – petroleum ether; HaCaT – human epidermal keratinocyte (human skin cell)

There were several plant products that exhibited antifungal activity comparable or superior to standard treatment. Antifungal activity against *M. furfur* varied widely as it was the most frequently tested microorganism. Aqueous extract of *Allium sativum* (Amaryllidaceae) performed moderately well with MIC_{90} of 125 μg/mL and MIC_{100} of 249.6 μg/mL as compared to ketoconazole (KTZ) MIC_{90} of 2 μg/mL (Gitanjali et al., 2014; Shams-Ghahfarokhi et al., 2006). Activity ranged as low as MIC_{90} of 32 μg/mL for methanolic extract of *Hypericum perforatum*, bark extract of *Cinnamomum verum*, leaf extract of *Murraya koenigii*, fruit extract of *Phyllanthus emblica*, and essential oil extract of *Melaleuca alternifolia* (Pooja et al., 2013; Sibi et al., 2014; Simonetti et al., 2015). In terms of fungicidal activity, Simonetti et al. (2017) exhibited MIC_{100} of 64 μg/mL and 81 μg/mL for MP 2012 A and ITA 2012 A variety, respectively, as compared to FLC MIC_{100} at 55 μg/mL. Rukayadi et al. (2007) determined MFC of 5 μg/mL for xanthorrhizol compound isolated from Curcuma xanthorrhiza as compared to MFC of 5 μg/mL for Zinc Pyrithione control. *Usnea barbata* extract also demonstrated potent MFC against *M. furfur* at 10 μg/mL (Weckesser et al., 2007). Mishra et al. (2016) demonstrated MFC of 3.12 μg/mL for ethanolic extract of *Nyctanthes arbor-tristis* (NAT). This varies strongly from the MFC of both the hexanolic and methanolic extracts of NAT at 50 mg/mL in a different study, likely due to the difference in organic solvent and the use of disk diffusion in the latter study, which is a less accurate methodology (Lalitha et al., 2016).

Several studies also tested botanicals against other *Malassezia* spp. that are implicated in common skin conditions including *M. globosa*, *M. restricta*, *M. sympodialis*, *M. obtusa*, and *M. slooffiae*. However, *M. obtusa* and *M. slooffiae* are not implicated in the pathogenesis of various skin conditions (Sugita et al., 2012); *M. obtusa* was only tested in one study (Sibi et al., 2014) and *M. slooffiae* was only reported in two studies (Brodin et al., 2007; Sibi et al., 2014).

Ethanolic extract of *Cladia aggreagata* (Cladoniaceae) exhibited moderate activity against *M. globosa* at 630 μg/mL as compared to fluconazole (FLC) control at 6 μg/mL (Pandey et al., 2013). Similarly, Onlom et al. (2014) tested *M. globosa* against *Asparagus racemosus* and showed MIC_{90} of 0.40 mg/mL (400 μg/mL). *Lawsonia inermis* (Lythraceae) exhibited potent activity against *M. globosa* with MIC_{90} of 64 μg/mL (Sibi et al., 2014). Further, *Acacia concinna* (Fabaceae) demonstrated antifungal activity against *M. globosa* with MIC_{90} of 32 μg/mL (Sibi et al., 2014). *Hibiscus rosa-sinensis* and *Azadirachta indica* both demonstrated MIC_{90} of 64 μg/mL against *M. sympodialis* (Sibi et al., 2014). As described with *M. furfur*, NAT extract demonstrated MFC of 3.12 μg/mL for *M. restricta*, *M. globosa,* and *M. sympodialis* as well (Mishra et al., 2016).

Based on the results, the ethanolic extract of the leaves of *Nyctanthes arbor-tristis* (Oleaceae) demonstrated the most effective antifungal activity against the pathogenic *Malassezia* spp. including *M. globosa*, *M. restricta*, *M. furfur*, and *M. sympodialis*. This concentration was more effective than Zinc pyrithione and Fluconazole and comparable to ketoconazole control, which exhibited MIC_{90} between 2-16 μg/mL (Filip et al., 2010; Pooja et al., 2013; Shams-Ghahfarokhi et al., 2006).

2.5 Clinical Research Utilizing Botanical Extracts

A few clinical trials with botanical extracts have also been conducted for individuals with AD, SD, and TV. Hon et al. (2007) studied the effects of a traditional Chinese medicine herbal formula for children with atopic dermatitis. 42 children were assigned to the treatment group (TCHM) and 43 children assigned to the control group for a 12-week trial. The treatment group was instructed to take three capsule of the herbal formula twice a day, while the control group

was given a placebo capsule. The herbal formula consisted of *Flos lonicerae* 2 g, *Herba menthae* 1 g, *Cortex moutan* 2 g, *Rhizoma atractylodis* 2 g, and *Cortex phellodendri* 2 g, for a total 9 g of raw herbs. Children were assessed using the SCORAD (Scoring Atopic Dermatitis) scale and CDLQI (Children's Dermatology Life Quality Index) at 4, 8, 12, and 16 weeks. A higher CDLQI score means the dermatological condition has a higher effect on the patient's life. The CDLQI scores significantly decreased for the treatment group at the end of the 12 weeks compared to placebo ($P = 0.008$). Further, topical corticosteroid use significantly decreased in the TCHM group by one-third ($P = 0.024$). However, the SCORAD score significantly decreased for both the TCHM group and the placebo ($P = 0.003$ and $P = 0.001$). Therefore, the results may provide some convincing evidence for efficacy in oral botanical treatment, but further research should be conducted.

There has also been anecdotal evidence that vinegar (acetic acid) plays a potent antimycotic role (Bassett et al., 2004; Cheung et al., 2014). It is likely the low pH that promotes antifungal activity, and not metabolites contained in the vinegar itself (Matousek et al., 2003). Our own analysis also confirmed this. We tested apple cider vinegar with "the mother" (unpasteurized vinegar that contains beneficial bacteria and live enzymes) and observed fungicidal activity at concentrations as low as 0.625% acetic acid. We then concentrated an extract of "the mother" polpyphenolic compounds that did not contain acetic acid, but failed to observe antifungal activity, even at concentrations as high as 512 μg/mL. Cheung et al. (2014) studied the effects of acetic acid against *Trichophyton rubrum*, a different yeast implicated in athlete's foot, ringworm, and fungal infections of the nails. They determined that a pH of 3.0 and lower exhibited fungicidal effects, however there is clinical concern about such a low pH disrupting the skin microbiome. Matousek et al. (2003), also studied the effect of acetic acid's

pH on growth of *M. pachydermatis* and similarly determined that a pH of 2.0 and 3.0 inhibited growth, but a pH of 4.0 was not acidic enough. However, this study used HCl to obtain the proper pH values, which also may have inhibited fungal growth (Matousek et al., 2003). There are also several relevant studies that tested plant extract and essential oil formulations topically for the treatment of either AD, SD, or TV. Results are displayed below in Table 2.

Table 2. Botanical Extract Formulations Tested Topically in Human and Animal Trials.

Formulation	Methods	Population	Results	Limitations	Source
5% tea tree oil shampoo	1. Two groups: 63 volunteers received 5% tea tree oil and 62 received placebo shampoo 2. Wash hair daily and leave shampoo in for 3 minutes	126 male and female patients aged 14 years or older with seborrheic dermatitis (scalp dandruff)	1. 41% improvement in quadrant-area-severity score compared with 11% in the placebo group ($p<0.001$). 2. Statistically significant improvement in itchiness sand greasiness based on patient self-assessment. 3. Statistically significant improvement in total area of involvement score and total severity score. 4. Scaliness improved in treatment group, but not statistically significant.	1. Longer study time needed to evaluate ongoing improvement of symptoms. 2. Shampoo not assessed in pediatric patients. 3. Different concentrations of tea tree oil shampoo not evaluated. 4. Did not use a positive control such as KTZ shampoo.	(Satchell et al., 2002)
8% *Ficus carica* (Fig) cream	1. Three treatment groups: 8% *F. carica* cream (n=16), hydrocortisone 1.0% (n=14), and placebo (n=15). 2. Patients instructed to apply cream twice a day for two weeks.	45 children aged 4 months to 14 years with mild to moderate AD.	1. SCORAD decreased from 33.85+10.05 to 14.85+8.83 after 2 weeks of treatment for the fig fruit extract cream ($p<0.0001$) and from 29.53+13.58 to 16.73 for hydrocortisone group ($p<0.001$). 2. Intensity score for AD decreased from 6.75+2.81 to 3.06+1.8 for the fig extract group ($p<0.0001$) vs. 6.28+2.84 to 3.28+1.77 for the hydrocortisone group ($p<0.001$). 3. Pruritus scores decreased from 5.31+2.70 to 1.93+1.91 for fig extract group ($p<0.001$) vs. 3.5 +2.76 to 2.35+1.98 for hydrocortisone group ($p<0.004$). 4. No significant differences were observed for placebo group.	1. Longer study time to evaluate ongoing improvement of symptoms 2. Small sample size 3. Although the bottles were identical, the creams inside were not and this could have affected blindness.	(Abbasi et al., 2017)

Natural honey, beeswax, and olive oil mixture	1. Treatment applied on left side and Vaseline control applied on right side. 2. Applied both therapies three times daily with gentle rubbing for two weeks.	21 total patients with AD: 1. Group 1: 10 patients with no treatment at time of inclusion. 2. 11 patients using topical corticosteroids 3-6 months prior to inclusion.	1. Group 1: At week 1, significant improvement in lesions as compared to right-side body Vaseline control ($p=0.022$). At week 2, significant improvement in lesions from baseline and compared to control ($p<0.05$ and $p=0.0129$). At week 3 and 4 follow-up significant improvement in lesions in 8/10 patients as compared to baseline ($p<0.0001$). 2. Group 2: Honey mixture enabled patients to reduce dose of corticosteroid, but decrease in skin lesions was not statistically significant from Vaseline control or baseline at any time points.	1. Small sample size 2. Short treatment length 3. Researchers were not blinded to the treatment locations on the body.	(Al-Waili, 2003)
2% boric acid and 2% acetic acid cleaning solution	1. Treatment: Applied once daily for first week and two times in second week. 2. Prophylaxis: applied once a week for two weeks. 3. Both groups instructed to massage ear canal for 5 minutes after application.	26 dogs with *Malassezia* spp. causing otitis externa: 18 dogs in treatment group and 8 in prophylaxis group	1. Otitis externa resolved in 88% (16/18). 2. Relapse was seen in 75% (6/8) using ear cleaner as prophylaxis. Effective treatment initially, but not appropriate for long-term prevention of recurrence.	1. Small sample size 2. Short treatment length 3. No control group 4. Solution was not tested on *Malassezia*-related skin conditions in humans.	(Bassett et al., 2004)
Shampoo and cream containing *Cymbopogon citratus* essential oil (lemon grass) at 1.25 ul/mL	1. Shampoo 3x a week and cream twice a day for 40 days. 2. Control group given same instructions, but contained 2% ketoconazole.	30 patients with TV received essential oil treatment and 18 received standard ketoconazole treatment.	1. 60% cure rate for treatment group as compared to over 80% cure rate for control group using ketoconazole shampoo.	1. Participants and researchers were not blinded to treatment 2. Did not specify method of detecting disease clearance 3. Only tested one concentration of treatment	(Carmo et al., 2013)

Cassia alata aqueous extract (semaiagathi)	1. Applied one single application at concentration between 70-100%	200 individuals with TV between the age of 16-60.	1. After one single application, mild irritation in first week. 2. Infection began to disappear at week 3 and original skin color restored within 4-10 months. However, TV began to recur at months 11-12. 3. Follow-up revealed one-time application every four months for 3 years resulted in complete cure.	1. Did not specify demographic information of patients 2. Patients were not blinded to treatment. 3. Not specified if patients utilized other treatments concomitantly during the study.	(Damodaran and Venkataraman, 1994)
4% *Quassia amara* (QX) gel	1. Applied approximately 0.05mL/cm2 twice a day for 28 days.	60 patients with SD: 20 patients received 4% QX gel, 20 received 2% ketoconazole (KTZ) gel, and 20 received 1% ciclopirox olamine (CIC) gel.	1. At day 7, only the QX and CIC groups significantly decreased SD severity scores. 2. At day 14, 21, and 28, all three products significantly decreased the mean SD severity score ($p<0.01$). 3. At the end of treatment, lowest severity score was 5.1 in QX group vs. 6.7 in CIC group vs. 6.8 in KTZ group. 4. At day 56, 1 month after discontinuing products, significant difference in SD severity score: 3.8 for QX group vs. 5.6 CIC group vs. 6.4 KTZ group ($p<0.01$).	1. Small sample size. 2. Only severity scores were analyzed. An index filled out by the patients would have provided an interesting comparison of treatment efficacy.	(Diehl and Ferrari, 2013)
Virgin coconut oil and mineral oil	1. Apply 5 mL of assigned oil twice daily.	117 pediatric patients with mild-to-moderate AD: 58 individuals in mineral oil group and 59 individuals in virgin coconut oil	1. Reduction of SCORAD index in VCO-treated group (68.23%) and mineral oil-treated group (38.13%) compared to baseline ($p<0.001$). 2. Decrease in transepidermal water loss in VCO-group and mineral oil treated groups compared to baseline. 3. Improvement in skin capacitance from 32.0 to 42.3 in VCO-treated group and from 31.31 to 37.49 in mineral-oil treated group	1. Blinding may have been affected by the scent of virgin coconut oil, although both oils were placed in opaque bottles.	(Evangelista et al., 2014)

Kanuka Honey	1. Patients applied medical-grade honey to a lesion on one side and aqueous cream BP to the other side every night for 2 weeks.	15 adult patients with AD.	1. Kanuka honey did not cause a significant decrease in SCORAD lesion from baseline to 2-week follow-up (4.6 vs. 4.4). 2. Three item severity score was not significantly different between baseline and follow-up (1.9 vs. 2.0). 3. Itching decreased from 41.7 at baseline to 26.4 at follow-up, however, this decrease was not statistically significant.	1. Incomplete blinding to the characteristics of honey. 2. Small sample size 3. Short treatment time	(Fingleton et al., 2014)
5% *Camellia sinensis* (green tea) extract solution	1. 30-minute bath in 700 mL of green tea extract solution mixed with 150 mL filtered tap water 3x/week for 1 month.	4 patients aged 5-9 with AD.	1. Mean SCORAD value decreased from 47.05+7.94 to 23.4+3.83 at week 4, a 50.3% reduction. 2. Visual analogue scale for pruritus decreased from 8.83+0.99 at baseline to 2.12+0.94 at week 4, but results were not statistically significant. 3. Initial mycological PCR test identified *M. sympodialis*, but at week 4, a new detection kit did not identify any *Malassezia* species.	1. Small sample size 2. Longer-treatment length may have further improved symptoms.	(Kim et al., 2012)
Shampoo containing 0.01% *Rosa centifolia* petals, 0.005% epigallocatechin gallate (EGCG), 0.3% zinc pyrithione, 0.45% climbazole	1. Three treatment groups: new formula shampoo, 2% ketoconazole shampoo, and 1% zinc-pyrithione shampoo. 2. Instructed to massage scalps for at least 5 minutes three times a week for 4 weeks.	72 patients with SD.	1. Clinical severity score improved significantly in all three groups at week 2 and 4 ($p<0.05$), but the changes in scores not significantly different between the groups. 2. Subjective improvement at weeks 2 and 4 not significantly different between the groups.	1. Shampoo formula contained well-known antidandruff agents and was not only made of plant extracts. 2. Would have been interesting to test a higher concentration formula to evaluate if efficacy changed.	(Kim et al., 2014)

Dano (poly-herbal oil containing *Wrightia tinctoria*, *Cassia alata*, and *Azadirachta indica*).	1. Apply oil to hair daily for 21 days.	10 volunteers with severe dandruff aged 18-22.	1. After 8 days of use, all volunteers had reduced scale rating from severe to mild. 2. At 10 days of use, there was no evidence of scaling in all participants. 3. At the beginning of the study, *M. furfur* was isolated in 100% of volunteers, but by day 8, none of the scale samples contained *M. furfur* in culture.	1. Small sample size 2. Did not specify the concentration of extracts in the hair oil 3. A quality index completed by the participants would have been useful to rate the efficacy 4. There was no control group.	(Krishnamoorthy et al., 2006)
BSASM, a multi-compound preparation containing Carnosic acid (*Rosmarinus officinalis*), apigenin (*Matricaria recubita*), Epigallocatechin-3-gallate (*Camellia sinensis*), Glycyrrhetinic acid (*Glycyrrhiza glabra*), Resveratrol (*Polygonum cuspidatum*), Baicalin (*Scutellaria baicalensis*), and Asiaticoside (*Centella asiatica*).	Patients all received three preparations: 1. Face and body wash used when taking a bath with lukewarm water for 10 minutes daily. 2. Lotion applied to entire body immediately after bathing. 3. Serum applied additionally to severely dry skin or lesions twice a day.	Thirty children (11 boys and 19 girls, 6-15 years) with mild AD symptoms	1. Statistically significant reduction in Eczema Area Severity Index (EASI) at 2 weeks and 4 weeks compared to before treatment (p= 0.001). 2. Pruritus decreased significantly at 2 weeks and 4 weeks (p=0.022). 3. Transepidermal water loss decreased significantly after treatment, but only the abdominal area was statistically significant (p=0.01).	1. Small sample size. 2. Only assessed children with mild AD. 3. No control group, control cream, or blinding in the study	(Lee et al., 2005)

Polyherbal formulation containing *Plumbugo zeylanicum*, *Brassica nigra*, *Dregea volubis*, *Rubia cordifolia*, *Raphanus sativus*, and vinegar.	1. Treatment: apply polyherbal formulation twice daily. 2. Control group: apply sodium thiosulphate lotion (20%) twice daily.	40 patients total with TV: 20 patients in treatment group and 20 patients in control group.	1. Improvement in scaling significant reduced in both groups as compared to baseline score. 2. Significant reduction in itching in both groups as compared to baseline score. 3. At 30-day assessment, all participants were negative for fungal colonization with KOH examination. 4. Total Sign Symptom Score significantly reduced in both groups from baseline ($p<0.001$).	1. No negative control utilized in study. 2. Small sample size. 3. Symptom severity not assessed after discontinuation of treatment to assess for recurrence. 4. Single-blinded.	(Lone et al., 2012)
Dill seed extract ointment (6 mg/g)	1. Apply two times daily for 2 weeks.	Ten patients with TV in treatment group.	1. Complete healing of lesions after 2 weeks of treatment	1. Small sample size. 2. No control and no blinding. 3. Symptom severity not assessed after discontinuation of treatment to assess for recurrence.	(Mahmoud et al., 2015)
7% *Acalypha wilkesiana* ointment	1. Apply two times daily for at least 4 weeks.	Three patients with TV.	1. Complete healing of lesions after 4 weeks of treatment	1. Small sample size. 2. No control and no blinding. 3. Symptom severity not assessed after discontinuation of treatment to assess for recurrence.	(Oyelami et al., 2003)
Artemisia sieberi essential oil lotion (5% concentration)	1. Control group prescribed 1% topical clotrimazole. 2. Both groups: apply twice daily for two weeks. 3. Assessment at two weeks and four weeks.	117 patients with TV: 51 patients in treatment and 49 in control group.	1. At end of 2 weeks, 86.3% improvement in treatment group as compared to 65.3% improvement in control ($p<0.05$). 2. Lab exam at end of 2 weeks showed 92.2% improvement in treatment group vs. 73.5% in control group ($p<0.01$). 3. Lab examination at four weeks (two weeks after stopping treatment) showed 96.1% clearance for treatment vs. 65.3% control ($p<0.01$).	1. Only checked recurrence two weeks after stopping treatment. 2. Recurrence over the following year would have been relevant. 3. Comparison of treatment times (1 week or 3 week) would have been useful as well.	(Rad et al., 2008)

1% and 2% *Glycyrrhiza glabra* (licorice) gel	1. Three groups given either 1% gel, 2% gel, or placebo. 2. Applied gel three times a day for two weeks.	90 patients total with AD— 1% gel: 30 patients 2% gel: 30 patients Placebo: 30 patients	1. Treatment with 1% and 2% gel resulted in statistically significant reduction in scores for erythema after two weeks ($p<0.05$). 2. Licorice extract significantly more effective than baseline in reduce edema and itching after one and two weeks ($p<0.05$). 3. 2% extract more effect than 1% extract at the end of first and second weeks. 4. Statistically significant improvement in erythema, edema, and itching for both gels as compared to placebo. No significant difference in scaling scores for either gel as compared to placebo.	1. Longer study time to evaluate ongoing improvement of symptoms. 2. Small sample size. 3. Follow-up after discontinuation of treatment.	(Saeedi et al., 2003)
1.5% hyperforin cream (constituent of *Hypericum perforatum*)	1. Treatment and placebo randomLy allocated to left or right side of patient's body. 2. Patient applied treatment and placebo twice daily for 4 weeks.	18 patients with mild-to-moderate AD	1. Intensity of eczematous lesions improved on both sides, but hyperforin cream significantly better than placebo at all follow-up visits ($p<0.05$). 2. Colonization with *Staphylococcus aureus* reduced by both hyperforin cream and placebo, but hyperforin cream showed better antibacterial activity ($p=0.064$).	1. Small sample size. 2. Used the same patient for both treatment and control.	(Schempp et al., 2003)
Aloe vera emulsion (30% Aloe vera crude extract)	1. Apply cream/emulsion by gentle massage to affected areas twice daily for 4-6 weeks.	44 adults with SD: 24 in treatment group and 20 in placebo group.	1. Significant decrease in scaling (36.6% treatment vs. 17.6% placebo), pruritus (21.5% vs. 5.3%), and number of involved sites (40.3% vs. 12.6%) as compared to baseline. 2. Decrease in erythema was not significant. 3. Complete resolution or significant improvement in 58% of patients.	1. Small sample size. 2. No follow-up to assess symptom severity after discontinuing treatment.	(Vardy et al., 1999)
Extra virgin coconut oil (VCO) and extra virgin olive oil (VOO)	1. Apply 5 mL oil two times a day for 4 weeks. 2. Apply oil to sites not infected with *S. aureus*, and massage for several seconds.	52 subjects with AD: 26 subjects received VCO and 26 subjects received VOO.	1. At follow-up 95% (19/20) VCO subjects were negative for *S. aureus* colonization vs. 50% (6/12) of VOO subjects. 2. Post-intervention SCORAD scores were significantly decreased for both oils, but effect was greater with VCO.	1. Small sample size	(Verallo-Rowell et al., 2008)

2.6 Relevance of Studying Botanicals

The search for novel botanical therapies that exhibit fungistatic and fungicidal effects is a relevant issue. With such a limited number of antifungal drugs and antifungal resistance increasing, we must look to alternative therapies. Botanicals offer several advantages as compared to standard antifungal, steroid, and TCI treatment for AD, SD, and TV.

Plants have formed the basis of sophisticated traditional medicine systems, such as Ayurvedic and Chinese medicine, for thousands of years (Gurib-Fakim, 2006). It is important to uphold and preserve this ethnobotanical knowledge since approximately 80% of the population still relies completely on traditional medicine systems for their healthcare (Quave and Pieroni, 2015; WHO, 2002). Further, natural products and their derivatives make up more than 50% of all pharmaceutical drugs currently in use today (Gurib-Fakim, 2006). An analysis of US prescriptions from 1959 to 1980 found that 25% contained plant extracts or active compounds derived from plants, pointing to the importance of botanical medicine in constituting effective therapies (Farnsworth et al., 1985). Therefore, it is important to utilize ethnobotanical knowledge in discovering potential therapies, especially since there is an increasing need for new compounds that do not develop similar mechanisms of resistance (Balkis et al., 2012).

There are thousands of plants available that possess a unique and adaptive chemistry and this variety is important in contrast to the limited option of antifungals currently available (Bourgaud et al., 2001). Botanicals do not offer risk of cross-resistance as observed in azole antifungal treatment because of their unique mechanism of action (Ebiloma et al., 2017). Further, botanical therapies utilize plant secondary metabolites, potent defenses that have evolved over thousands of years to fight off harmful bacteria and fungi (Bourgaud et al., 2001). In addition to concern about antifungal resistance, other current treatments including corticosteroid and TCI

use can have adverse effects and long-term use is not recommended (Borda and Wikramanayake, 2015). Therefore, with a plethora of medicinal plants available, we must search for novel therapies that exhibit antifungal effects and can be used long-term.

Chapter 3 Materials and Methods

3.1 Collection and Extraction

The Quave Natural Products Library (QNPL) was tested for activity against *Malassezia furfur*. Nearly 1,000 botanical extracts (000011-001275) were made from plants obtained from the Balkans, Italy, Georgia, and Florida. All plants were collected following WHO good agricultural practices (WHO, 2003) and voucher specimens were deposited at local herbaria and the Emory University Herbarium (GEO). Once a plant with potential medicinal activity is identified by interviews or literature reviews, different parts of the plant were separated. The bulk collection of each plant specimen is field dried and then further processed at the phytochemistry lab at Emory University under an USDA permit. The materials are quarantined and frozen at -80^{o}C for at least 48 hours. The plant material is then dried to a constant mass and ground into a powder using a Wiley Mill with a 2 mm screen (Thomas Scientific). The ground material is extracted by either a double maceration or decoction. Maceration is an extraction technique using an organic solvent and decoction is an aqueous extraction – the type of solvent utilized determines which secondary metabolites and compounds are extracted from the samples (Azwanida, 2015).

For macerations the powdered plant material is soaked in a closed container with organic solvent, 80-95% methanol or ethanol, at a ratio of 1:10 (w:v) for 3 days. Afterwards, the mixture is filtered into a flask. The initial plant material is then macerated for a second 3-day period

using the same solvent as the initial maceration. The maceration is again filtered and the filtrates from the first and second macerations are combined (Azwanida, 2015). After 6 days, the mixture is strained by filtration into a round-bottom flask (Azwanida, 2015). The extract is then concentrated on a rotary evaporatory (rotovap), which slowly turns the flask in a water bath at temperatures under 40^{o}C and reduced pressure (Azwanida, 2015). The concentrated extract is dissolved in a small amount of water and shell frozen (spin freeze) in a dry ice and acetone bath. This is done by slowly rotating the flask in the bath of acetone and dry ice until the sides of the flask form an icy sheet. The frozen extract is covered with aluminum foil and stored in the -80^{o}C freezer until lyophilization. Lyophilization is the final step to obtain pure plant powder with complete removal of water and solvent. Samples are loaded onto the lyophilizer and typically take about 24 hours to dry. The dry product is scraped into a scintillation vial and stored in the -20 ^{o}C freezer.

For decoctions, the ground plant powder is boiled for 20 minutes in deionized (DI) water. Afterwards, the sample is centrifuged, filtered, and concentrated on the rotovap at less than 40^{o}C. The dry or nearly dry extract is redissolved in DI water and shell frozen as specified above, then lyophilized for 24 hours. Once the extract is freeze dried, it is scraped into a scintillation vial and stored at -20^{o}C.

When a botanical extract is required for microbiological testing, approximately 10 milligrams of the powder are weighed into a 2 mL snap-cap tube and dissolved in an appropriate solvent (either DMSO or dH_2O) depending on the initial solvent used for maceration or decoction. For initial MIC testing, extracts were tested at 10 mg/mL. For XTT assay testing and initial serial dilution testing, samples were prepared at a concentration of 50 mg/mL. Sonication was utilized to ensure samples were completely dissolved.

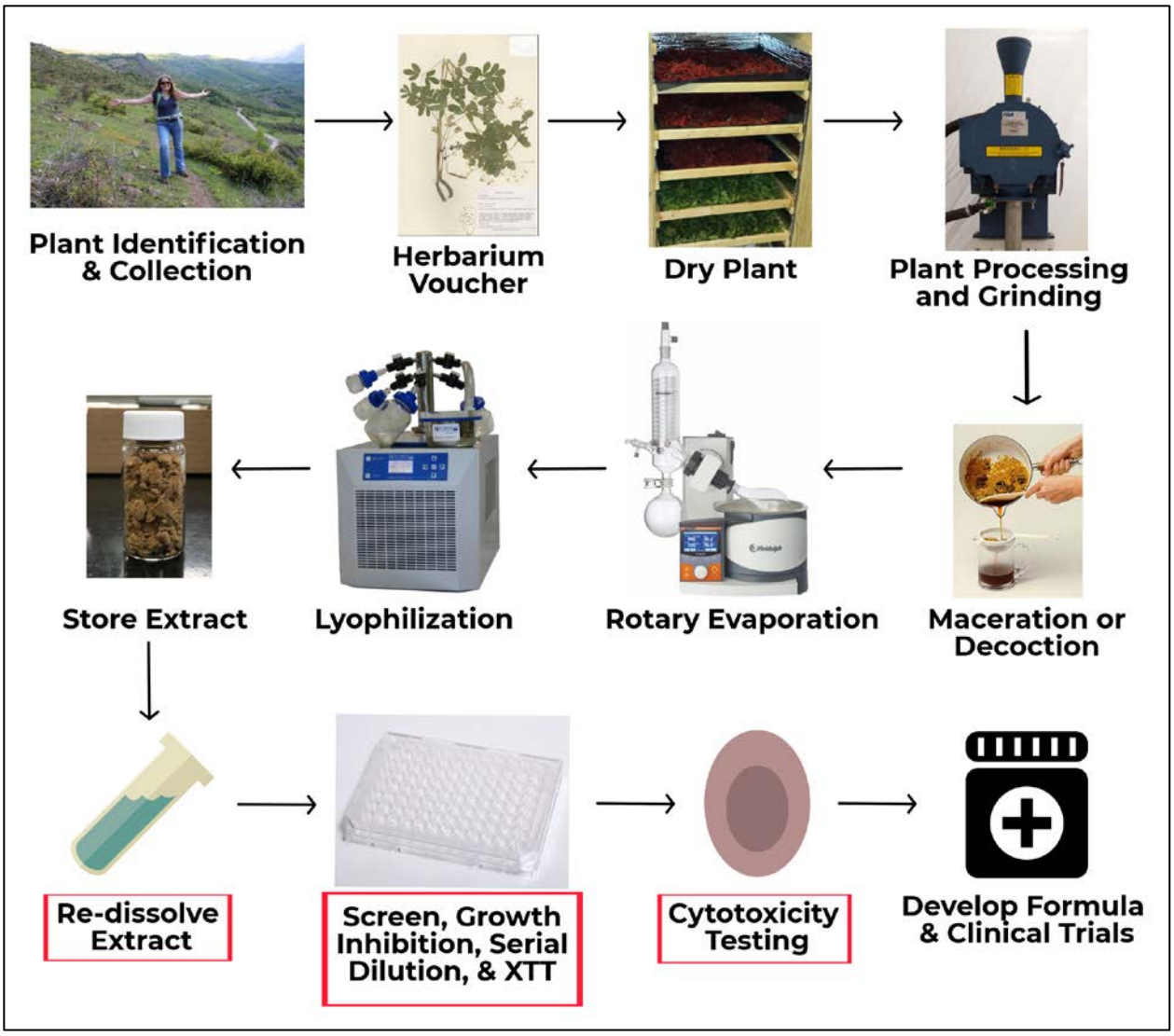


Figure 3. Plant Collection, Chemical Processing, and Microbiological Experimentation. Steps involved in identifying, collecting and preparing plants into botanical extracts for antimicrobial experimentation. Red outline around the step signifies microbiological methodology that took place specifically with *M. furfur* antifungal testing.

3.2 Growing *Malassezia* species: Agar and Broth Microdilution Method

M. furfur is lipophilic and therefore must be grown on lipid-supplemented media. Experiments were conducted using ATCC 12078 strain of *M. furfur* and grown on ATCC Medium 2737: Leeming & Notman agar (bacteriological peptone, glucose, yeast extract, ox bile, glycerol, glycerol monostearate, Tween 40, olive oil, agar, and deionized water). The LNA media calls for Tween 60, but the formula was adjusted and Tween 40 was substituted because it dissolves better in the media as shown from previous experiments (Rincón et al., 2006). LNA media is autoclaved at 110^{o}C for 20 minutes. *M. furfur* requires 72-96 hours at 32$\pm$2^{o}C incubation for proper growth of colonies.

There was much difficulty determining the most effective broth microdilution method. Previous studies attempted to test *M. furfur* antifungal susceptibility by dissolving colonies in phosphate-buffered saline (PBS) and using a supplemented RPMI media for growth during assay inhibition testing (Rincón et al., 2006). However, *M. furfur* colonies do not dissolve well in PBS solution, even after extensive vortex application. Further, adding colonies to PBS results in clumping, with certain wells containing higher fungal density and therefore skewing optical density results.

This study tested two different broth microdilution protocols for more uniform growth and assay testing of *M. furfur* as well as lower variation in results (Rincón et al., 2006; Shibata et al., 2009). Rincon et al. (2006) previously tested antifungal susceptibility of *Malassezia* spp. by growing yeasts in supplemented RPMI. Shibata et al. (2009) tested antifungal susceptibility by using yeast peptone dextrose (YPD) broth supplemented with 1% olive oil. During the initial experiments, *M. furfur* colonies dissolved in PBS and RPMI media adhered to the bottom of wells during assay testing causing incorrect optical density (OD) values. Further, *M. furfur* grew

faster in supplemented YPD versus RPMI (48 vs. 96 hours). Therefore, the protocol outlined in Shibata et al. (2009) was used for both initial screening of master extract plate layouts and serial dilution testing.

3.3 Microbiological Assay: Initial Screen

Plant extracts were obtained from the Quave Natural Products Library. All extracts were dissolved at 10 mg/mL in either DMSO or dH_2O unless otherwise specified. There were eleven master extract layouts that were tested corresponding to extract ID 000011 through 001275. Extracts were tested on *M. furfur* in 96-well microtiter plates in triplicate (Falcon 35-1172). *M. furfur* was grown on LNA for 72 hours. One colony of *M. furfur* was dissolved in 50 mL of autoclaved YPD supplemented with 1% olive oil in an Erlenmeyer flask. The flask was agitated at 320 rpm in an incubator set to 32^{o}C for 48 hours until the OD value was greater than or equal to 0.430 (corresponding to 3.2-5.41 x 10^6 CFU/mL as determined by quantitative plate count). Once the correct CFU/mL was obtained, the inoculum was further diluted in fresh supplemented YPD, standardized to 0.430 at 530 nm, in preparation for assay testing (Rincón et al., 2006).

M. furfur was utilized for the pre-screen testing of plant extracts. Initially, the QNPL was screened at 16 μg/mL. DMSO and dH_2O were tested as negative controls; ketoconazole (KTZ), amphotericin B (AmB) and apple cider vinegar (ACV) were tested as positive controls. A few plant extracts exhibited potential antifungal activity, however further analysis with serial dilution and XTT assay did not produce significant results. Further, the microbiological lab experienced a contamination issue, which may have produced inaccurate antifungal activity for these extracts. Therefore, after thorough decontamination of the lab, the QNPL was re-screened at 128 μg/mL and demonstrated significant antifungal activity for several botanical extracts. These extracts

were further tested with serial dilution to determine MIC_{90} and minimum fungicidal concentration (MFC) via agar plate test.

Ketoconazole was obtained from Sigma-Aldrich and dissolved in DMSO warmed to 37^{o}C to obtain a final concentration of 20 mg/mL, which was further diluted in DMSO to 10 mg/mL and 1 mg/mL for assay testing. Amphotericin B (AmB) was obtained from MP biomedicals and dissolved in DMSO for a final concentration of 20 mg/mL which was further diluted to 10 mg/mL and 1 mg/mL in DMSO for assay testing. Apple cider vinegar was purchased from Bragg Organic Apple Cider Vinegar. It was prepared through sterile filtration. Previous analysis determined the pH to be 3.075 with 5.14% acetic acid content (2017b). Apple cider vinegar was either tested at this concentration or further diluted with DMSO and sterile filtered to achieve 2.5% and 1.25% acetic acid content.

After the preparation outlined above, three replicates were prepared for master extract testing of the QNPL. Working culture was obtained from supplemented YPD grown for 48 hours (Shibata et al., 2009). The working culture was standardized to an OD absorbance value of 0.430 nm (Rincón et al., 2006). 197.44 μL of working culture was added to each well. Next, 2.56 μL of extract or DMSO at a final concentration of 128 μg/mL was added to each well, pipetting up and down twice to ensure proper dissolving of solution for a total well volume of 200 μL. 2.56 μL of AmB was added at a final concentration of 128 μg/mL. 3.2 μL of KTZ (initial concentration 1 mg/mL) was added at final concentration of 16 μg/mL. Plates were incubated at 32^{o}C for 24 and 48 hours. Optical density (OD_{600}) was measured at 0, 24, and 48 hours using a BioTek Cytation3 plate reader. Prior to each read, plates were agitated at 500 rpm for 2-3 minutes.

Extracts that exhibited $\leq$ 0% change from 0 hour OD value at both 24 and 48-hour plate reads were further tested using serial dilution to determine the lowest concentrations that exhibited MIC_{50}, MIC_{90}, and MFC (Table 3 and Table 5).

3.4 Serial Dilution and Fungicidal Plate Test

Extracts 000031, 000055, 000066, 000637, and 001251 and pure compounds 000448, 000449, and 000451 exhibited $\leq$ 0% change at both 24 and 48-hour optical density reads during the initial QNPL screen. These extracts were further tested using 2-fold serial dilution in replicates of four (2 to 256 μg/mL). One colony of *M. furfur* was added to 50 mL of YPD supplemented with 1% olive oil. The inoculum was grown for 48 hours at 32ºC and standardized to an absorbance value of 0.430, as stated previously. 150 uL of inoculum was added to each well. An additional 147 uL of inoculum was added to the first row of the 96-well microtiter plates (Falcon 35-1172). 3 μL of extract (initial concentration 25 mg/mL) was added at a final concentration of 256 μg/mL to the first row of wells as shown in the layout below, for a final volume of 300 μL. 3 μL of DMSO was added to the first row as a negative control. 3.84 μL of KTZ at an initial concentration of 20 mg/mL was added to the first row as a positive control for a starting concentration of 256 μg/mL. 3.84 μL of AmB at an initial concentration of 20 mg/mL was added to the first row in triplicate as a positive control for a final concentration of 256 μg/mL. 150 μL of supplemented YPD was added to the first four rows of column 12 as a negative control. 150 μL of untreated inoculum was added to the last four rows of column 12. Plates were serially diluted, pipetting up and down twice. Plates were incubated at 32ºC and read at 24 and 48 hours. Optical density (OD_{600}) was measured using a BioTek Cytation3 plate reader

at initial and final timepoints, to account for extract color. Growth inhibition was calculated using the formula:

$$\left(1 - \left(\frac{\Delta ODtest}{\Delta ODvehicle}\right)\right) * 100 = \%\text{ growth inhibition.}$$

MIC_{50} was defined as the lowest concentration at which an extract displayed $\geq$ 50% inhibition. MIC_{90} was defined as the lowest concentration at which an extract displayed $\geq$ 90% inhibition. Once MIC_{90} was identified at the lowest concentration for each extract, the fungicidal plate test was conducted in triplicate. LNA plates were divided into zones and labeled according to extract number and concentration. Next, one 15 μL sample was taken from each of the corresponding wells and plated onto an LNA plate. Plates were incubated at 32°C for 96 hours, after which results were documented by visual identification of colonies and photographs of results. Zones with < 3 colonies were determined to be a fungicidal concentration, while zones with $\geq$ 3 colonies were a fungistatic concentration.

	1	2	3	4	5	6	7	8	9	10	11	12
256 µg/mL	DMSO				KTZ				AmB			YPD blank
128												
64												
32												
16												Inoculum (No Tx)
8												
4												
2												

Figure 4. Control Plate Layout for Serial Dilution Testing. DMSO used as negative control. KTZ and AmB used as positive controls, tested at starting concentration of 256 ug/mL. Controls tested in quadruplicate (AmB tested in triplicate) for 24 and 48 hours at 32ºC.

	1	2	3	4	5	6	7	8	9	10	11	12
256 µg/mL	Extract 1				Extract 2				Extract 3			
128												
64												
32												
16												
8												
4												
2												

Figure 5. Treatment Plate Layout for Serial Dilution Testing. All extracts tested at an initial concentration of 256 µg/mL in quadruplicate for 24 and 48 hours at 32ºC.

3.5 XTT Assay

The initial screen of the QNPL at 16 μg/mL was utilized to discover potential botanical extracts with antifungal activity. However, it was challenging to plot accurate dose response graphs because of high levels of deviation caused by filament formation in the bottom of the wells. I tried various methods to prevent filament formation, including shaking the plates prior to each read, shaking the plates overnight during incubation, and using a breathable seal instead of a hard, plastic cover over the 96-well microtiter plates. While these methods helped, white clumps and filament still formed at the bottom of the wells. A spectrophotometer determines optical density (OD) by sending light through each well—a cloudier well will have a higher OD value, signifying bacterial or fungal growth. Since there were clumps at the bottom of the wells, however, this disturbed OD reads and contributed to large standard deviation values. Further, because of these variations it was hard to calculate growth inhibition based on DMSO control values. Therefore, after the initial QNPL screen at 16 μg/mL, we utilized XTT colorimetric assay in concurrence with serial dilution testing. XTT colorimetric assay is more expensive and therefore was not amenable for the initial screen, however there is more precision with this assay method (Figueredo et al., 2013). Since a contamination issue was discovered in the lab, it was determined that initial promising XTT results were not accurate. The new QNPL screen at a higher concentration of 128 μg/mL contained lower standard deviation values, signifying more precision. Therefore, because of greater precision and time constraints, the XTT assay was not tested on extracts 000031, 000055, 000066, 000448, 000449, 000451, 000637, and 001251. However, the XTT assay will be an important experiment to conduct in follow-up studies with these extracts and therefore an explanation of its methodology is warranted.

The XTT assay is used to detect cell metabolism and growth (Kuhn et al., 2003). XTT is a colorless tetrazolium salt that is reduced to an orange derivative in the presence of metabolic activity (2011; Kuhn et al., 2003). Therefore, the XTT assay provides a more accurate visual method for determining fungicidal activity: a darker orange-red color signals increased frequency of cells in the well and mitochondrial activity, while a clear-yellow well demonstrates botanical extract effectiveness at the specified concentration.

The XTT assay is conducted after the 48-hour read for the serial dilution experiments. The assay is light-sensitive so must be completed with the hood light off; low lights in the background will not affect the assay. One vial containing 50 μl of PMS activation reagent is added to 10 mL of XTT reagent and vortexed. Then 25 μl is added to each well and plates are placed in complete darkness in the incubator at 32^{o}C for three hours. After 3 hours, 80 μl is transferred to another plate and absorbance is measured at 490 nm using the microtiter plate reader (Figueredo et al., 2013). OD values of the extracts are compared to DMSO control. Results of a control plate example are shown below in Figure 8.

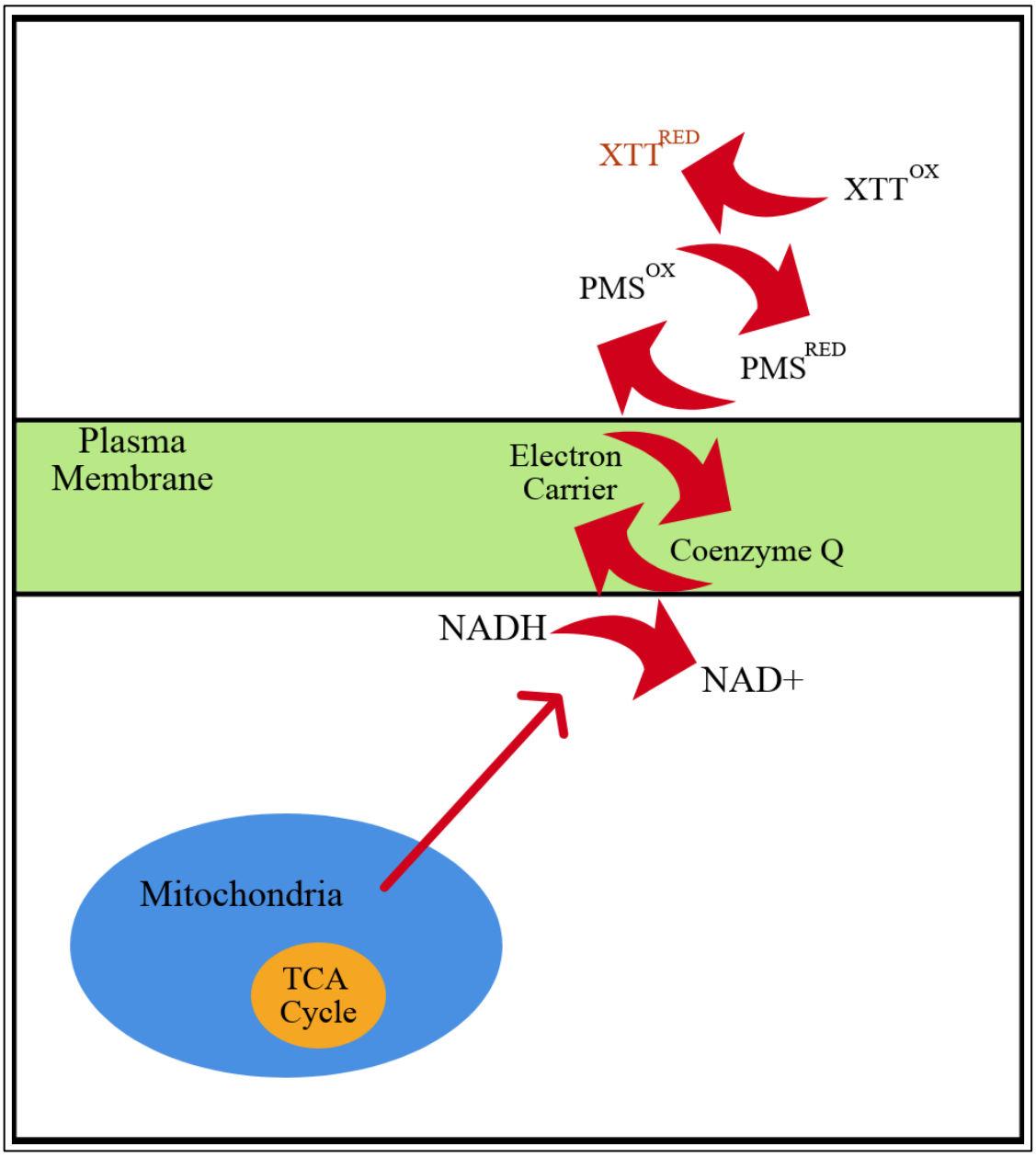


Figure 6. Colorimetric Reduction of XTT by Cellular Enzymes. The color change signifies metabolic activity of cells and occurs when the positively-charged tetrazole ring in XTT is broken apart using energy from previous reactions. Image adapted from ATCC XTT Cell Proliferation Assay Kit Instruction Manual (2011).

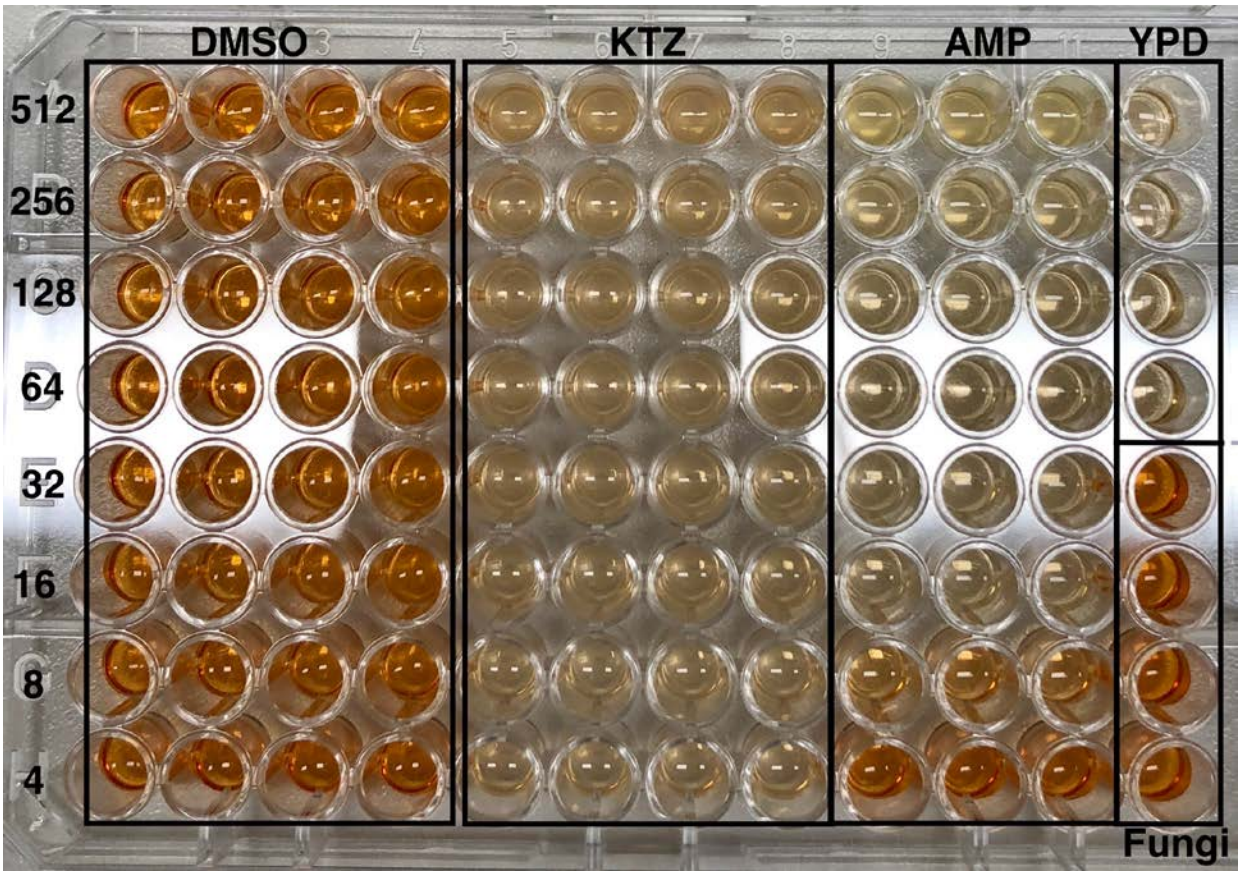


Figure 7. XTT Results of Control Plate Tested against *M. furfur*. In this XTT assay example, serial dilution was conducted with a starting concentration 512 μg/mL. KTZ was effective against *M. furfur* at the lowest concentration of 4 μg/mL as can be observed by the light-yellow wells. AmB exhibited growth inhibition at concentrations as low as 16 μg/mL. DMSO and untreated inoculum dyed orange as expected, revealing metabolic activity of *M. furfur* in the wells. Supplemented YPD blank remained clear, since no cells were ever present in these control wells.

3.6 Cytotoxicity Assay

Human immortalized keratinocytes (HaCaT cell line) were maintained in Dulbecco's modified Eagle's medium with L-glutamine and 4.5 g/L^{-1} glucose (Corning, Corning, NY) supplemented with 10% heat-inactivated fetal bovine serum (Seradigm, Randor, PA) and 1X solution of 100 IU Penicillin and 100 µg/mL Streptomycin (Corning, Corning, NY) at 37°C, 5% CO_2 in 75 cm^2 flasks (Greiner Bio-One) (Quave et al., 2015). Upon reaching suitable confluency (90–95%), cells were detached from the flask bottom for cell splitting and plating using 0.25% trypsin, 0.1% ethylenediaminetetraacetic acid (EDTA) in Hanks' balanced salt solution (HBSS) without Ca^{2+}, Mg^{2+}, or $NaHCO_3$ (Corning, Corning, NY) (Quave et al., 2015).

Toxicity of extracts was evaluated with the LDH cytotoxicity assay (G-Biosciences, St. Louis, MO) (Quave et al., 2015). Briefly, the cell culture was standardized to 4 x 10^4 cells/mL using a hemocytometer and 200 µL added per well in a 96 well tissue culture treated microtiter plate (Falcon 35–3075) (Quave et al., 2015). Plates were incubated for 48 hours to allow for seeding, prior to media aspiration. Either media containing extracts or vehicle were serially diluted 2-fold (4-512 µg/mL) and were processed 24 hours later following manufacturer's protocol for chemical induced cytotoxicity (Quave et al., 2015). Cytotoxicity was calculated using the growth inhibition formula.

Chapter 4 Results

4.1 Screening Outcomes

Crude extracts 000031, 000055, 000066, 000637, 001251 and pure compounds 000448, 000449, and 000451 showed decrease in optical density value at both 24 and 48 hours as compared to 0 hour OD. These extracts were further tested via serial dilution to determine minimum inhibitory concentration. Results of initial QNPL screen at 128 μg/mL are displayed below in Table 3.

Table 3. Summary of Growth Inhibition Results for Quave Natural Products Library Screen

Extract	0 hour average OD	SD	24 hour average OD	SD	48 hour average OD	SD	Difference in 0 and 24 hr OD	Difference in 0 and 48 hr OD
11	0.577	0.003	0.871	0.352	1.009	0.348	50.87	74.71
14	0.573	0.009	0.502	0.033	0.592	0.155	-12.34	3.32
26	0.585	0.020	1.089	0.394	1.267	0.256	86.15	116.58
27	0.586	0.007	0.837	0.204	0.999	0.398	42.78	70.53
31	**0.557**	0.017	**0.422**	0.041	**0.249**	0.056	-24.19	-55.33
33	0.551	0.007	0.559	0.048	0.640	0.057	1.45	16.21
35	0.583	0.022	1.068	0.155	1.229	0.061	83.35	110.98
36	0.575	0.014	0.558	0.025	0.578	0.090	-2.96	0.52
38	0.567	0.012	0.719	0.146	0.687	0.386	26.88	21.18
39	0.629	0.046	0.891	0.145	0.940	0.326	41.65	49.50
40	0.607	0.037	0.693	0.135	0.786	0.357	14.11	29.43
41	0.551	0.007	0.771	0.174	0.685	0.416	39.90	24.30
42	0.581	0.009	0.952	0.173	1.268	0.106	64.01	118.37
43	0.571	0.026	0.483	0.095	0.658	0.185	-15.41	15.29
44	0.656	0.065	1.064	0.374	1.094	0.363	62.16	66.63
45	0.593	0.029	0.799	0.452	0.596	0.325	34.79	0.51
48	0.635	0.043	0.677	0.056	0.983	0.425	6.61	54.80
50	0.579	0.016	0.601	0.039	0.608	0.068	3.74	4.89
51	0.601	0.062	0.961	0.379	0.907	0.484	59.76	50.78
52	0.565	0.001	0.653	0.031	0.670	0.074	15.45	18.46
53	0.599	0.093	0.734	0.010	0.930	0.223	22.59	55.20
54	0.577	0.015	0.581	0.048	1.092	0.654	0.81	89.31
55	**0.610**	0.059	**0.523**	0.023	**0.543**	0.116	-14.16	-10.93
56	0.565	0.018	0.529	0.063	0.756	0.168	-6.43	33.75
57	0.586	0.011	0.570	0.056	0.586	0.073	-2.73	0.06
58	0.569	0.016	0.555	0.020	0.756	0.056	-2.46	33.00
59	0.617	0.043	0.581	0.050	0.713	0.061	-5.78	15.61
60	0.588	0.015	0.720	0.085	0.916	0.199	22.58	55.93
61	0.629	0.027	0.590	0.083	0.699	0.088	-6.20	11.24
62	0.590	0.007	0.600	0.067	1.070	0.184	1.64	81.25
63	0.297	0.010	0.488	0.086	0.756	0.067	64.38	154.94
64	0.569	0.023	0.943	0.442	0.788	0.293	65.57	38.35
65	0.316	0.008	0.635	0.107	0.867	0.110	100.84	173.97
66	**0.582**	0.011	**0.568**	0.066	**0.505**	0.125	-2.41	-13.17
67	0.631	0.066	0.526	0.103	0.687	0.097	-16.74	8.82
68	0.623	0.073	0.644	0.178	1.265	0.253	3.48	103.10
69	0.302	0.008	0.382	0.036	1.212	0.151	26.63	301.66
70	0.605	0.036	0.678	0.169	0.789	0.092	12.07	30.47
71	0.629	0.084	0.614	0.087	0.670	0.142	-2.33	6.52

74	0.588	0.002	0.755	0.446	0.867	0.454	28.46	47.39
76	0.610	0.018	0.644	0.053	0.733	0.095	5.63	20.16
77	0.583	0.024	0.570	0.075	0.754	0.202	-2.23	29.26
78	0.567	0.019	0.625	0.060	0.578	0.050	10.24	2.00
79	0.634	0.097	0.822	0.323	0.909	0.335	29.60	43.38
80	0.303	0.008	0.318	0.035	0.763	0.138	4.95	151.71
81	0.297	0.007	0.366	0.039	0.517	0.221	23.34	74.19
82	0.603	0.054	0.579	0.015	0.756	0.311	-4.09	25.36
83	0.651	0.039	0.594	0.043	0.864	0.259	-8.80	32.60
84	0.674	0.091	0.768	0.214	0.540	0.034	13.84	-19.87
85	0.589	0.015	0.779	0.398	1.054	0.411	32.31	78.89
86	0.611	0.015	0.788	0.126	0.755	0.176	28.90	23.56
87	0.610	0.033	0.709	0.140	1.057	0.227	16.17	73.13
88	0.580	0.014	0.742	0.251	0.918	0.414	27.86	58.18
89	0.564	0.031	0.700	0.234	1.070	0.269	24.10	89.55
90	0.618	0.053	0.976	0.312	1.147	0.366	57.84	85.44
91	0.575	0.015	0.896	0.094	0.967	0.397	55.88	68.23
92	0.588	0.011	0.672	0.029	0.689	0.402	14.34	17.23
93	0.321	0.022	0.718	0.280	0.925	0.078	124.01	188.36
94	0.590	0.021	0.784	0.250	1.197	0.207	33.01	103.05
95	0.588	0.020	0.771	0.097	1.115	0.140	31.14	89.73
96	0.671	0.027	0.885	0.147	0.967	0.508	31.83	44.04
97	0.600	0.032	0.612	0.059	0.900	0.203	1.94	49.86
98	0.632	0.028	0.570	0.138	0.782	0.240	-9.86	23.72
100	0.596	0.003	0.762	0.309	0.853	0.080	27.87	43.26
101	0.622	0.061	0.821	0.449	0.904	0.480	32.06	45.36
102	0.577	0.036	0.848	0.196	0.709	0.198	46.97	22.88
103	0.579	0.021	0.717	0.034	0.798	0.046	23.85	37.90
104	0.561	0.020	0.646	0.044	0.512	0.157	15.16	-8.62
105	0.609	0.033	0.669	0.237	0.908	0.341	9.80	49.04
106	0.613	0.011	0.745	0.106	0.851	0.277	21.47	38.75
107	0.592	0.021	0.613	0.060	0.956	0.145	3.66	61.63
108	0.627	0.016	0.936	0.303	1.096	0.174	49.41	74.89
109	0.603	0.025	0.726	0.029	0.871	0.022	20.40	44.50
110	0.604	0.015	0.589	0.072	0.607	0.077	-2.48	0.44
112	0.614	0.009	0.622	0.097	0.682	0.115	1.30	11.01
113	0.654	0.025	0.589	0.046	0.721	0.206	-9.93	10.24
114	0.698	0.038	0.957	0.169	1.267	0.086	36.99	81.38
115	0.584	0.006	0.653	0.186	0.729	0.118	11.82	24.84
116	0.581	0.021	0.691	0.147	0.906	0.317	18.88	55.94
117	0.578	0.019	0.642	0.144	0.908	0.126	11.19	57.18
118	0.661	0.077	0.600	0.055	0.698	0.125	-9.23	5.55
119	0.610	0.024	0.700	0.144	0.661	0.083	14.81	8.42
120	0.615	0.017	0.810	0.119	1.058	0.135	31.64	71.89

121	0.627	0.033	0.752	0.052	0.814	0.060	20.05	29.95
122	0.656	0.016	0.774	0.118	0.887	0.281	18.05	35.28
123	0.606	0.012	0.758	0.129	1.122	0.206	25.03	85.20
124	0.587	0.034	0.571	0.038	0.653	0.091	-2.84	11.12
125	0.578	0.009	0.680	0.027	0.979	0.179	17.65	69.32
126	0.586	0.017	0.606	0.058	0.634	0.090	3.30	8.19
128	0.573	0.014	0.637	0.054	0.821	0.229	11.11	43.34
129	0.573	0.010	0.717	0.084	0.534	0.078	25.13	-6.81
131	0.350	0.030	0.607	0.058	0.793	0.049	73.50	126.79
132	0.334	0.048	0.553	0.083	0.808	0.090	65.73	142.16
133	0.450	0.019	0.833	0.098	0.859	0.121	85.11	90.96
134	0.367	0.031	0.615	0.079	0.512	0.091	67.33	39.38
135	0.368	0.032	0.645	0.101	0.740	0.219	75.52	101.36
136	0.370	0.018	0.561	0.108	0.485	0.070	51.40	30.96
137	0.362	0.027	0.801	0.027	0.896	0.135	121.18	147.51
138	0.354	0.021	0.488	0.055	0.519	0.188	38.08	46.75
139	0.337	0.007	0.524	0.125	0.675	0.236	55.59	100.20
140	0.330	0.010	0.679	0.063	0.811	0.038	105.55	145.51
141	0.334	0.017	0.657	0.043	0.786	0.096	96.61	135.23
142	0.338	0.013	0.654	0.256	0.719	0.209	93.68	112.83
143	0.377	0.034	0.549	0.059	0.553	0.124	45.66	46.81
144	0.347	0.037	0.604	0.085	0.755	0.035	74.23	117.79
145	0.364	0.009	0.627	0.084	0.762	0.079	72.32	109.53
146	0.347	0.017	0.592	0.095	0.677	0.088	70.70	95.20
147	0.337	0.019	0.563	0.045	0.746	0.054	67.23	121.58
148	0.329	0.024	0.577	0.108	0.657	0.024	75.30	99.60
149	0.336	0.017	0.604	0.054	0.641	0.083	79.76	90.77
150	0.331	0.014	0.666	0.081	0.741	0.110	101.11	123.97
151	0.331	0.017	0.621	0.036	0.731	0.022	87.70	120.97
152	0.348	0.003	0.670	0.056	0.751	0.035	92.25	115.60
153	0.393	0.066	0.632	0.067	0.782	0.055	61.04	99.15
154	0.337	0.027	0.638	0.151	0.766	0.143	89.22	127.30
155	0.356	0.014	0.743	0.080	0.959	0.076	108.61	169.48
157	0.346	0.012	0.728	0.143	0.823	0.290	110.20	137.73
158	0.309	0.043	0.590	0.197	0.852	0.198	90.63	175.54
159	0.333	0.026	0.645	0.236	0.850	0.088	93.79	155.26
160	0.302	0.024	0.599	0.023	0.679	0.077	98.24	124.70
161	0.297	0.012	0.535	0.155	0.712	0.152	80.45	140.11
162	0.312	0.037	0.603	0.252	0.691	0.353	93.48	121.60
163	0.304	0.012	0.593	0.048	0.723	0.053	95.18	137.72
164	0.362	0.033	0.593	0.063	0.696	0.138	63.57	92.00
165	0.356	0.005	0.672	0.120	0.812	0.139	88.86	128.00
166	0.360	0.025	0.569	0.105	0.768	0.049	58.15	113.33
167	0.351	0.029	0.602	0.068	0.612	0.113	71.67	74.52

168	0.335	0.020	0.600	0.029	0.674	0.090	78.93	100.89
169	0.311	0.005	0.603	0.041	0.796	0.107	93.99	156.33
170	0.318	0.014	0.539	0.056	0.707	0.262	69.21	122.09
171	0.317	0.019	0.526	0.049	0.710	0.011	65.76	123.84
172	0.304	0.011	0.576	0.055	0.736	0.023	89.79	142.26
173	0.304	0.035	0.771	0.168	0.895	0.162	154.01	194.84
174	0.312	0.009	0.641	0.043	0.770	0.030	105.56	146.90
175	0.323	0.020	0.746	0.187	0.901	0.235	131.20	179.34
176	0.378	0.038	0.682	0.058	0.716	0.280	80.49	89.59
177	0.458	0.026	0.697	0.092	0.779	0.162	52.18	70.01
178	0.370	0.004	0.518	0.051	0.526	0.180	40.00	42.07
179	0.333	0.013	0.581	0.021	0.781	0.032	74.20	134.40
180	0.333	0.024	0.656	0.047	0.802	0.072	97.09	140.98
181	0.339	0.031	0.710	0.126	0.973	0.076	109.74	187.20
182	0.325	0.017	0.581	0.041	0.974	0.058	78.87	199.69
183	0.307	0.040	0.681	0.193	0.840	0.137	121.96	173.91
184	0.301	0.015	0.636	0.030	0.774	0.034	110.95	156.86
185	0.349	0.053	0.637	0.109	0.798	0.054	82.79	128.97
186	0.357	0.008	0.700	0.035	1.117	0.115	95.99	212.59
187	0.333	0.024	0.709	0.035	1.208	0.053	112.60	262.50
188	0.352	0.012	0.673	0.033	1.050	0.077	91.47	198.48
189	0.330	0.008	0.559	0.138	0.667	0.258	69.12	102.02
190	0.327	0.022	0.573	0.032	0.799	0.082	75.41	144.69
191	0.330	0.026	0.618	0.044	1.127	0.055	87.36	241.86
193	0.313	0.017	0.471	0.042	0.643	0.053	50.64	105.65
194	0.318	0.012	0.650	0.084	0.778	0.110	104.30	144.65
195	0.314	0.017	0.637	0.046	0.781	0.008	102.76	148.62
197	0.306	0.025	0.708	0.137	1.071	0.114	131.01	249.51
198	0.317	0.045	0.582	0.105	0.760	0.102	83.68	140.11
199	0.375	0.009	0.614	0.056	0.776	0.096	63.68	106.66
200	0.348	0.002	0.626	0.012	0.733	0.023	80.15	110.93
201	0.343	0.003	0.512	0.087	0.661	0.098	49.37	92.71
202	0.356	0.017	0.624	0.027	1.075	0.107	75.54	202.16
203	0.337	0.022	0.590	0.015	0.740	0.056	75.00	119.37
204	0.330	0.012	0.856	0.173	1.119	0.113	159.49	238.99
205	0.321	0.032	0.736	0.098	0.974	0.102	129.52	203.64
207	0.352	0.041	0.621	0.051	0.781	0.103	76.35	121.76
215	0.356	0.024	0.676	0.021	0.764	0.124	89.80	114.31
216	0.355	0.029	0.588	0.079	0.775	0.064	65.70	118.52
217	0.318	0.017	0.764	0.206	0.938	0.202	140.61	195.38
220	0.385	0.031	0.722	0.112	1.001	0.198	87.61	160.31
221	0.333	0.019	0.752	0.099	0.791	0.133	126.05	137.88
222	0.335	0.013	0.671	0.053	0.800	0.114	100.60	139.14
223	0.373	0.037	0.525	0.089	0.475	0.066	40.97	27.55

224	0.361	0.028	0.627	0.089	0.953	0.126	73.78	163.99
225	0.329	0.018	0.603	0.124	0.768	0.115	83.18	133.54
226	0.354	0.038	0.633	0.163	0.812	0.019	78.91	129.28
227	0.398	0.038	0.780	0.033	0.864	0.065	95.98	117.09
228	0.328	0.006	0.578	0.134	0.757	0.126	75.94	130.66
229	0.364	0.038	0.699	0.116	0.759	0.214	92.12	108.71
230	0.354	0.036	0.484	0.040	0.451	0.070	36.76	27.43
231	0.337	0.020	0.619	0.043	0.890	0.080	83.58	164.00
232	0.323	0.022	0.500	0.047	0.729	0.022	54.74	125.36
233	0.340	0.011	0.678	0.031	1.147	0.020	99.22	237.12
234	0.314	0.010	0.733	0.026	0.951	0.062	133.58	203.08
235	0.327	0.013	0.612	0.268	0.906	0.281	87.26	176.91
237	0.315	0.014	0.722	0.105	0.825	0.090	129.31	161.80
239	0.318	0.003	0.757	0.039	0.926	0.220	138.05	191.30
242	0.305	0.021	0.608	0.071	0.623	0.137	99.45	104.60
243	0.307	0.019	0.761	0.070	0.782	0.205	147.77	154.72
244	0.325	0.021	0.610	0.118	0.748	0.054	87.79	130.15
245	0.311	0.024	0.576	0.126	0.842	0.117	85.21	170.85
246	0.305	0.019	0.583	0.025	0.737	0.042	90.94	141.38
247	0.320	0.023	0.574	0.028	0.775	0.016	79.48	142.29
249	0.298	0.032	0.621	0.074	0.790	0.057	108.62	165.29
250	0.302	0.015	0.576	0.048	0.728	0.044	90.63	140.79
251	0.285	0.014	0.585	0.101	0.907	0.160	105.15	218.13
252	0.306	0.027	0.617	0.063	1.074	0.236	101.96	251.47
256	0.309	0.055	0.597	0.065	0.472	0.167	93.41	52.92
257	0.297	0.011	0.670	0.048	0.799	0.055	125.96	169.33
259	0.333	0.030	0.600	0.103	0.799	0.067	80.18	139.84
261	0.312	0.008	0.549	0.091	0.724	0.055	75.96	132.05
263	0.334	0.021	0.596	0.069	0.742	0.036	78.17	122.03
264	0.320	0.018	0.654	0.116	0.836	0.107	104.69	161.42
265	0.312	0.029	0.519	0.134	0.693	0.055	66.17	121.77
267	0.328	0.022	0.574	0.076	0.785	0.184	75.28	139.47
269	0.276	0.021	0.607	0.068	0.707	0.092	119.66	155.97
270	0.299	0.022	0.743	0.045	0.997	0.094	148.38	233.44
272	0.301	0.026	0.578	0.044	0.685	0.017	92.14	127.69
273	0.307	0.012	0.656	0.025	0.745	0.068	113.45	142.41
274	0.337	0.019	0.658	0.057	1.058	0.107	95.35	213.95
281	0.336	0.034	0.651	0.012	0.900	0.143	93.85	167.76
282	0.339	0.018	0.580	0.064	0.783	0.052	71.02	130.75
285	0.331	0.020	0.667	0.099	1.048	0.058	101.71	217.04
286	0.323	0.030	0.570	0.162	0.983	0.085	76.39	204.02
287	0.311	0.015	0.673	0.068	0.827	0.073	116.51	166.02
287	0.323	0.006	0.679	0.149	0.941	0.253	110.11	191.23
288	0.298	0.020	0.598	0.087	0.734	0.031	100.45	146.15

288	0.315	0.020	0.606	0.032	0.747	0.061	92.28	136.79
289	0.308	0.017	0.581	0.059	0.984	0.034	88.95	219.93
290	0.323	0.013	0.595	0.114	0.970	0.103	84.31	200.31
290	0.307	0.009	0.669	0.036	1.118	0.055	118.26	264.57
292	0.301	0.033	0.542	0.077	0.514	0.048	80.18	70.65
292	0.323	0.011	0.667	0.028	0.873	0.130	106.19	170.10
293	0.300	0.012	0.691	0.065	0.786	0.103	130.44	162.11
294	0.305	0.018	0.668	0.064	0.968	0.102	118.78	217.03
296	0.321	0.010	0.643	0.012	0.737	0.046	100.52	129.83
297	0.298	0.029	0.641	0.033	1.049	0.087	115.34	252.41
299	0.282	0.024	0.592	0.021	0.745	0.069	110.06	164.50
310	0.347	0.019	0.684	0.025	0.968	0.117	97.21	178.87
311	0.350	0.022	0.718	0.081	0.971	0.177	105.05	177.43
313	0.327	0.019	0.616	0.042	1.130	0.021	88.47	245.82
317	0.305	0.010	0.696	0.110	0.977	0.014	128.09	220.22
318	0.304	0.029	0.633	0.055	0.988	0.110	108.11	225.00
319	0.318	0.016	0.619	0.096	0.929	0.188	94.86	192.44
320	0.290	0.015	0.585	0.055	0.738	0.043	101.84	154.60
321	0.315	0.016	0.709	0.076	0.917	0.188	124.95	190.91
323	0.306	0.024	0.597	0.034	0.746	0.018	95.42	144.06
324	0.326	0.034	0.680	0.013	0.781	0.023	108.38	139.22
325	0.326	0.026	0.653	0.169	0.871	0.217	100.20	167.18
326	0.334	0.015	0.458	0.150	0.373	0.134	36.89	11.47
327	0.322	0.021	0.593	0.060	0.743	0.061	84.16	130.64
328	0.326	0.010	0.690	0.083	0.890	0.109	111.66	172.90
329	0.330	0.017	0.728	0.103	0.958	0.011	120.93	190.60
332	0.294	0.011	0.658	0.075	0.881	0.024	123.56	199.21
334	0.338	0.017	0.744	0.153	0.917	0.068	119.90	171.03
336	0.320	0.033	0.793	0.291	0.830	0.079	147.97	159.54
353	0.323	0.019	0.559	0.069	0.538	0.259	73.14	66.84
357	0.302	0.021	0.780	0.139	0.791	0.366	158.28	161.92
367	0.320	0.040	0.630	0.026	0.490	0.110	96.57	53.07
418	0.302	0.012	0.301	0.034	0.335	0.076	-0.22	10.94
419	0.262	0.001	0.376	0.056	0.608	0.129	43.46	131.77
420	0.265	0.007	0.320	0.030	0.546	0.188	20.91	106.42
421	0.257	0.003	0.299	0.040	0.470	0.071	16.21	82.88
423	0.324	0.017	0.390	0.068	0.825	0.347	20.60	154.79
424	0.267	0.005	0.448	0.070	0.749	0.215	67.67	180.65
425	0.283	0.009	0.373	0.098	0.679	0.196	32.08	140.33
429	0.322	0.024	0.462	0.151	0.590	0.166	43.48	83.13
430	0.291	0.004	0.345	0.054	0.801	0.102	18.81	175.46
432	0.274	0.012	0.346	0.050	0.734	0.134	26.12	167.68
433	0.314	0.002	0.556	0.111	0.854	0.337	76.96	171.87
434	0.268	0.003	0.344	0.075	0.814	0.336	28.20	203.35

435	0.269	0.003	0.452	0.134	0.601	0.070	67.70	123.02
436	0.280	0.008	0.277	0.016	0.287	0.080	-1.07	2.50
437	0.267	0.003	0.276	0.008	0.375	0.129	3.12	40.15
438	0.271	0.003	0.349	0.021	0.973	0.058	28.82	259.61
439	0.268	0.001	0.340	0.059	0.806	0.099	26.99	200.62
440	0.288	0.005	0.312	0.029	0.532	0.273	8.32	84.39
441	0.304	0.001	0.360	0.064	0.624	0.028	18.29	105.15
442	0.272	0.002	0.321	0.027	0.882	0.224	18.01	224.14
443	0.281	0.019	0.415	0.141	1.011	0.357	47.69	259.79
444	0.282	0.001	0.363	0.044	0.683	0.160	28.69	141.91
445	0.289	0.009	0.372	0.084	0.820	0.144	28.60	183.62
446	0.289	0.012	0.370	0.072	0.947	0.136	28.18	227.94
447	0.335	0.017	0.359	0.067	0.652	0.150	7.17	94.72
448	**0.490**	0.134	**0.342**	0.016	**0.430**	0.081	-30.18	-12.37
449	**0.292**	0.006	**0.290**	0.006	**0.249**	0.025	-0.57	-14.51
450	0.324	0.017	0.371	0.055	0.782	0.112	14.61	141.46
451	**0.273**	0.002	**0.263**	0.014	**0.251**	0.025	-3.66	-8.05
452	0.310	0.007	0.312	0.069	0.676	0.098	0.65	118.19
453	0.345	0.013	0.499	0.054	0.982	0.253	44.87	184.82
454	0.273	0.005	0.251	0.010	0.299	0.073	-7.94	9.65
455	0.482	0.067	0.411	0.055	0.851	0.097	-14.72	76.43
456	0.298	0.006	0.393	0.063	0.957	0.139	31.88	221.25
457	0.306	0.006	0.393	0.045	0.823	0.125	28.40	168.77
458	0.303	0.016	0.489	0.130	1.071	0.397	61.67	253.74
459	0.278	0.010	0.410	0.097	0.888	0.138	47.19	218.92
460	0.288	0.005	0.477	0.058	1.051	0.305	65.32	264.62
461	0.329	0.010	0.339	0.082	0.454	0.339	3.25	38.13
470	0.291	0.012	0.413	0.078	0.977	0.327	41.76	235.47
471	0.293	0.010	0.424	0.018	0.992	0.076	44.55	238.07
472	0.281	0.001	0.341	0.053	0.579	0.269	21.23	105.93
481	0.298	0.005	0.364	0.045	0.749	0.181	22.40	151.62
482	0.273	0.004	0.311	0.044	0.664	0.157	14.04	143.10
483	0.267	0.002	0.308	0.061	0.646	0.257	15.50	142.13
484	0.283	0.010	0.282	0.032	0.436	0.052	-0.35	54.36
485	0.269	0.002	0.280	0.028	0.647	0.052	4.09	140.52
486	0.285	0.007	0.403	0.117	0.732	0.284	41.45	157.26
487	0.318	0.016	0.500	0.026	1.148	0.069	57.13	261.01
488	0.293	0.009	0.438	0.087	0.973	0.065	49.54	232.46
489	0.306	0.012	0.373	0.070	0.905	0.037	21.92	196.07
490	0.272	0.004	0.281	0.011	0.323	0.060	3.56	18.77
491	0.317	0.002	0.361	0.029	0.643	0.113	14.11	102.95
492	0.293	0.020	0.345	0.059	0.662	0.212	17.77	126.31
493	0.287	0.004	0.361	0.048	0.893	0.224	25.52	210.67
494	0.283	0.002	0.391	0.123	0.708	0.358	38.12	149.88

495	0.284	0.002	0.340	0.023	0.646	0.070	19.60	127.46
496	0.292	0.002	0.445	0.075	0.751	0.128	52.22	156.90
497	0.287	0.008	0.386	0.038	0.806	0.167	34.65	181.16
499	0.285	0.007	0.343	0.042	0.800	0.086	20.49	181.15
508	0.287	0.027	0.424	0.116	0.923	0.483	47.62	221.60
512	0.315	0.013	0.350	0.049	0.695	0.124	11.10	120.40
514	0.293	0.012	0.477	0.162	1.020	0.273	62.61	247.61
515	0.327	0.015	0.397	0.068	0.803	0.118	21.53	145.82
516	0.312	0.028	0.355	0.050	0.601	0.024	13.90	92.73
517	0.287	0.003	0.345	0.058	0.726	0.148	20.07	152.55
518	0.280	0.006	0.380	0.077	0.553	0.097	35.88	97.62
519	0.290	0.008	0.307	0.030	0.555	0.115	5.98	91.71
520	0.298	0.001	0.331	0.069	0.536	0.129	10.84	79.55
521	0.306	0.009	0.355	0.039	0.630	0.023	16.03	106.11
522	0.288	0.004	0.324	0.068	0.546	0.048	12.62	89.58
523	0.309	0.010	0.405	0.075	0.697	0.089	31.21	125.81
524	0.296	0.019	0.305	0.025	0.471	0.235	2.93	59.23
525	0.278	0.010	0.386	0.043	0.807	0.321	39.02	190.76
526	0.287	0.009	0.380	0.049	0.888	0.189	32.52	209.52
527	0.282	0.005	0.348	0.049	0.586	0.064	23.26	107.56
528	0.277	0.003	0.371	0.121	0.710	0.149	34.10	156.63
529	0.314	0.007	0.518	0.103	0.818	0.164	65.14	160.68
530	0.337	0.020	0.482	0.024	0.810	0.149	43.13	140.26
531	0.328	0.016	0.518	0.027	0.943	0.034	57.77	187.21
532	0.326	0.011	0.504	0.119	0.958	0.115	54.76	194.27
533	0.316	0.003	0.528	0.033	0.934	0.140	67.19	195.68
534	0.326	0.009	0.610	0.150	0.932	0.272	86.82	185.70
538	0.314	0.006	0.515	0.089	0.938	0.029	63.73	198.52
539	0.339	0.014	0.478	0.067	0.888	0.169	40.77	161.69
541	0.376	0.013	0.534	0.053	0.745	0.351	41.90	97.96
542	0.393	0.009	0.511	0.040	0.949	0.085	30.03	141.39
543	0.375	0.013	0.538	0.088	0.901	0.280	43.25	140.14
544	0.386	0.007	0.479	0.028	0.797	0.215	24.01	106.56
545	0.399	0.007	0.506	0.031	0.922	0.083	26.90	130.99
546	0.357	0.007	0.504	0.048	0.911	0.129	41.14	155.04
547	0.320	0.004	0.591	0.121	0.820	0.083	84.78	156.62
548	0.311	0.011	0.542	0.022	0.847	0.212	73.98	172.06
549	0.319	0.001	0.528	0.101	0.468	0.011	65.45	46.56
550	0.308	0.010	0.318	0.054	0.534	0.162	3.36	73.46
551	0.340	0.018	0.582	0.093	0.845	0.097	71.44	148.87
552	0.310	0.010	0.277	0.005	0.465	0.027	-10.54	50.00
553	0.325	0.009	0.540	0.047	0.943	0.084	66.05	190.05
554	0.307	0.002	0.520	0.072	0.626	0.061	69.57	104.13
555	0.321	0.001	0.514	0.174	0.727	0.134	60.29	126.61

556	0.366	0.014	0.458	0.048	1.034	0.201	25.02	182.35
557	0.331	0.012	0.587	0.127	0.956	0.122	77.42	189.11
558	0.311	0.013	0.337	0.011	0.767	0.194	8.47	146.62
559	0.329	0.003	0.602	0.112	1.006	0.077	82.69	205.57
560	0.324	0.012	0.516	0.058	0.840	0.089	59.16	159.16
561	0.322	0.006	0.607	0.030	0.943	0.021	88.61	192.75
562	0.354	0.021	0.456	0.116	0.919	0.133	28.60	159.27
563	0.326	0.007	0.529	0.068	0.843	0.106	62.17	158.69
564	0.330	0.016	0.594	0.041	1.028	0.161	79.72	211.20
566	0.320	0.004	0.465	0.076	0.531	0.079	45.57	66.11
567	0.335	0.019	0.661	0.224	0.978	0.158	97.02	191.75
569	0.352	0.018	0.491	0.060	1.051	0.019	39.53	198.77
571	0.380	0.004	0.508	0.081	0.947	0.015	33.89	149.43
572	0.337	0.010	0.398	0.071	0.669	0.091	18.08	98.42
573	0.329	0.012	0.454	0.060	0.663	0.159	38.10	101.62
574	0.349	0.021	0.559	0.166	0.834	0.330	60.27	139.06
575	0.319	0.003	0.582	0.141	1.072	0.350	82.25	235.70
576	0.343	0.010	0.466	0.102	0.533	0.054	35.86	55.49
578	0.371	0.016	0.562	0.155	0.688	0.098	51.53	85.61
579	0.338	0.003	0.543	0.095	0.764	0.067	60.81	126.26
580	0.348	0.011	0.650	0.009	1.070	0.014	86.78	207.38
581	0.348	0.007	0.670	0.073	1.110	0.052	92.81	219.18
582	0.351	0.007	0.589	0.077	0.887	0.408	67.71	152.80
583	0.348	0.019	0.839	0.229	1.067	0.377	140.96	206.32
584	0.347	0.021	0.617	0.223	1.130	0.140	77.91	225.55
585	0.358	0.010	0.566	0.051	1.051	0.137	57.86	193.21
586	0.356	0.011	1.193	0.077	1.374	0.138	235.02	285.96
587	0.338	0.008	0.490	0.063	0.799	0.311	44.83	136.06
588	0.335	0.017	0.570	0.139	0.898	0.105	70.15	167.96
589	0.342	0.011	0.626	0.094	0.983	0.132	82.86	187.24
590	0.363	0.014	0.616	0.075	0.979	0.165	69.94	169.94
591	0.346	0.003	0.463	0.089	0.774	0.441	33.94	123.82
592	0.366	0.009	0.495	0.054	0.441	0.228	35.25	20.49
593	0.352	0.012	0.517	0.035	0.597	0.470	47.01	69.86
594	0.344	0.016	0.598	0.196	0.985	0.209	74.10	186.71
595	0.347	0.012	0.495	0.067	0.376	0.166	42.56	8.45
596	0.396	0.040	0.559	0.085	0.525	0.142	41.28	32.69
597	0.347	0.012	0.564	0.111	0.554	0.384	62.44	59.56
598	0.347	0.012	0.455	0.041	0.456	0.265	31.03	31.32
599	0.336	0.011	0.509	0.103	0.789	0.299	51.49	134.72
600	0.352	0.003	0.603	0.076	0.911	0.045	71.47	158.96
603	0.333	0.019	0.630	0.121	0.970	0.128	89.48	191.48
604	0.388	0.011	0.618	0.021	0.836	0.009	59.23	115.19
606	0.331	0.014	0.492	0.115	0.951	0.095	48.59	187.02

607	0.388	0.027	0.549	0.071	0.789	0.101	41.41	103.35
609	0.336	0.009	0.616	0.040	0.922	0.077	83.61	174.58
610	0.377	0.019	0.555	0.072	0.884	0.138	47.08	134.36
612	0.341	0.024	0.592	0.035	0.827	0.155	73.61	142.42
613	0.414	0.031	0.662	0.119	0.879	0.300	59.95	112.49
617	0.316	0.007	0.740	0.187	0.915	0.329	134.04	189.15
618	0.336	0.022	0.522	0.129	0.859	0.136	55.41	155.81
619	0.353	0.016	0.540	0.022	0.831	0.121	52.74	135.28
620	0.373	0.010	0.235	0.013	0.451	0.132	-36.94	21.02
621	0.340	0.021	0.492	0.052	0.596	0.149	44.47	75.02
630	0.340	0.017	0.546	0.052	0.967	0.029	60.84	184.79
632	0.350	0.013	0.556	0.034	0.839	0.297	58.76	139.62
633	0.341	0.008	0.467	0.123	0.646	0.049	36.95	89.44
634	0.358	0.026	0.598	0.094	0.821	0.409	66.95	129.24
635	0.382	0.092	0.342	0.057	0.516	0.048	-10.55	34.87
636	0.339	0.003	0.446	0.074	0.911	0.027	31.34	168.57
637	**0.690**	0.029	**0.656**	0.089	**0.652**	0.099	-4.88	-5.56
638	0.688	0.085	0.864	0.057	0.864	0.305	25.47	25.57
639	0.328	0.008	0.681	0.055	0.963	0.134	107.72	193.60
640	1.105	0.078	1.128	0.045	1.115	0.058	2.14	0.91
641	0.341	0.013	0.413	0.076	0.700	0.056	21.21	105.38
642	0.304	0.003	0.492	0.096	0.820	0.132	61.84	169.85
643	0.328	0.008	0.698	0.106	0.930	0.126	112.49	183.35
644	0.330	0.016	0.429	0.062	0.514	0.119	30.23	55.81
645	0.310	0.006	0.529	0.152	0.807	0.120	70.46	160.15
646	0.320	0.017	0.554	0.156	1.002	0.247	73.23	213.02
647	0.319	0.009	0.628	0.096	0.874	0.079	97.07	174.16
648	0.331	0.006	0.517	0.030	0.612	0.044	56.25	85.08
649	0.326	0.006	0.446	0.052	0.440	0.264	36.95	35.21
651	0.320	0.008	0.408	0.157	0.981	0.305	27.60	206.46
652	0.326	0.036	0.492	0.140	0.744	0.144	50.92	128.12
653	0.341	0.022	0.575	0.186	1.217	0.107	68.46	256.45
654	0.326	0.017	0.451	0.074	0.763	0.017	38.49	134.19
655	0.322	0.013	0.493	0.104	0.847	0.264	53.16	163.32
656	0.311	0.028	0.371	0.050	0.722	0.162	19.42	132.51
657	0.308	0.012	0.522	0.057	0.955	0.066	69.41	209.73
658	0.325	0.007	0.537	0.066	0.968	0.241	65.16	197.44
659	0.327	0.011	0.514	0.108	1.074	0.287	57.45	228.78
660	0.310	0.007	0.490	0.099	0.761	0.132	58.06	145.48
661	0.331	0.023	0.652	0.296	0.921	0.322	97.08	178.53
666	0.330	0.002	0.357	0.028	0.777	0.236	8.39	135.69
668	0.332	0.008	0.427	0.043	0.769	0.136	28.39	131.39
670	0.334	0.018	0.394	0.022	0.746	0.216	17.98	123.58
672	0.333	0.010	0.580	0.280	0.974	0.385	74.35	192.69

674	0.315	0.010	0.524	0.151	0.910	0.145	66.63	189.30
677	0.308	0.008	0.483	0.093	0.935	0.264	56.88	203.90
678	0.312	0.003	0.445	0.061	0.760	0.244	42.58	143.33
679	0.329	0.014	0.487	0.132	0.772	0.436	48.02	134.55
680	0.367	0.009	0.421	0.069	0.642	0.284	14.62	75.02
681	0.317	0.008	0.579	0.122	1.077	0.233	82.54	239.75
687	0.329	0.011	0.615	0.191	0.893	0.220	86.83	171.53
688	0.330	0.010	0.530	0.074	0.933	0.123	60.51	182.73
689	0.331	0.012	0.352	0.029	0.383	0.134	6.55	15.83
725	0.348	0.007	0.489	0.085	1.003	0.168	40.75	188.40
726	0.318	0.009	0.710	0.157	0.809	0.261	123.61	154.56
728	0.349	0.008	0.491	0.052	0.727	0.110	40.59	108.31
729	0.339	0.009	0.581	0.108	0.680	0.125	71.56	100.89
730	0.341	0.017	0.482	0.023	0.673	0.164	41.31	97.17
731	0.325	0.007	0.454	0.030	0.550	0.093	39.45	69.16
732	0.344	0.014	0.521	0.058	0.721	0.106	51.70	109.80
733	0.330	0.006	0.519	0.082	0.545	0.253	57.01	64.98
734	0.325	0.008	0.513	0.142	0.730	0.325	57.85	124.51
735	0.357	0.040	0.538	0.075	0.831	0.291	50.75	132.99
737	0.341	0.015	0.562	0.046	0.860	0.197	65.07	152.35
744	0.597	0.021	0.785	0.065	1.020	0.271	31.62	71.01
745	0.563	0.012	0.686	0.041	1.166	0.040	21.86	107.29
746	0.540	0.035	0.991	0.272	0.853	0.416	83.57	58.00
747	0.618	0.083	0.957	0.478	0.789	0.322	54.91	27.62
748	0.567	0.009	0.956	0.208	1.110	0.134	68.57	95.71
750	0.530	0.007	0.814	0.256	0.996	0.174	53.58	87.92
751	0.547	0.015	0.858	0.331	0.894	0.505	56.95	63.60
752	0.594	0.019	0.981	0.229	1.138	0.180	65.24	91.69
753	0.605	0.023	1.067	0.138	1.258	0.191	76.21	107.76
754	0.560	0.036	0.993	0.201	0.797	0.353	77.22	42.24
755	0.549	0.015	1.043	0.249	1.226	0.298	90.10	123.51
756	0.532	0.013	0.705	0.054	0.549	0.086	32.50	3.07
757	0.572	0.011	0.671	0.108	0.576	0.179	17.31	0.70
758	0.548	0.012	0.802	0.079	1.199	0.156	46.38	118.93
759	0.539	0.009	0.992	0.517	0.845	0.537	83.93	56.61
760	0.577	0.010	0.732	0.072	0.906	0.134	26.99	57.11
761	0.596	0.043	0.780	0.406	1.018	0.421	30.95	70.90
762	0.627	0.024	1.007	0.236	0.979	0.533	60.74	56.28
763	0.542	0.031	0.971	0.352	1.258	0.098	79.32	132.15
769	0.548	0.009	0.576	0.027	0.738	0.361	5.17	34.67
770	0.558	0.012	0.547	0.037	0.630	0.135	-2.03	12.90
771	0.540	0.023	0.558	0.068	0.632	0.076	3.40	17.17
772	0.550	0.019	0.854	0.266	1.183	0.224	55.43	115.28
773	0.572	0.046	0.672	0.166	0.726	0.157	17.47	26.79

774	0.616	0.023	0.820	0.252	1.098	0.095	33.13	78.29
775	0.543	0.014	0.849	0.439	1.111	0.334	56.26	104.42
776	0.601	0.004	0.724	0.075	0.857	0.176	20.34	42.46
777	0.543	0.009	0.771	0.257	0.899	0.034	41.90	65.40
778	0.540	0.010	0.932	0.287	1.181	0.291	72.42	118.63
779	0.555	0.023	0.807	0.380	1.099	0.308	45.32	97.96
780	0.542	0.018	0.741	0.151	1.087	0.069	36.57	100.37
781	0.564	0.015	0.794	0.050	1.006	0.129	40.78	78.43
783	0.532	0.021	0.621	0.108	0.971	0.102	16.79	82.58
784	0.307	0.001	0.486	0.150	0.736	0.193	58.03	139.48
785	0.560	0.008	0.621	0.113	0.820	0.206	10.77	46.28
786	0.280	0.005	0.568	0.149	0.833	0.148	102.73	197.03
787	0.287	0.007	0.846	0.151	0.965	0.293	194.89	236.12
788	0.299	0.011	0.437	0.083	1.108	0.160	46.32	271.09
789	0.289	0.013	0.758	0.149	0.932	0.153	162.10	222.24
790	0.290	0.013	0.698	0.130	0.918	0.020	140.85	216.92
791	0.530	0.010	0.742	0.168	1.122	0.067	40.15	111.89
792	0.545	0.015	1.066	0.244	1.111	0.383	95.66	103.91
793	0.560	0.009	0.579	0.022	0.683	0.127	3.45	21.90
796	0.290	0.012	0.643	0.034	0.832	0.076	121.58	186.68
797	0.578	0.015	0.911	0.334	1.341	0.182	57.67	132.01
798	0.558	0.018	0.790	0.229	1.067	0.164	41.43	91.04
799	0.573	0.037	0.686	0.048	1.037	0.212	19.72	81.04
800	0.566	0.015	0.660	0.066	0.904	0.057	16.54	59.62
801	0.566	0.006	0.565	0.056	0.738	0.226	-0.12	30.41
802	0.571	0.027	0.712	0.077	1.000	0.381	24.68	74.97
804	0.533	0.018	0.657	0.110	0.362	0.283	23.26	-32.02
805	0.613	0.013	0.806	0.159	1.189	0.285	31.50	94.07
806	0.548	0.016	0.553	0.049	0.747	0.114	0.79	36.17
807	0.539	0.006	0.741	0.070	1.015	0.092	37.39	88.26
808	0.545	0.003	1.054	0.233	1.256	0.128	93.46	130.52
809	0.566	0.019	0.783	0.235	1.200	0.151	38.40	112.01
810	0.570	0.014	0.749	0.023	0.852	0.125	31.40	49.42
812	0.584	0.009	0.614	0.046	0.823	0.058	5.25	41.06
813	0.632	0.037	0.782	0.067	0.721	0.388	23.67	13.97
814	0.595	0.037	0.672	0.052	1.086	0.104	13.00	82.58
815	0.583	0.028	0.672	0.091	0.955	0.174	15.32	63.81
816	0.566	0.017	0.549	0.066	0.834	0.044	-2.95	47.50
817	0.570	0.024	0.851	0.182	1.199	0.223	49.24	110.35
818	0.620	0.015	0.667	0.036	1.056	0.434	7.58	70.32
819	0.548	0.009	0.729	0.230	1.158	0.035	33.01	111.25
820	0.549	0.004	0.633	0.063	1.108	0.103	15.24	101.82
821	0.599	0.008	0.785	0.044	0.994	0.448	31.18	66.09
822	0.577	0.034	0.862	0.297	0.898	0.412	49.31	55.54

823	0.575	0.019	0.875	0.383	1.014	0.456	52.14	76.25
824	0.554	0.005	0.649	0.088	1.091	0.263	17.02	96.87
825	0.534	0.019	0.608	0.023	0.584	0.180	13.92	9.43
826	0.574	0.014	0.711	0.237	1.140	0.330	24.00	98.72
827	0.594	0.038	0.729	0.056	1.048	0.213	22.74	76.47
828	0.563	0.016	0.820	0.139	1.210	0.019	45.59	114.86
829	0.599	0.027	0.714	0.095	1.081	0.250	19.32	80.62
830	0.573	0.010	0.824	0.366	1.001	0.321	43.66	74.59
831	0.558	0.009	0.903	0.373	1.216	0.297	61.86	118.11
832	0.554	0.005	0.883	0.389	0.953	0.537	59.29	71.86
834	0.663	0.058	1.000	0.363	0.903	0.616	50.78	36.25
835	0.601	0.030	0.711	0.071	0.746	0.151	18.42	24.20
836	0.573	0.009	0.621	0.119	0.867	0.103	8.44	51.37
837	0.570	0.031	0.721	0.251	0.932	0.019	26.36	63.41
838	0.532	0.006	0.668	0.111	0.717	0.119	25.58	34.86
839	0.588	0.021	0.552	0.013	0.849	0.208	-6.12	44.44
840	0.568	0.009	0.555	0.065	0.904	0.147	-2.17	59.19
841	0.589	0.008	0.843	0.386	1.060	0.369	43.10	79.92
842	0.577	0.014	0.629	0.113	0.817	0.114	9.13	41.68
843	0.601	0.050	0.699	0.129	0.847	0.227	16.25	40.99
844	0.550	0.009	0.486	0.050	0.900	0.149	-11.52	63.67
845	0.558	0.021	0.878	0.369	1.150	0.288	57.50	106.28
846	0.576	0.018	1.032	0.440	1.181	0.546	79.11	105.09
847	0.559	0.006	0.733	0.110	0.865	0.250	31.19	54.80
848	0.581	0.013	0.831	0.253	1.145	0.268	42.89	96.90
849	0.326	0.011	0.477	0.192	0.965	0.111	46.32	196.01
850	0.556	0.005	0.782	0.182	0.994	0.086	40.56	78.73
851	0.580	0.004	0.611	0.034	0.593	0.225	5.35	2.30
853	0.563	0.017	0.642	0.056	0.706	0.257	14.16	25.47
854	0.284	0.004	0.623	0.369	0.861	0.427	119.37	203.05
855	0.283	0.001	0.692	0.139	0.874	0.115	144.93	209.08
856	0.288	0.004	0.654	0.175	0.850	0.159	127.08	195.25
857	0.281	0.010	0.356	0.044	0.954	0.168	26.81	239.50
858	0.290	0.012	0.794	0.094	0.783	0.106	173.48	169.69
859	0.284	0.015	0.665	0.129	0.765	0.076	134.15	169.25
860	0.293	0.010	0.589	0.149	0.866	0.258	101.14	195.56
861	0.303	0.012	0.716	0.081	0.918	0.097	135.93	202.64
862	0.314	0.021	0.528	0.099	0.887	0.334	68.05	182.38
863	0.311	0.007	0.535	0.082	0.781	0.287	72.21	151.50
864	0.298	0.007	0.632	0.166	1.019	0.015	112.19	241.95
865	0.307	0.021	0.483	0.112	0.889	0.417	57.44	189.47
866	0.288	0.003	0.541	0.113	0.941	0.183	88.18	226.94
869	0.290	0.004	0.757	0.200	0.951	0.093	160.73	227.44
870	0.285	0.005	0.522	0.087	1.064	0.086	83.06	273.01

871	0.292	0.006	0.646	0.154	0.892	0.167	120.98	205.25
872	0.285	0.006	0.673	0.233	1.161	0.125	135.75	306.78
874	0.282	0.008	0.724	0.252	0.865	0.326	156.86	206.86
875	0.293	0.006	0.544	0.168	0.773	0.139	85.78	163.71
876	0.295	0.007	0.720	0.036	1.096	0.100	144.46	271.95
877	0.298	0.004	0.806	0.101	1.107	0.044	170.66	271.78
879	0.299	0.010	0.900	0.076	1.042	0.104	201.00	248.38
880	0.295	0.004	0.591	0.070	0.938	0.062	100.45	218.33
881	0.290	0.003	0.721	0.276	0.966	0.139	149.02	233.60
883	0.289	0.004	0.718	0.141	0.923	0.098	148.61	219.75
884	0.293	0.011	0.563	0.107	0.997	0.212	92.04	240.16
885	0.292	0.004	0.402	0.008	0.985	0.192	37.56	237.21
886	0.296	0.009	0.761	0.225	1.092	0.075	157.09	268.81
887	0.286	0.004	0.583	0.114	0.827	0.151	103.85	189.04
888	0.304	0.005	0.556	0.067	0.947	0.078	82.69	211.06
889	0.303	0.010	0.645	0.154	0.994	0.155	112.64	227.58
890	0.324	0.038	0.924	0.082	1.093	0.163	185.38	237.69
891	0.311	0.009	0.784	0.121	1.020	0.104	152.25	228.33
892	0.308	0.006	0.653	0.218	1.132	0.073	112.24	267.93
893	0.313	0.011	0.424	0.021	0.954	0.203	35.61	205.12
894	0.321	0.008	0.437	0.048	1.062	0.153	36.38	231.29
895	0.301	0.008	0.796	0.087	1.011	0.101	164.34	235.88
896	0.315	0.011	0.438	0.091	1.091	0.107	38.79	245.98
897	0.310	0.005	0.462	0.045	1.087	0.125	48.76	250.27
898	0.293	0.005	0.445	0.133	1.067	0.027	52.05	264.46
899	0.580	0.025	0.702	0.123	0.738	0.111	20.98	27.24
901	0.564	0.004	0.631	0.040	0.621	0.045	11.82	10.11
902	0.696	0.048	0.933	0.052	0.644	0.152	34.07	-7.38
903	0.584	0.008	0.882	0.073	1.116	0.147	51.00	91.04
904	0.551	0.007	0.595	0.049	0.700	0.242	7.99	26.98
905	0.307	0.011	0.688	0.079	0.830	0.199	124.46	170.76
906	0.559	0.024	0.985	0.327	1.148	0.298	76.37	105.43
907	0.588	0.013	0.776	0.034	1.095	0.074	31.84	86.18
908	0.289	0.002	0.824	0.173	0.791	0.143	185.57	174.13
909	0.324	0.035	0.960	0.364	1.135	0.350	195.99	249.85
910	0.318	0.011	0.733	0.266	1.164	0.085	130.50	266.14
911	0.329	0.015	0.696	0.040	1.196	0.067	111.76	263.79
912	0.314	0.026	0.641	0.385	0.699	0.105	104.25	122.95
914	0.290	0.004	0.749	0.125	1.070	0.059	158.16	268.85
915	0.298	0.013	0.570	0.034	0.818	0.031	91.39	174.38
916	0.317	0.006	0.445	0.058	1.032	0.179	40.63	225.89
919	0.424	0.029	0.719	0.174	0.976	0.199	69.50	130.19
920	0.302	0.002	0.591	0.092	1.123	0.059	96.02	272.15
921	0.346	0.023	0.753	0.169	1.006	0.128	117.32	190.57

922	0.337	0.006	0.813	0.165	1.114	0.135	141.11	230.14
923	0.330	0.018	0.618	0.306	0.842	0.202	87.17	155.15
924	0.292	0.007	1.003	0.287	1.082	0.226	243.77	271.09
925	0.290	0.007	0.727	0.058	0.711	0.187	150.57	145.17
926	0.300	0.011	0.771	0.072	0.800	0.278	156.83	166.26
927	0.307	0.004	0.517	0.171	0.904	0.120	68.22	194.14
928	0.303	0.015	0.542	0.040	1.007	0.137	78.99	232.23
929	0.308	0.014	0.997	0.027	1.100	0.160	223.94	257.64
932	0.310	0.009	0.776	0.175	1.072	0.202	150.32	245.91
933	0.302	0.020	0.707	0.245	1.123	0.059	134.48	272.38
934	0.314	0.033	1.015	0.318	1.334	0.155	222.91	324.28
936	0.338	0.009	0.636	0.170	0.934	0.236	88.45	176.51
937	0.402	0.008	0.686	0.173	1.241	0.028	70.42	208.37
938	0.302	0.021	0.859	0.159	1.212	0.122	184.33	301.16
939	0.296	0.005	0.888	0.184	1.175	0.160	200.23	297.52
940	0.304	0.009	0.793	0.127	0.888	0.012	160.75	192.11
941	0.420	0.033	0.660	0.090	1.197	0.122	57.14	185.08
942	0.303	0.008	0.633	0.226	0.901	0.353	108.57	197.14
943	0.291	0.008	0.662	0.148	1.016	0.193	127.12	248.86
944	0.320	0.007	0.360	0.069	0.877	0.036	12.38	173.67
945	0.330	0.017	0.649	0.059	1.033	0.217	96.97	213.25
946	0.367	0.018	0.701	0.201	1.157	0.118	91.01	215.35
947	0.340	0.011	1.028	0.326	1.216	0.158	202.75	258.00
948	0.295	0.008	0.945	0.055	1.210	0.069	219.98	309.71
949	0.292	0.012	0.741	0.188	0.795	0.179	153.88	172.37
950	0.310	0.029	0.969	0.248	1.066	0.314	212.14	243.39
951	0.332	0.022	0.735	0.130	0.670	0.511	121.51	102.01
952	0.298	0.013	0.758	0.025	0.832	0.148	154.76	179.40
954	0.300	0.009	0.754	0.154	1.097	0.128	151.17	265.15
1005	0.391	0.030	0.817	0.094	1.025	0.075	109.13	162.29
1006	0.305	0.013	0.496	0.078	1.128	0.070	62.51	269.73
1007	0.292	0.002	0.599	0.240	0.983	0.085	105.26	236.91
1008	0.296	0.012	0.357	0.050	1.041	0.126	20.86	251.97
1009	0.286	0.006	0.889	0.129	1.082	0.070	211.09	278.88
1010	0.283	0.004	0.716	0.220	0.833	0.080	152.82	193.88
1011	0.312	0.022	0.495	0.033	0.955	0.136	58.72	206.31
1012	0.312	0.008	0.602	0.346	1.244	0.094	92.95	298.72
1013	0.310	0.010	0.671	0.117	0.880	0.083	116.58	184.28
1014	0.300	0.013	0.683	0.271	0.976	0.209	128.03	225.81
1015	0.303	0.008	0.571	0.090	0.820	0.234	88.34	170.74
1016	0.317	0.012	0.454	0.045	0.877	0.244	42.96	176.26
1017	0.295	0.005	0.562	0.068	0.843	0.120	90.61	185.97
1018	0.308	0.022	0.646	0.112	0.950	0.103	109.41	208.22
1019	0.303	0.022	0.824	0.173	1.012	0.256	172.06	233.88

1020	0.301	0.016	0.827	0.245	0.947	0.326	175.06	214.97
1021	0.292	0.016	0.826	0.409	1.129	0.245	182.55	286.32
1022	0.317	0.008	0.818	0.057	0.875	0.113	158.32	176.32
1023	0.384	0.034	0.846	0.404	1.071	0.261	120.42	179.06
1024	0.323	0.002	0.948	0.082	0.938	0.106	193.09	190.10
1025	0.324	0.010	0.843	0.128	0.890	0.275	159.82	174.51
1025	0.327	0.018	0.286	0.077	1.011	0.206	-12.45	209.39
1026	0.304	0.010	0.298	0.048	0.680	0.064	-1.87	123.93
1026	0.309	0.007	0.801	0.153	1.015	0.126	158.84	228.23
1027	0.320	0.009	0.704	0.070	1.028	0.057	119.90	221.25
1028	0.384	0.032	0.755	0.157	1.234	0.123	96.36	221.08
1029	0.286	0.003	0.698	0.141	1.029	0.199	143.94	259.91
1030	0.292	0.007	0.436	0.116	0.971	0.005	49.32	232.42
1031	0.286	0.004	0.432	0.162	0.990	0.122	50.93	246.04
1033	0.282	0.003	0.652	0.157	0.741	0.069	131.21	162.88
1034	0.313	0.009	0.774	0.168	1.128	0.218	147.65	260.87
1035	0.325	0.010	0.427	0.053	0.562	0.031	31.35	72.85
1036	0.295	0.011	0.786	0.118	1.010	0.056	166.14	242.10
1038	0.297	0.006	0.790	0.164	1.021	0.112	165.99	243.77
1039	0.295	0.008	0.655	0.011	0.974	0.034	121.67	229.68
1040	0.364	0.032	0.900	0.181	1.012	0.188	147.39	178.19
1041	0.322	0.019	0.763	0.098	1.060	0.249	136.71	228.85
1043	0.297	0.018	0.689	0.246	0.969	0.150	131.73	225.78
1044	0.305	0.002	0.758	0.192	0.864	0.150	148.63	183.28
1045	0.300	0.008	0.780	0.106	0.971	0.296	160.40	223.92
1046	0.297	0.005	0.687	0.131	0.963	0.123	130.94	223.99
1047	0.309	0.024	0.845	0.266	0.973	0.274	173.06	214.44
1048	0.297	0.008	0.438	0.046	1.016	0.132	47.59	242.20
1049	0.297	0.006	0.565	0.074	1.012	0.206	89.91	240.36
1050	0.290	0.010	0.930	0.075	1.033	0.205	220.94	256.62
1051	0.307	0.022	0.334	0.097	1.187	0.153	8.80	287.17
1052	0.342	0.017	0.595	0.070	1.118	0.071	73.98	226.80
1053	0.297	0.018	0.365	0.104	0.936	0.143	23.15	215.62
1054	0.307	0.012	0.544	0.055	0.998	0.128	77.39	225.33
1055	0.317	0.010	0.514	0.161	0.918	0.231	62.15	189.70
1056	0.313	0.027	0.632	0.159	0.856	0.172	101.60	173.19
1057	0.401	0.044	0.668	0.075	1.120	0.089	66.72	179.45
1058	0.326	0.034	0.804	0.352	1.033	0.310	146.37	216.55
1059	0.333	0.018	0.302	0.078	0.850	0.021	-9.32	155.51
1060	0.365	0.064	0.832	0.114	1.205	0.162	128.04	230.05
1061	0.333	0.022	0.649	0.063	1.054	0.216	94.60	216.10
1062	0.351	0.012	0.709	0.185	0.976	0.098	101.71	177.89
1063	0.310	0.008	0.929	0.218	1.228	0.090	200.00	296.56
1064	0.302	0.008	0.688	0.193	0.843	0.114	127.45	178.72

1065	0.296	0.006	0.459	0.119	0.801	0.229	54.89	170.19
1066	0.298	0.005	0.684	0.152	0.837	0.048	129.16	180.67
1069	0.300	0.024	0.686	0.258	0.815	0.231	128.30	171.48
1071	0.343	0.032	0.941	0.116	1.213	0.103	174.17	253.20
1072	0.339	0.041	0.792	0.349	0.914	0.117	133.86	169.78
1073	0.322	0.012	0.801	0.009	1.073	0.152	149.02	233.47
1074	0.320	0.007	0.855	0.314	1.054	0.249	167.19	229.48
1075	0.300	0.018	0.709	0.119	1.063	0.117	136.48	254.62
1076	0.299	0.009	0.853	0.169	1.137	0.189	185.60	280.80
1077	0.299	0.018	0.860	0.317	1.194	0.083	187.42	298.89
1078	0.316	0.013	0.652	0.329	0.989	0.224	106.44	213.31
1080	0.296	0.007	0.836	0.185	1.195	0.047	182.23	303.37
1081	0.304	0.020	0.683	0.136	0.911	0.119	124.81	200.00
1082	0.339	0.029	0.818	0.349	1.011	0.320	141.44	198.43
1083	0.296	0.001	0.675	0.294	0.781	0.354	127.78	163.67
1084	0.292	0.010	0.763	0.228	0.841	0.170	161.00	187.69
1085	0.282	0.002	0.652	0.091	0.797	0.105	131.60	182.84
1086	0.287	0.002	0.751	0.045	0.863	0.094	161.86	200.93
1087	0.293	0.006	0.635	0.038	0.832	0.070	116.72	183.96
1088	0.289	0.004	0.649	0.242	0.865	0.198	124.31	198.85
1089	0.295	0.009	0.872	0.381	0.793	0.207	195.15	168.62
1090	0.348	0.004	0.892	0.097	1.063	0.250	156.66	205.66
1091	0.299	0.008	0.900	0.105	1.023	0.148	201.11	242.03
1092	0.313	0.005	0.780	0.071	0.958	0.087	149.47	206.50
1093	0.334	0.041	0.610	0.200	0.838	0.089	82.73	150.90
1093	0.320	0.012	0.880	0.171	1.159	0.160	174.82	261.71
1094	0.378	0.024	0.855	0.235	1.321	0.171	126.30	249.78
1095	0.300	0.003	0.809	0.013	0.778	0.286	169.56	159.22
1096	0.408	0.040	0.582	0.019	1.146	0.222	42.76	181.03
1097	0.313	0.001	0.336	0.029	0.564	0.072	7.34	80.11
1098	0.311	0.016	0.760	0.358	1.040	0.302	144.37	234.30
1099	0.534	0.037	0.606	0.114	0.956	0.114	13.35	78.91
1100	0.306	0.014	0.870	0.069	1.195	0.086	184.20	290.52
1101	0.302	0.008	0.759	0.241	0.950	0.250	151.71	215.03
1102	0.339	0.008	0.709	0.116	1.267	0.068	109.45	274.21
1103	0.305	0.006	0.735	0.172	1.043	0.253	140.61	241.59
1104	0.447	0.038	0.558	0.016	0.538	0.156	24.74	20.27
1105	0.321	0.003	0.897	0.055	1.186	0.054	179.05	269.19
1106	0.309	0.022	1.015	0.074	1.182	0.106	228.73	283.05
1107	0.323	0.009	0.770	0.158	1.098	0.399	138.74	240.29
1108	0.307	0.003	0.861	0.115	0.970	0.089	180.26	215.62
1109	0.310	0.015	0.493	0.036	0.563	0.108	58.75	81.31
1111	0.311	0.005	0.336	0.028	0.668	0.125	8.15	114.68
1112	0.385	0.040	0.764	0.097	0.854	0.179	98.27	121.63

1114	0.301	0.006	0.814	0.147	0.954	0.016	170.54	216.94
1115	0.304	0.001	0.760	0.100	1.035	0.160	149.62	240.09
1116	0.510	0.091	0.552	0.120	0.743	0.107	8.10	45.59
1117	0.317	0.028	0.925	0.101	1.202	0.142	191.60	278.78
1118	0.400	0.017	0.661	0.117	1.131	0.295	65.30	182.99
1119	0.330	0.008	0.374	0.038	0.743	0.106	13.23	125.25
1120	0.302	0.008	0.743	0.143	1.275	0.096	145.64	321.72
1121	0.327	0.025	0.869	0.088	0.899	0.241	165.92	175.20
1122	0.337	0.017	0.353	0.067	0.727	0.358	4.95	115.94
1123	0.312	0.014	0.790	0.255	1.045	0.114	153.37	235.40
1124	0.300	0.006	0.897	0.119	1.059	0.225	199.44	253.28
1125	0.292	0.010	0.917	0.147	1.071	0.160	213.68	266.36
1126	0.369	0.038	0.378	0.098	0.426	0.286	2.53	15.54
1128	0.319	0.016	0.604	0.156	0.285	0.088	89.54	-10.67
1129	0.369	0.011	0.428	0.058	0.710	0.159	15.79	92.24
1130	0.358	0.018	0.626	0.074	0.818	0.006	74.79	128.37
1131	0.400	0.022	0.600	0.098	0.793	0.082	49.92	98.33
1132	0.320	0.005	0.368	0.056	0.595	0.184	15.02	86.24
1133	0.298	0.006	0.319	0.079	0.941	0.088	7.28	216.24
1134	0.430	0.036	0.469	0.088	0.604	0.169	9.15	40.57
1135	0.403	0.016	0.497	0.027	0.731	0.054	23.43	81.62
1136	0.435	0.059	0.432	0.030	0.973	0.082	-0.69	123.93
1137	0.391	0.041	0.435	0.101	0.817	0.165	11.35	109.04
1141	0.313	0.005	0.365	0.083	0.790	0.232	16.61	152.50
1142	0.337	0.011	0.433	0.043	0.455	0.205	28.49	35.01
1143	0.314	0.013	0.367	0.022	0.885	0.052	16.86	181.44
1144	0.304	0.020	0.348	0.032	0.961	0.092	14.46	215.88
1145	0.299	0.004	0.611	0.226	0.381	0.064	104.69	27.68
1147	0.338	0.004	0.491	0.067	0.614	0.135	45.27	81.66
1148	0.341	0.010	0.452	0.135	0.755	0.078	32.68	121.62
1149	0.335	0.009	0.432	0.122	0.540	0.104	29.08	61.35
1150	0.468	0.063	0.504	0.065	0.886	0.213	7.84	89.52
1151	0.499	0.023	0.509	0.070	0.801	0.129	1.94	60.35
1152	0.583	0.013	0.598	0.046	0.668	0.327	2.52	14.64
1153	0.344	0.013	0.395	0.012	0.834	0.391	14.94	142.77
1154	0.484	0.045	0.427	0.025	0.927	0.214	-11.65	91.59
1155	0.408	0.022	0.557	0.113	0.505	0.183	36.52	23.77
1156	0.339	0.011	0.413	0.035	0.897	0.140	21.95	164.96
1157	0.382	0.016	0.470	0.042	0.941	0.076	23.06	146.64
1158	0.379	0.003	0.545	0.050	0.817	0.139	43.93	115.85
1160	0.338	0.012	0.345	0.036	0.800	0.085	1.87	136.55
1161	0.316	0.010	0.337	0.036	0.907	0.102	6.65	187.03
1162	0.318	0.026	0.336	0.033	0.793	0.232	5.67	149.53
1163	0.310	0.012	0.557	0.075	0.973	0.137	79.59	213.43

1164	0.311	0.009	0.399	0.096	0.810	0.165	28.05	160.06
1165	0.315	0.003	0.439	0.107	0.587	0.090	39.26	86.35
1166	0.300	0.006	0.369	0.096	0.770	0.223	23.25	156.95
1167	0.393	0.043	0.473	0.119	0.328	0.013	20.36	-16.45
1168	0.345	0.014	0.463	0.069	1.018	0.024	34.43	195.45
1169	0.308	0.020	0.524	0.077	0.609	0.173	69.84	97.62
1170	0.340	0.006	0.527	0.112	0.493	0.035	54.90	45.10
1171	0.323	0.012	0.414	0.067	0.765	0.297	28.20	137.19
1172	0.357	0.015	0.502	0.068	1.032	0.134	40.71	188.98
1173	0.379	0.005	0.472	0.101	0.796	0.122	24.74	110.30
1174	0.454	0.040	0.559	0.050	0.948	0.158	23.14	108.96
1175	0.398	0.013	0.553	0.023	0.754	0.070	38.86	89.53
1176	0.426	0.061	1.014	0.128	0.729	0.061	137.76	70.99
1177	0.419	0.014	0.495	0.020	0.924	0.138	18.22	120.53
1178	0.369	0.033	0.480	0.073	0.704	0.094	30.11	91.05
1179	0.308	0.011	0.572	0.140	0.715	0.022	85.62	131.89
1180	0.549	0.193	0.527	0.194	0.823	0.261	-4.00	49.82
1181	0.338	0.019	0.786	0.090	0.997	0.047	132.68	195.36
1182	0.415	0.042	0.535	0.111	0.778	0.075	28.92	87.47
1183	0.362	0.007	0.526	0.067	0.776	0.063	45.17	114.08
1184	0.334	0.020	0.554	0.035	0.755	0.014	65.70	125.82
1185	0.386	0.009	0.477	0.070	0.374	0.050	23.77	-3.11
1186	0.330	0.019	0.576	0.034	0.401	0.112	74.82	21.74
1187	0.379	0.006	0.537	0.044	0.956	0.203	41.48	152.11
1188	0.338	0.030	0.392	0.047	0.797	0.056	15.98	135.90
1189	0.337	0.025	0.452	0.051	0.450	0.029	34.36	33.56
1190	0.752	0.110	0.840	0.116	0.526	0.043	11.66	-30.05
1191	0.319	0.025	0.591	0.106	0.923	0.118	85.37	189.34
1192	0.381	0.020	0.448	0.024	0.754	0.034	17.48	97.64
1193	0.348	0.010	0.602	0.110	0.856	0.100	72.99	145.88
1194	0.463	0.009	0.569	0.100	0.661	0.092	22.82	42.76
1195	0.512	0.090	0.855	0.067	1.015	0.080	66.82	98.18
1196	0.517	0.016	0.844	0.058	1.074	0.084	63.35	107.87
1197	0.481	0.012	0.923	0.055	1.141	0.045	91.89	137.28
1198	0.449	0.050	0.867	0.126	0.998	0.067	93.02	122.20
1199	0.514	0.027	0.914	0.098	1.062	0.125	77.82	106.61
1200	0.434	0.030	0.889	0.091	0.916	0.059	105.00	111.15
1201	0.555	0.230	1.060	0.282	1.253	0.261	91.05	125.84
1202	0.492	0.143	0.926	0.274	1.057	0.232	88.27	114.92
1203	0.513	0.127	0.980	0.084	1.079	0.105	91.22	110.53
1204	0.511	0.145	0.925	0.155	1.031	0.137	81.08	101.83
1205	0.470	0.087	0.838	0.139	0.971	0.044	78.35	106.81
1206	0.554	0.084	1.127	0.272	1.216	0.283	103.31	119.42
1207	0.551	0.024	0.890	0.085	1.050	0.048	61.56	90.62

1208	0.444	0.067	0.881	0.151	1.085	0.142	98.20	144.19
1209	0.551	0.065	1.067	0.086	1.235	0.060	93.65	124.08
1210	0.545	0.088	0.905	0.194	1.085	0.147	65.89	98.90
1211	0.505	0.035	0.919	0.024	1.063	0.041	82.05	110.43
1212	0.564	0.193	1.058	0.298	1.239	0.258	87.76	119.81
1213	0.535	0.138	0.957	0.257	1.076	0.230	78.83	101.06
1214	0.502	0.121	0.960	0.226	1.059	0.217	91.17	110.89
1215	0.428	0.024	0.864	0.096	0.924	0.052	102.03	116.13
1216	0.485	0.081	0.764	0.227	0.850	0.275	57.63	75.31
1217	0.501	0.055	0.811	0.208	1.019	0.276	61.94	103.33
1218	0.514	0.091	0.948	0.137	1.122	0.103	84.25	118.15
1219	0.535	0.069	0.976	0.070	1.217	0.025	82.48	127.68
1220	0.515	0.016	0.933	0.042	1.104	0.069	80.98	114.17
1221	0.515	0.043	0.885	0.226	1.070	0.253	72.02	107.97
1222	0.504	0.036	0.991	0.064	1.138	0.050	96.56	125.79
1223	0.532	0.100	1.018	0.175	1.143	0.191	91.29	114.79
1224	0.548	0.134	1.001	0.144	1.112	0.104	82.66	102.98
1225	0.525	0.143	0.982	0.146	1.074	0.249	86.87	104.38
1226	0.507	0.105	1.065	0.126	1.228	0.075	110.26	142.37
1227	0.656	0.134	1.077	0.275	1.113	0.136	64.13	69.66
1228	0.838	0.163	1.105	0.222	1.272	0.183	31.81	51.77
1229	0.481	0.113	0.940	0.102	1.141	0.077	95.43	137.28
1230	0.513	0.051	1.016	0.019	1.127	0.052	98.24	119.83
1231	0.497	0.029	0.918	0.044	1.067	0.069	84.77	114.90
1232	0.476	0.045	0.976	0.074	1.128	0.030	104.90	136.74
1233	0.502	0.157	0.956	0.076	1.072	0.134	90.31	113.40
1234	0.490	0.120	0.937	0.183	1.144	0.212	91.03	133.24
1235	0.451	0.121	0.720	0.171	0.808	0.168	59.84	79.36
1236	0.546	0.112	0.900	0.246	0.962	0.165	64.84	76.19
1237	0.479	0.065	1.018	0.058	1.102	0.065	112.45	129.90
1238	0.449	0.169	0.858	0.190	0.972	0.211	91.09	116.48
1239	0.507	0.057	1.056	0.072	1.292	0.056	108.22	154.77
1240	0.558	0.062	1.017	0.028	1.174	0.046	82.20	110.39
1241	0.524	0.057	0.933	0.129	1.095	0.147	78.23	109.17
1242	0.480	0.025	0.873	0.052	1.057	0.040	81.88	120.14
1243	0.545	0.133	0.989	0.152	1.252	0.084	81.52	129.80
1244	0.509	0.102	0.926	0.124	1.086	0.162	81.86	113.43
1245	0.456	0.059	1.096	0.181	1.280	0.149	140.45	180.91
1246	0.445	0.010	0.870	0.039	0.991	0.050	95.58	122.94
1247	0.647	0.036	1.106	0.060	1.123	0.049	70.99	73.57
1248	0.498	0.023	1.093	0.127	1.159	0.192	119.48	132.80
1249	0.494	0.048	0.945	0.121	1.022	0.162	91.36	106.82
1250	0.477	0.054	0.882	0.096	1.023	0.126	85.10	114.62
1251	**0.477**	0.060	**0.418**	0.030	**0.376**	0.039	-12.31	-21.05

1252	0.439	0.060	0.887	0.131	1.028	0.130	102.20	134.42
1253	0.476	0.064	0.894	0.040	1.076	0.062	88.02	126.14
1254	0.516	0.143	0.959	0.153	1.172	0.188	85.79	127.13
1255	0.389	0.072	0.887	0.102	0.996	0.044	127.83	155.74
1256	0.505	0.068	0.935	0.124	1.025	0.024	85.20	103.17
1257	0.495	0.048	0.854	0.075	0.911	0.106	72.41	83.85
1259	0.445	0.093	0.896	0.147	1.055	0.125	101.42	137.26
1260	0.499	0.041	0.951	0.028	1.073	0.016	90.78	115.24
1261	0.508	0.077	0.923	0.145	1.099	0.061	81.51	116.13
1262	0.531	0.072	0.938	0.124	1.111	0.102	76.76	109.36
1263	0.451	0.058	0.802	0.082	0.942	0.122	77.96	109.10
1264	0.521	0.146	0.994	0.098	1.109	0.088	90.73	112.79
1265	0.422	0.078	0.837	0.087	0.985	0.088	98.18	133.23
1266	0.443	0.082	0.839	0.091	0.985	0.052	89.61	122.44
1267	0.487	0.048	0.859	0.093	0.990	0.096	76.51	103.49
1268	0.538	0.033	0.923	0.051	1.008	0.047	71.50	87.30
1269	0.545	0.045	0.965	0.134	1.121	0.115	77.00	105.63
1270	0.474	0.093	0.946	0.046	1.069	0.063	99.51	125.46
1271	0.694	0.093	0.861	0.134	0.866	0.124	24.05	24.68
1272	0.493	0.030	0.965	0.134	0.975	0.097	95.67	97.84
1273	0.457	0.078	0.818	0.093	0.974	0.078	78.99	113.13
1274	0.549	0.171	0.811	0.165	0.960	0.171	47.81	75.03
1275	0.493	0.139	0.986	0.146	1.068	0.215	100.14	116.78
213A	0.331	0.018	0.650	0.040	0.894	0.091	96.37	169.99
214A	0.335	0.026	0.688	0.084	1.021	0.182	105.27	204.88
215A	0.318	0.022	0.675	0.106	0.758	0.069	112.37	138.36
216A	0.325	0.005	0.688	0.068	0.745	0.081	111.79	129.13
218A	0.330	0.038	0.729	0.091	0.782	0.059	120.91	136.87
219A	0.378	0.018	0.736	0.053	0.818	0.059	94.63	116.12
220D-F2	0.369	0.011	0.773	0.168	1.113	0.083	109.67	201.81
224C	0.339	0.032	0.694	0.074	1.072	0.128	104.92	216.54
224C-F2	0.321	0.028	0.622	0.157	0.846	0.170	93.67	163.45
305*	0.331	0.025	0.718	0.081	0.910	0.048	116.80	174.65
306*	0.346	0.049	0.572	0.017	0.624	0.074	65.26	80.17
307*	0.347	0.023	0.636	0.063	0.770	0.071	83.37	122.02
429B	0.332	0.014	0.446	0.095	0.799	0.096	34.47	141.01
429C	0.291	0.007	0.358	0.024	0.419	0.056	23.02	43.87
429D	0.285	0.003	0.368	0.081	0.768	0.234	29.27	169.91
429E	0.325	0.011	0.410	0.064	0.768	0.009	26.02	135.96
430B	0.305	0.028	0.356	0.028	0.921	0.137	16.72	202.08
430C	0.301	0.006	0.327	0.015	0.495	0.039	8.53	64.45
430D	0.315	0.020	0.402	0.119	0.934	0.234	27.38	196.19
430E	0.266	0.008	0.308	0.040	0.861	0.004	15.79	223.81

568a	0.345	0.014	0.611	0.103	0.819	0.317	77.20	137.49
570(a)	0.338	0.005	0.443	0.039	0.679	0.254	31.07	100.79
577(a)	0.359	0.018	0.556	0.015	0.663	0.226	54.73	84.60
725C	0.340	0.018	0.546	0.165	0.907	0.272	60.49	166.86
725D	0.334	0.017	0.500	0.049	0.761	0.261	49.95	128.07
725E	0.325	0.006	0.646	0.168	0.898	0.252	98.67	176.02
726B	0.382	0.007	0.455	0.012	0.323	0.010	18.92	-15.52
726C	0.326	0.006	0.466	0.050	0.745	0.210	42.90	128.40
726D	0.326	0.014	0.723	0.285	1.190	0.309	121.78	265.03
726E	0.328	0.006	0.518	0.102	0.750	0.176	57.99	128.99
728B	0.319	0.004	0.384	0.055	0.997	0.155	20.48	212.43
728C	0.341	0.012	0.494	0.091	0.871	0.142	44.82	155.18
728D	0.360	0.018	0.594	0.116	1.024	0.131	65.06	184.80
728E	0.335	0.014	0.529	0.051	0.701	0.197	58.17	109.46
729C	0.337	0.012	0.541	0.104	0.842	0.218	60.53	149.95
729D	0.351	0.023	0.622	0.072	0.966	0.334	77.30	175.21
729E	0.335	0.009	0.553	0.119	0.978	0.265	65.34	192.23
730C	0.334	0.022	0.521	0.078	0.813	0.268	55.83	143.17
730D	0.347	0.020	0.521	0.021	0.879	0.073	50.10	152.98
730E	0.336	0.010	0.584	0.097	0.912	0.172	73.74	171.26
731C	0.326	0.005	0.542	0.113	0.832	0.077	66.09	155.06
731D	0.327	0.009	0.543	0.105	0.939	0.151	66.12	187.35
731E	0.328	0.009	0.486	0.030	0.612	0.238	48.02	86.29
732C	0.354	0.011	0.567	0.073	0.616	0.264	60.23	74.08
732D	0.334	0.002	0.473	0.114	0.999	0.101	41.86	199.30
732E	0.329	0.012	0.494	0.036	0.579	0.035	50.10	75.71
733C	0.331	0.017	0.602	0.075	1.031	0.119	82.16	211.90
733D	0.330	0.008	0.449	0.032	0.632	0.411	35.82	91.22
733E	0.324	0.006	0.507	0.091	0.630	0.382	56.42	94.35
734B	0.336	0.014	0.719	0.117	0.576	0.059	113.88	71.26
734C	0.344	0.004	0.446	0.002	0.613	0.200	29.75	78.10
734D	0.343	0.011	0.490	0.071	0.549	0.201	42.72	59.81
734E	0.337	0.008	0.598	0.103	0.947	0.125	77.52	181.19
735B	0.401	0.042	0.551	0.198	0.871	0.147	37.44	117.30
735C	0.345	0.014	0.683	0.153	1.016	0.190	97.87	194.59
735D	0.337	0.012	0.651	0.176	1.075	0.242	93.27	219.41
735E	0.331	0.001	0.553	0.039	0.906	0.248	66.90	173.34
737B	0.341	0.009	0.578	0.077	0.705	0.057	69.50	106.84
737C	0.327	0.005	0.705	0.185	1.107	0.105	115.48	238.09
737E	0.338	0.011	0.517	0.051	0.770	0.024	52.96	127.71

* = Extracts diluted at 20 mg/mL instead of 10 mg/mL.

- Extracts with a letter after the number such as “730D” are partitions and not crude extract.

- Extracts labeled F2 signify different fractions and not crude extract.

- Optical density values are an average of three replicates tested at 128 μg/mL.

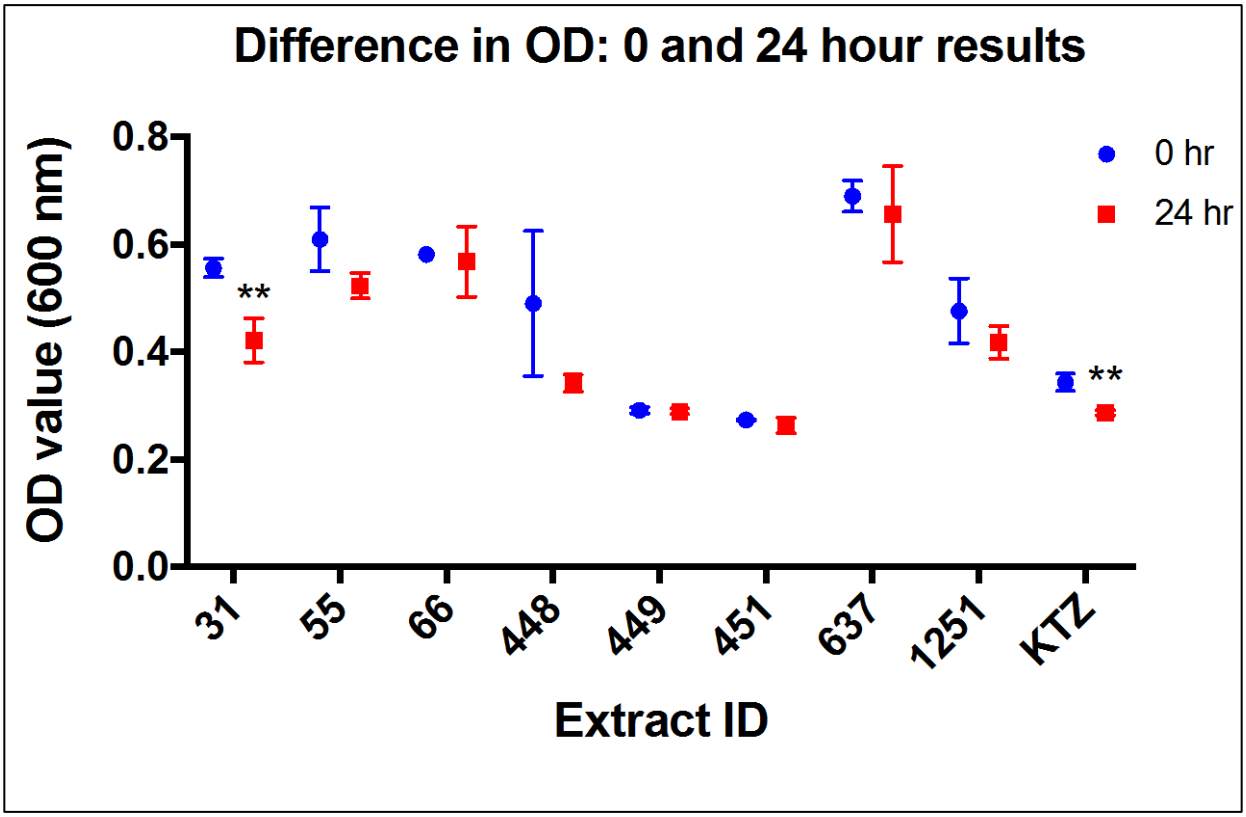


Figure 8. Difference in Optical Density at 0 and 24 Hours. This graph displays the initial optical density (OD) of extracts tested in triplicate at 128 μg/mL as compared to OD read at 24 hours. KTZ control tested at 16 μg/mL. Extract 31 had a statistically significant decreased in OD ($p= 0.006$), while all the other extracts did not have a significant change in OD from 0 hour read, signifying fungistatic activity. KTZ control had a statistically significant decrease in OD ($p= 0.004$). All extracts were further tested via serial dilution to determine lowest concentration (MIC) that displays fungistatic or fungicidal activity.

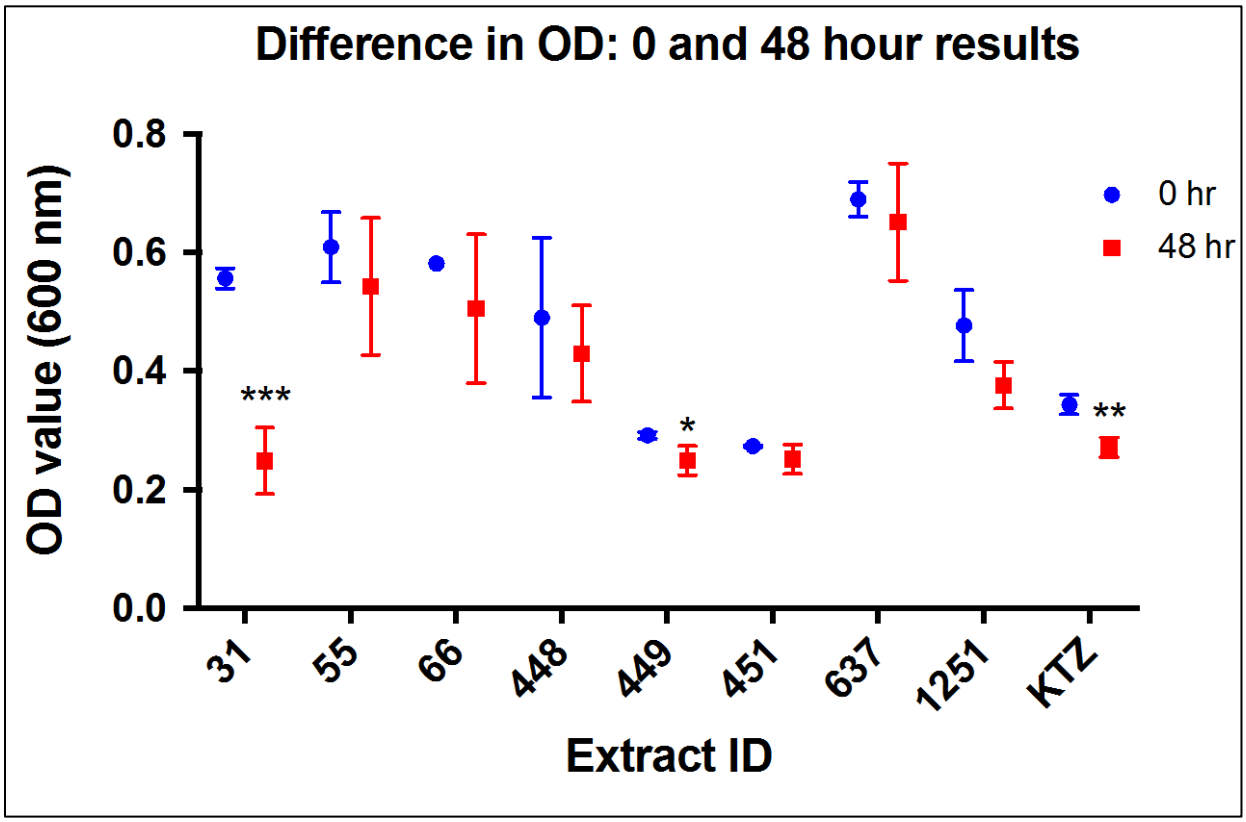


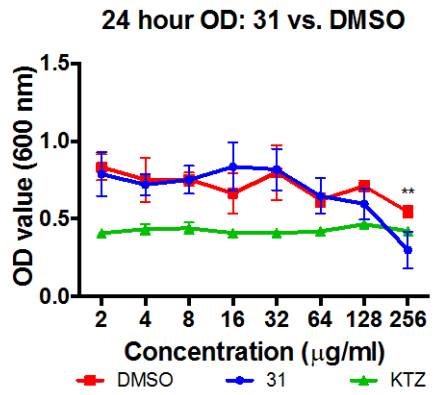
Figure 9. Difference in Optical Density at 0 and 48 Hours. This graph displays the initial optical density (OD) of extracts tested in triplicate at 128 µg/mL as compared to OD read at 48 hours. KTZ control tested at 16 µg/mL. Extract 31 and 449 had a statistically significant decreased in OD ($p= 0.0008$; $p= 0.04$), while all the other extracts did not have a significant change in OD from 0 hour read, signifying fungistatic activity. KTZ control had a statistically significant decrease in OD ($p= 0.006$). All extracts were further tested via serial dilution to determine lowest concentration (MIC) that displays fungistatic or fungicidal activity.

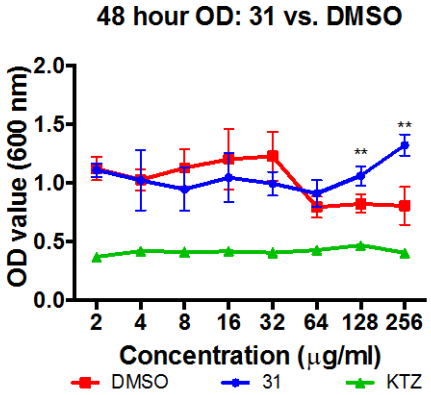
4.2 Antifungal Activities—MIC Results

Crude extracts 000031, 000055, 000066, 000637, 001251, and pure compounds 000448, 000449, and 000451were further tested via two-fold serial dilution to determine minimum inhibitory concentration. All extracts were tested in quadruplicate at starting concentration of 256 μg/mL. KTZ, AmB, and DMSO controls were also tested at the same initial starting concentration of 256 μg/mL. Samples from *Allium amethystinum* (extract 1251) were shown to be most active in inhibition of *M. furfur* growth at both 24 and 48 hours. Further, *Allium cepa* (crude extracts 158 and 237) was tested with serial dilution for potential antifungal activity since it is in the same genus as *A. amethystinum*. All extracts were also tested on human keratinocytes and did not show significant cytotoxicity.

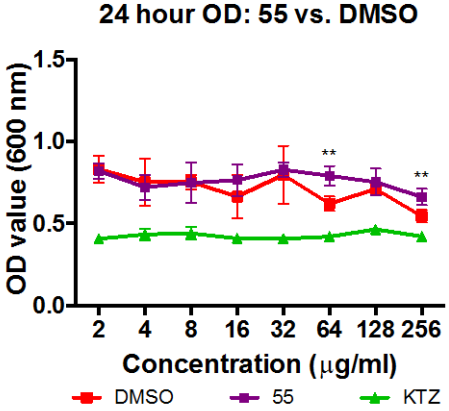
Extract 31 showed significant growth inhibition at 256 μg/mL at the 24-hour read ($p<0.01$), but failed to show any activity at 48 hours as compared to DMSO control. Crude extracts 55, 66, and 637 did not exhibit significant growth inhibition at any concentration for either the 24 or 48-hour timepoint as compared to DMSO control. Extract 1251 displayed significant growth inhibition at 128 and 256 μg/mL at both timepoints. Pure compounds 448 and 449 did not demonstrate growth inhibition at 24 hour, but did have significant antifungal activity at 48 hours: 128 and 256 μg/mL for 448 and 32-256 μg/mL for 449. Pure compound 451 revealed growth inhibition at 16, 64, and 128 μg/mL at 24 hours, but showed more demonstrable inhibition at 48 hours at 16-256 μg/mL as compared to DMSO control. Results are displayed below in figure 10.

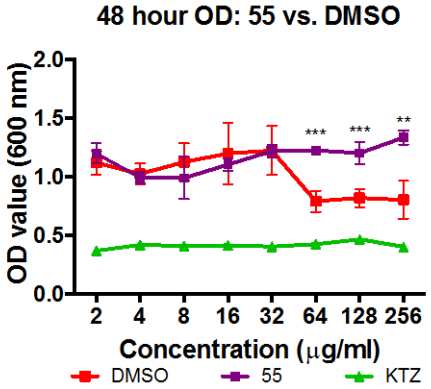
a


24 hour OD: 31 vs. DMSO
OD value (600 nm)
1.5
1.0
0.5
0.0
**
2 4 8 16 32 64 128 256
Concentration (μg/ml)
DMSO
31
KTZ

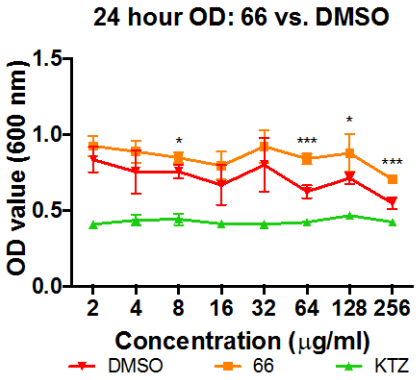

48 hour OD: 31 vs. DMSO
OD value (600 nm)
2.0
1.5
1.0
0.5
0.0
**
**
2 4 8 16 32 64 128 256
Concentration (μg/ml)
DMSO
31
KTZ

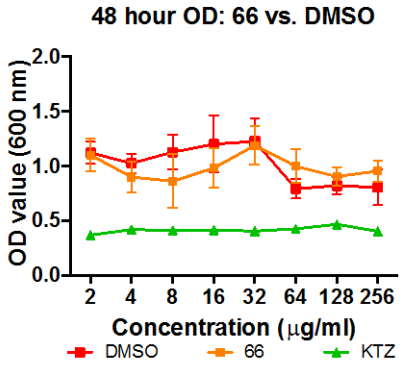
b


24 hour OD: 55 vs. DMSO
OD value (600 nm)
1.5
1.0
0.5
0.0
**
**
2 4 8 16 32 64 128 256
Concentration (μg/ml)
DMSO
55
KTZ

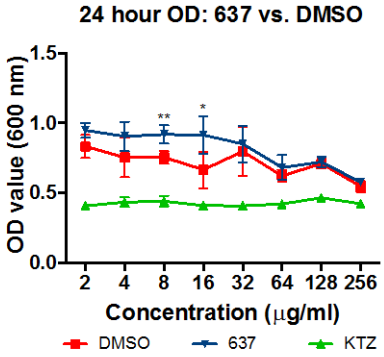

48 hour OD: 55 vs. DMSO
OD value (600 nm)
2.0
1.5
1.0
0.5
0.0
***
***
**
2 4 8 16 32 64 128 256
Concentration (μg/ml)
DMSO
55
KTZ

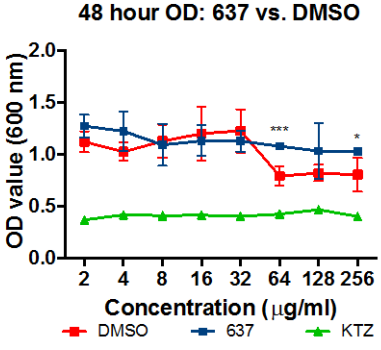
c


24 hour OD: 66 vs. DMSO
OD value (600 nm)
1.5
1.0
0.5
0.0
*
***
*
***
2 4 8 16 32 64 128 256
Concentration (μg/ml)
DMSO
66
KTZ


48 hour OD: 66 vs. DMSO
OD value (600 nm)
2.0
1.5
1.0
0.5
0.0
2 4 8 16 32 64 128 256
Concentration (μg/ml)
DMSO
66
KTZ

d


24 hour OD: 637 vs. DMSO
OD value (600 nm)
1.5
1.0
0.5
0.0
**
*
2 4 8 16 32 64 128 256
Concentration (μg/ml)
DMSO
637
KTZ


48 hour OD: 637 vs. DMSO
OD value (600 nm)
2.0
1.5
1.0
0.5
0.0
***
*
2 4 8 16 32 64 128 256
Concentration (μg/ml)
DMSO
637
KTZ

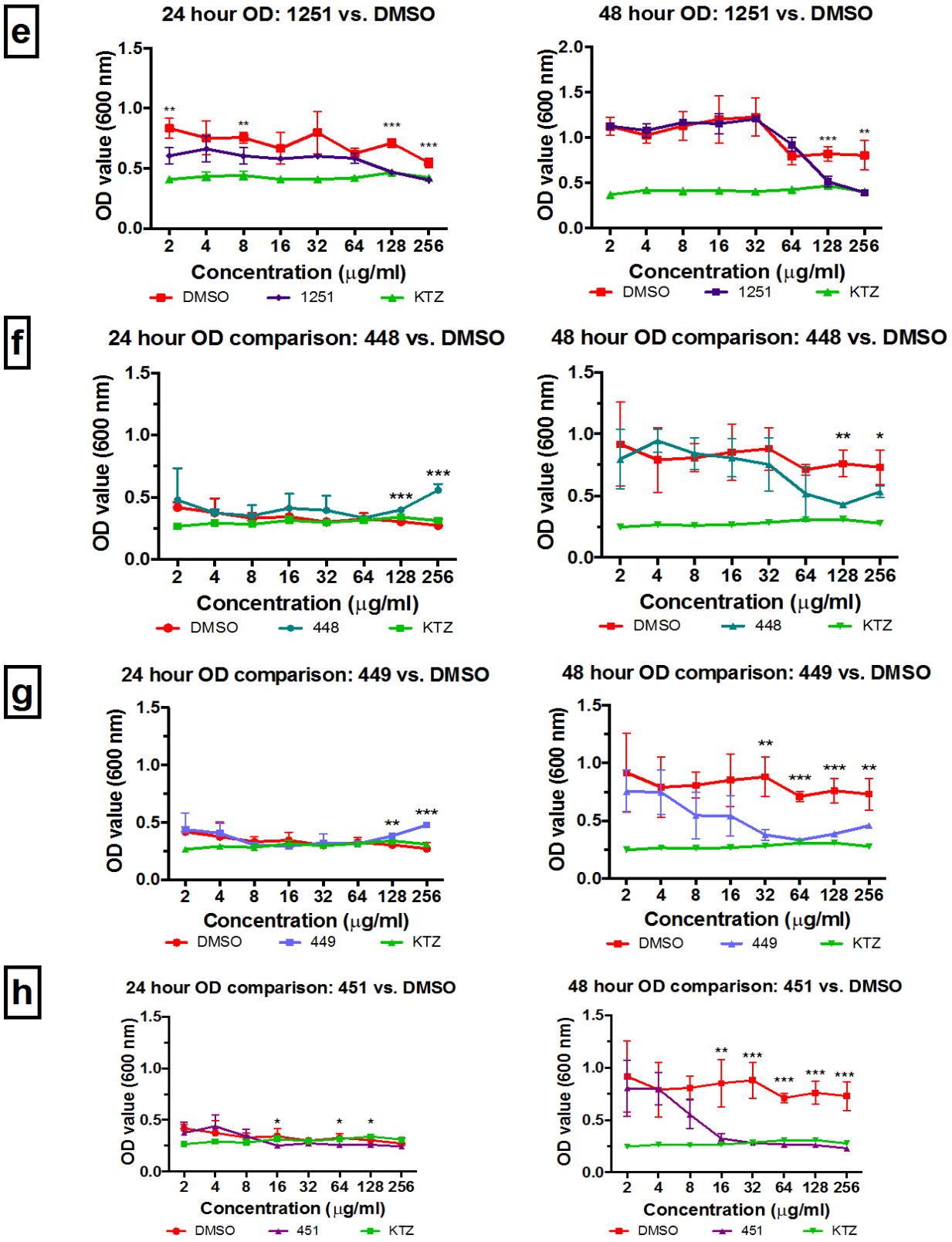


Figure 10. OD Results from Serial Dilution Testing of Botanical Extracts.
*p<0.05, **p<0.01, ***p<0.001.

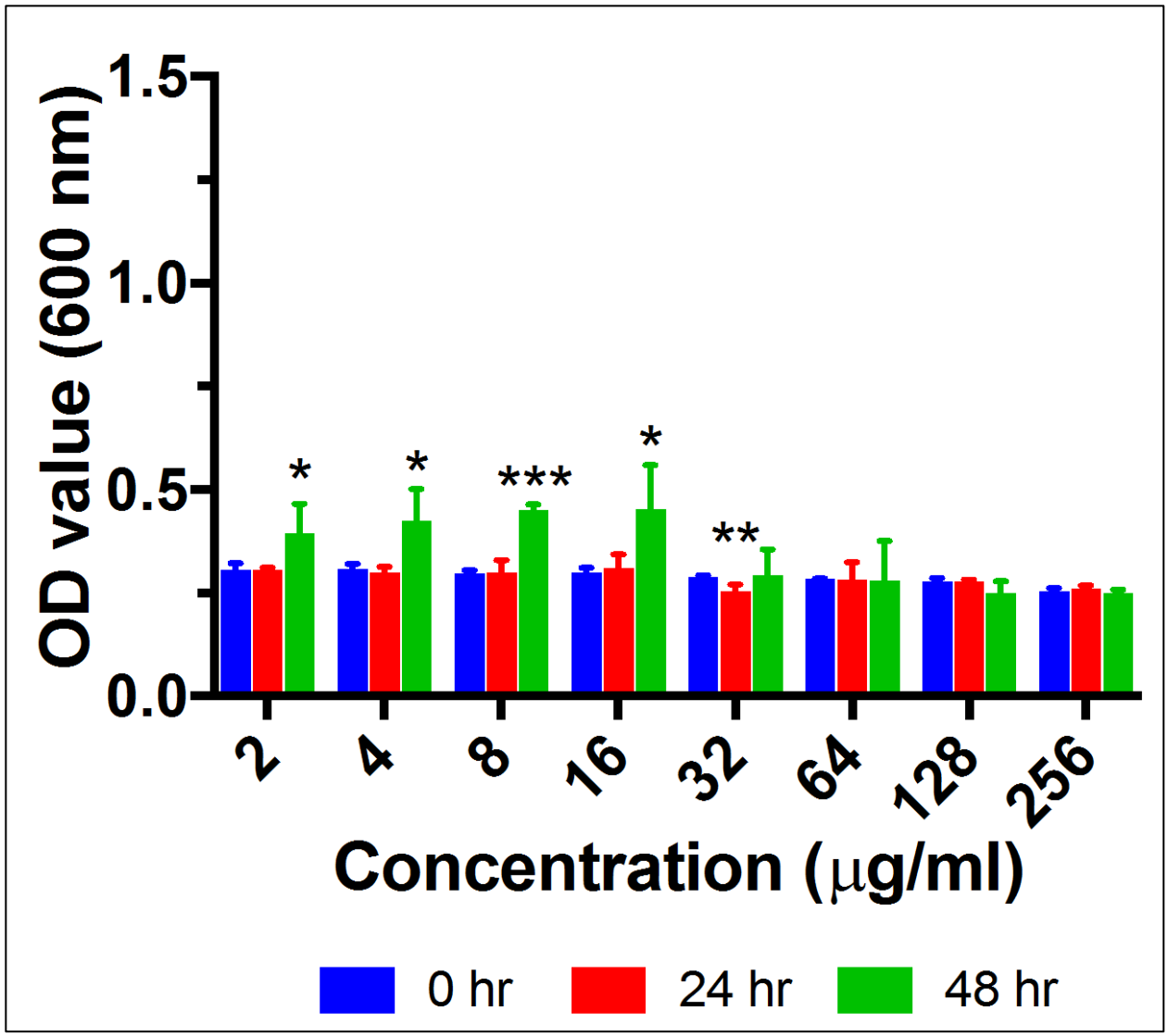


Figure 11. Comparison to 0 hour OD for Extract 1251. No significant difference in OD at all concentrations for 24 hour read, except at 32 μg/mL, which had a significant decrease in OD ($p<0.01$). There was a significant increase in OD for 48 hour read at 2 μg/mL ($p<0.05$), 4 μg/mL ($p<0.05$), 8 μg/mL ($p<0.001$), and 16 μg/mL ($p<0.05$). There was no significant difference in 24 and 48 hour OD at 64, 128, and 256 μg/mL as compared to 0 hour OD results.

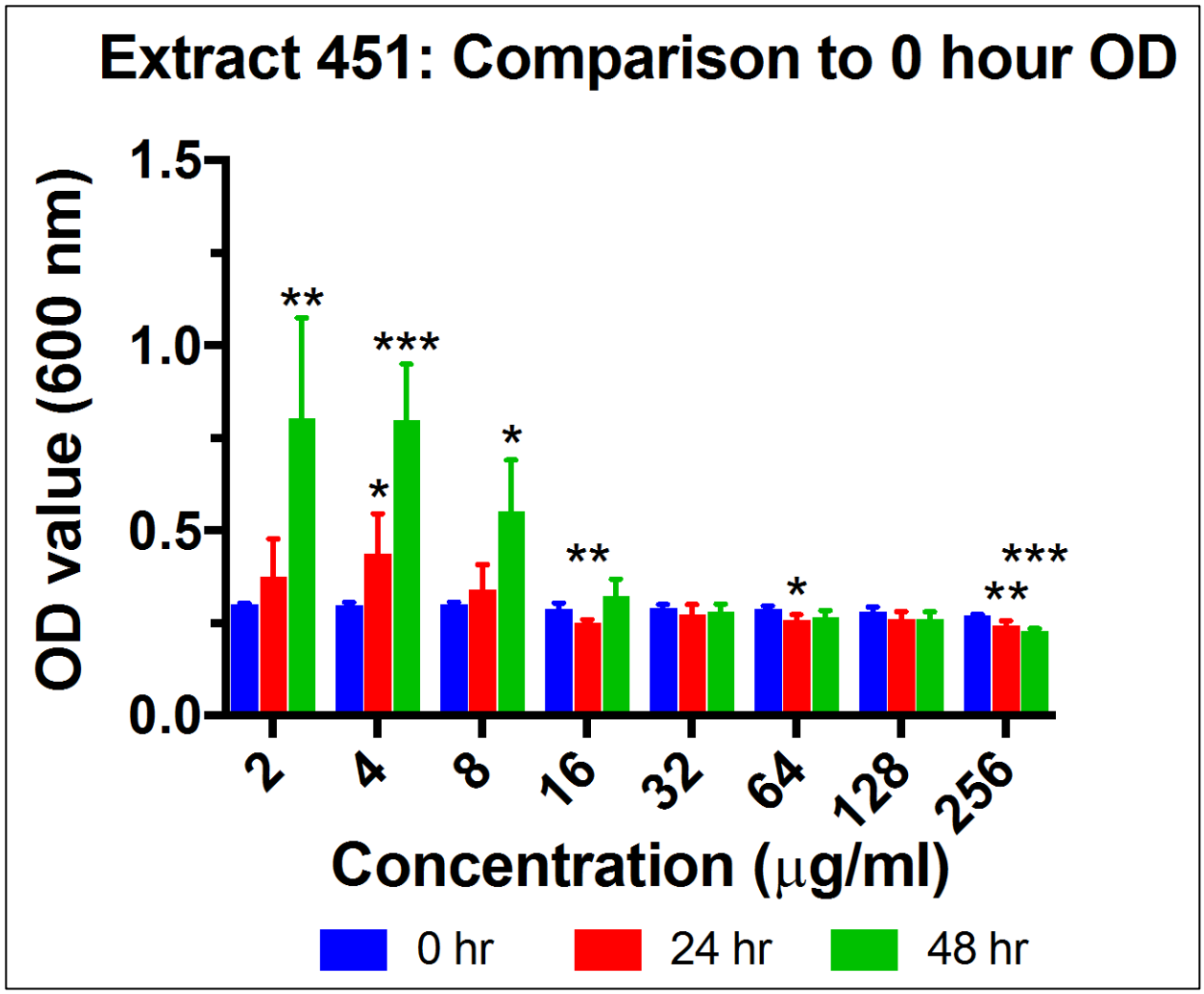


Figure 12. Comparison to 0 hour OD for Pure Compound 451. There was a significant increase in OD for 24 hour read at 4 μg/mL ($p<0.05$). There was a significant increase in OD for 48 hour read at 2 μg/mL ($p<0.01$), 4 μg/mL ($p<0.001$), and 8 μg/mL ($p<0.05$). There was a significant decrease in OD for 24 hour read at 16 μg/mL ($p<0.01$), 64 μg/mL ($p<0.05$), and 256 μg/mL ($p<0.01$). There was a significant decrease in OD for 48 hour read at 256 μg/mL ($p<0.001$). There was no significant change in OD at all other concentrations for both 24 and 48 hour timepoints.

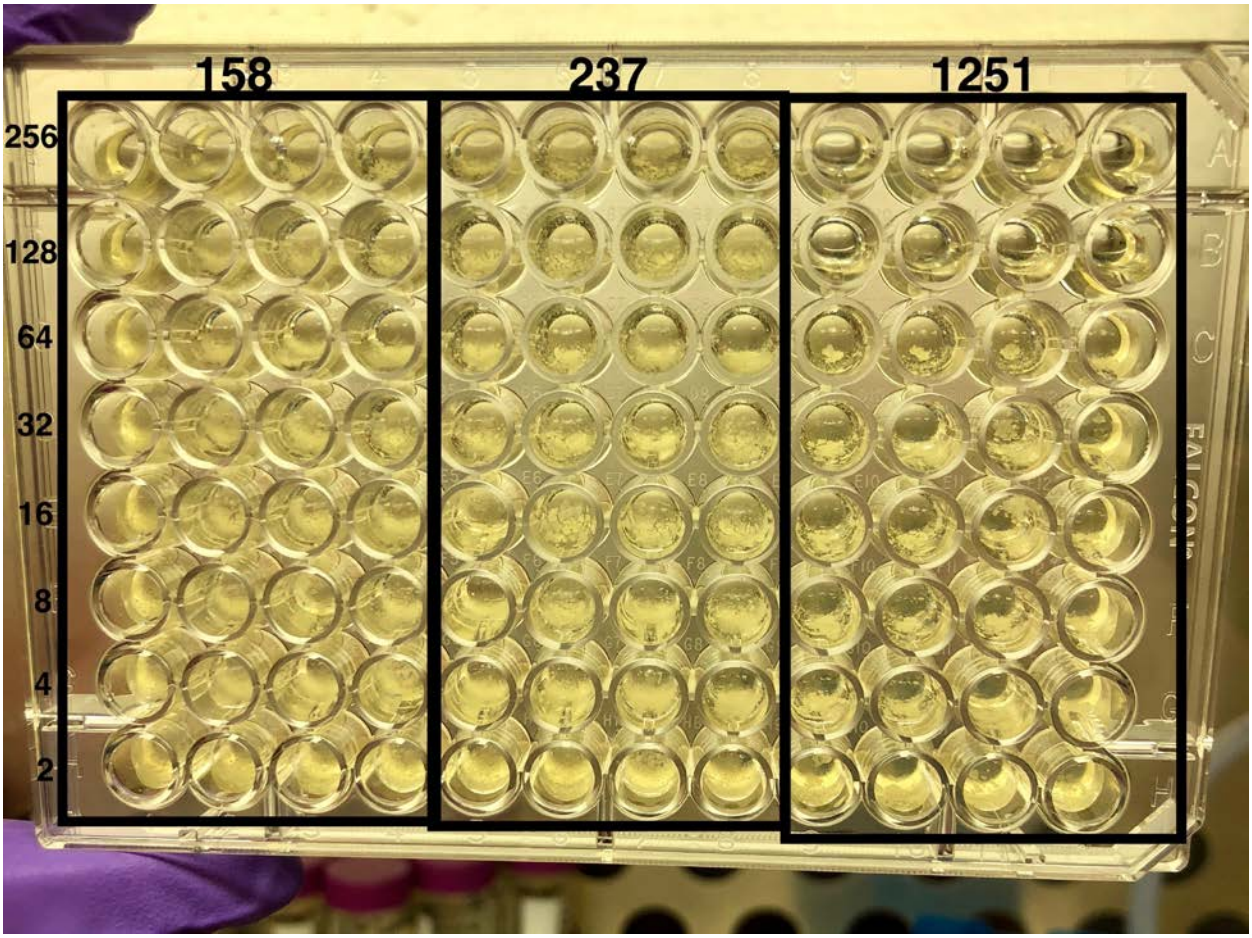


Figure 13. Serial Dilution Results of Botanical Extracts in the *Allium* Genus. Extract 158 is an ethanolic extract of the entire plant and extract 237 is a methanolic extract of all plant parts of *Allium cepa*. Both extracts 158 and 237 showed growth of *M. furfur* at highest concentration tested (256 μg/mL), as evident by clumping in the wells after 48 hours of incubation. Extract 1251 showed growth inhibition at 256 and 128 μg/mL, but had presence of clumps at 64 μg/mL and all lower concentrations.

4.3 Fungicidal Plate Results

After growth inhibition and serial dilution testing, 15 μL of inoculum was plated on LNA plates in triplicate and incubated for 4 days. Crude extracts 31, 55, 66, and 637, were plated at the highest concentration of 256 μg/mL, and extracts 31, 55, and 66 were also plated at 128 μg/mL. Crude extract 1251 was plated at 64, 128, and 256 μg/mL in triplicate. Pure compounds 448 and 449 were not tested due to time constraints and the study's focus on crude extracts. Pure compound 451 was plated at 16-256 μg/mL. Ketoconazole and YPD were also plated as controls. Extracts 31, 55, 66, and 637 did not demonstrate fungicidal activity at concentrations tested (>3 colonies in all zones). Extract 1251 demonstrated a fungicidal concentration at 256 μg/mL (average <3 colonies across replicates) and pure compound 451 exhibited a fungicidal concentration at 256 μg/mL (clear zones). Example pictures of colony growth are displayed below.

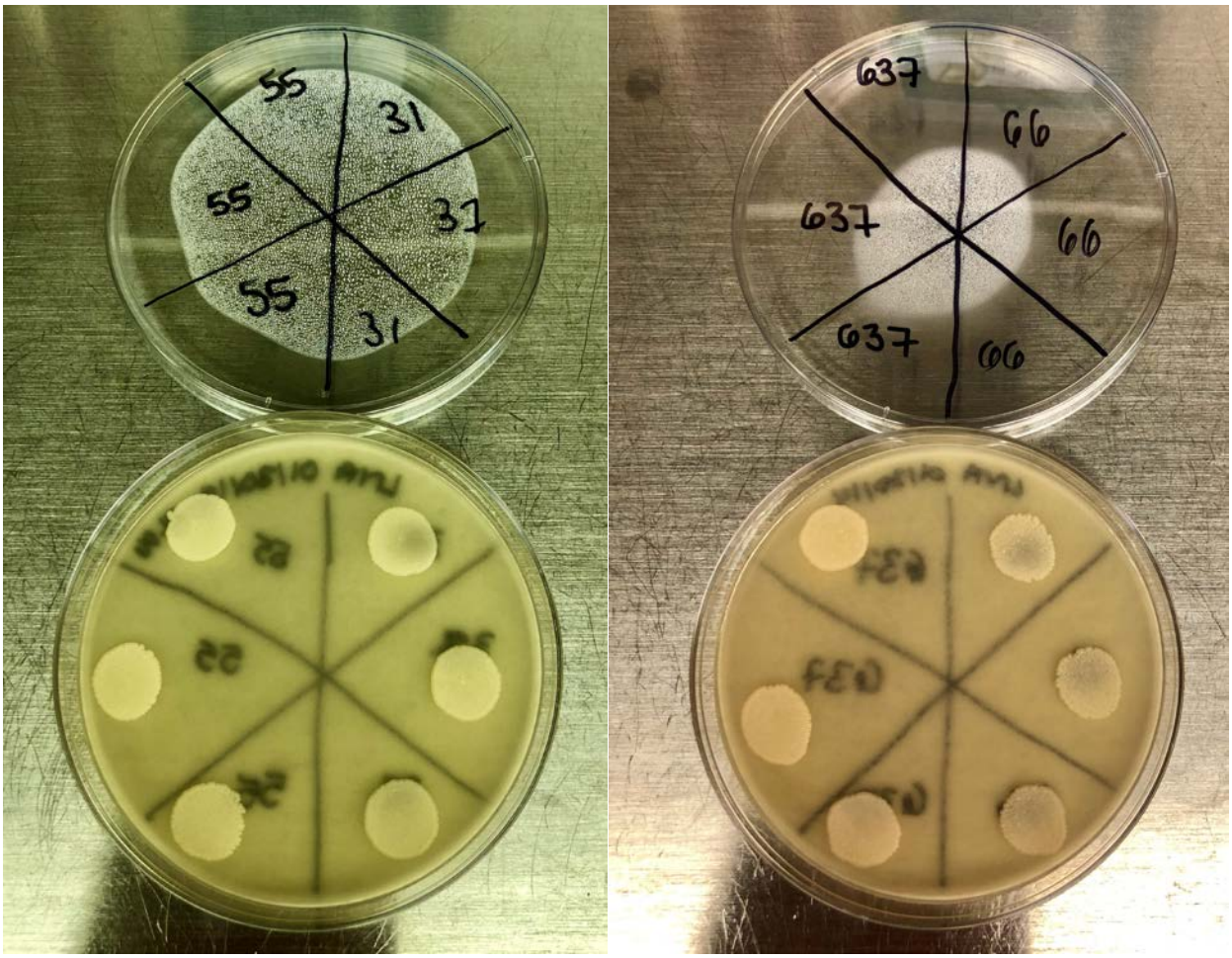


Figure 14. Fungicidal Plate Test for Extracts 31, 55, 66, and 637. The plates were divided into six zones and three separate samples of extract at 256 μg/mL were plated. After 96 hours of incubation, growth was assessed for fungicidal activity. As shown above, all extracts had lawn growth and did not exhibit fungicidal activity.

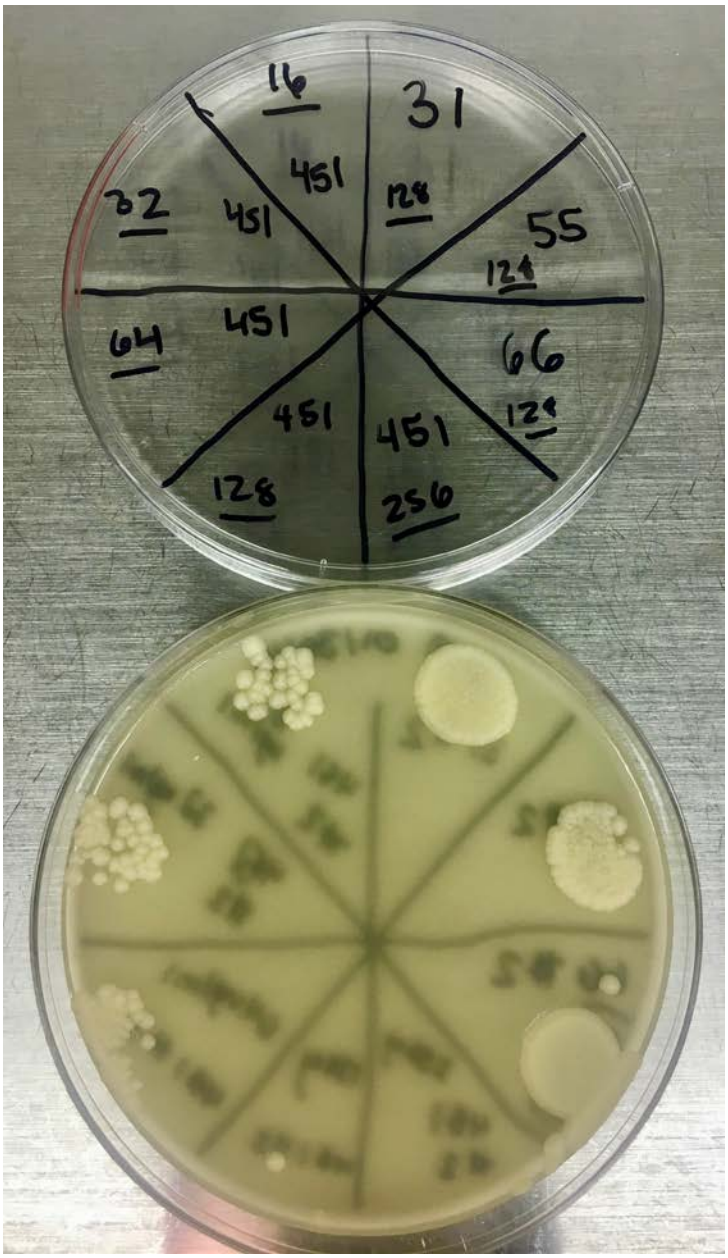


Figure 15. Fungicidal Plate Test for Extracts 31, 55, 66, and 451. The plate was divided into 8 zones and extract 451 was plated at a concentration of 256 μg/mL to 16 μg/mL in triplicate (other replicates not shown). Extracts 31, 55, and 66 were plated at 128 μg/mL. Pure compound 451 exhibited a fungicidal concentration at 256 μg/mL, based on average colony growth across all replicates (not shown here). Crude extracts 31, 55, and 66 had lawn growth suggesting that growth inhibition at 128 μg/mL is fungistatic.

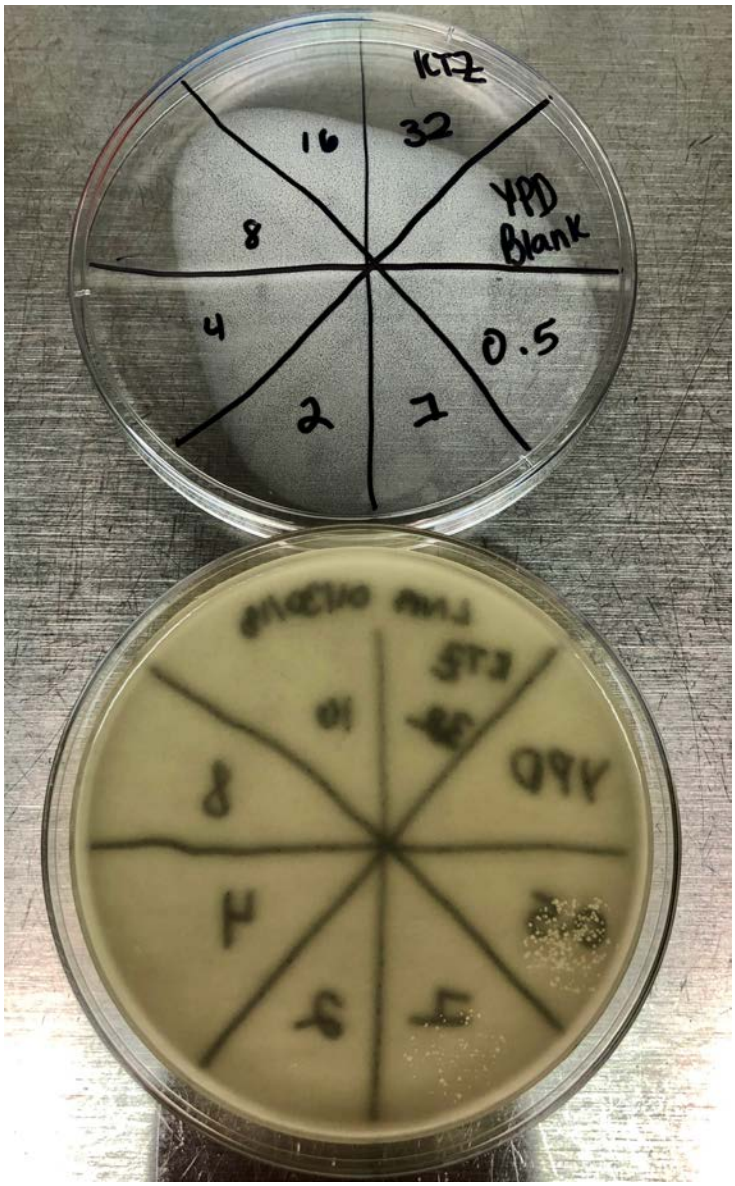


Figure 16. Fungicidal Plate Test for Ketoconazole and YPD control. The plate was divided into 8 zones and ketoconazole was plated at a concentration of 32 μg/mL to 0.5 μg/mL. Ketoconazole demonstrated a minimum fungicidal concentration at 2 μg/mL. As expected, YPD control did not have any colony growth.

4.4 LDH Cytotoxicity Results

Crude extracts 31, 55, 66, 637, and 1251 were tested on human keratinocytes to determine cytotoxicity. None of the tested extracts reached an IC_{50} ($\geq$50% cytotoxicity) at concentrations as high as 512 μg/mL and therefore are not cytotoxic against mammalian skin cells. Extract 31 demonstrated 17%, 43% and 10% cytotoxicity at 512, 256 and 128 μg/mL, respectively. Extract 55 demonstrated 21% and 5% cytotoxicity at 512 and 256 μg/mL, respectively. Extract 66 demonstrated 16% cytotoxicity at 512 μg/mL. Extract 637 demonstrated 11% cytotoxicity at 512 μg/mL. All other concentrations tested did not exhibit significant cytotoxicity.

Allium amethystinum (extract 1251) did not demonstrate IC_{50} at any test concentration (4 to 512 μg/mL). Cytotoxicity was 32% and 38% at 128 and 256 μg/mL, respectively. At 512 μg/mL, twice the fungicidal concentration, cytotoxicity was 19%. This data provides promising evidence for development of *A. amethystinum* as a topical skin treatment.

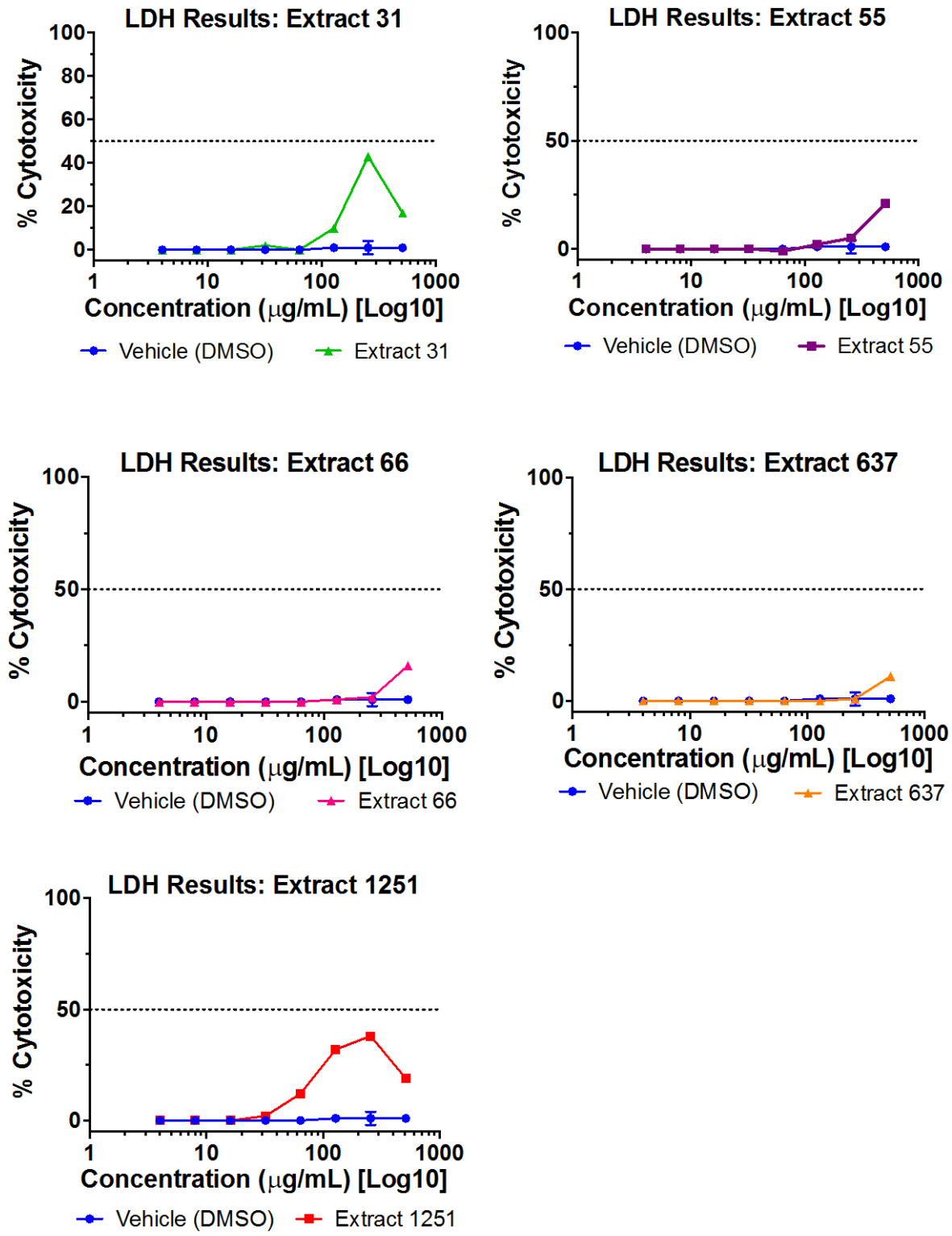


Figure 17. Results of HaCaT Testing with Crude Extracts.

Chapter 5 Discussion

Implications of Results

This chapter discusses the implications of results for the botanical extracts tested against *M. furfur*. Extract 1251 is recommended for future study on the basis of its antifungal activity. *Allium amethystinum* (Amaryllidaceae) displayed inhibitory and fungicidal activity against *M. furfur* which perpetuates symptoms of chronic skin disorders, substantiating its use as a topical antifungal treatment. This botanical extract may be useful in modern medicine as antifungal resistance of current treatments continues to increase. Characteristics of the botanical extracts that exhibited growth inhibition from QNPL screen are displayed below in Table 4. Minimum inhibitory concentrations, MIC_{50} and $MIC_{90,}$ determined from serial dilution and MFC determined from fungicidal plate testing are reported in Table 5.

After further serial dilution, it was determined that crude extracts 31, 55, 66, and 637 are not active at concentrations tested. Crude extract 1251 is the most active and showed fungicidal activity at 256 μg/mL and 90% growth inhibition at 128 μg/mL. Pure compound 451 is also biologically active with a fungicidal concentration of 256 μg/mL and 90% growth inhibition at 16 μg/mL. The current study, however, is more focused on finding biological activity of crude extracts since bioassay-guided fractionation may identify novel active compounds that exhibit MICs equal or superior to current antifungals. Further, isolated active compounds can also be tested with current antifungals for synergistic activity and decrease risk of resistance by using combination therapy.

Table 4. Characteristics of Botanical Extracts with Effective Growth Inhibition

Extract ID	Scientific Name	Common Name	Parts Extracted	Extraction Solvent	Inhibition (%) ± SD at 24 hr	Inhibition (%) ± SD at 48 hr
000031	*Erodium malacoides* (L.) L'Hér. (Geraniaceae)	Mediterranean Stork's Bill	Leaves, Stems, Flowers	Ethanol	161.92 ± 11.68	166.96 ± 10.46
000055	*Verbascum thapsus* L. (Scrophulariaceae)	Common Mullein	Inflorescence	Ethanol	139.69 ± 17.81	114.49 ± 12.38
000066	*Vicia faba* L. (Fabaceae)	Fava Bean	Whole plant	Ethanol	106.44 ± 29.22	116.67± 28.96
000448	-	-	Pure Compound	Pure Compound	216.15 ± 112.79	110.76 ± 9.68
000449	-	-	Pure Compound	Pure Compound	101.31 ± 6.58	107.51 ± 4.72
000451	-	-	Pure Compound	Pure Compound	107.85 ± 11.13	103.90 ± 4.24
000637	*Liquidambar styraciflua L.* (Hamamelidaceae)	Sweet gum	Fruits, Seeds	Methanol	117.42 ± 35.32	101.61 ± 13.10
001251	*Allium amethystinum* (Amaryllidaceae)	Red Mohican	Inflorescence	95% Ethanol	116.44 ± 9.06	121.65 ± 5.30

SD – serial dilution. Growth inhibition (%) reported from QNPL initial screen data as compared to DMSO control.

Table 5. MIC Results of Extracts from Serial Dilution Testing

Botanical Extract	MIC_{50}	MIC_{90}	MFC
31	128	>256	>256
55	128	>256	>256
66	128	>256	>256
448	32	128	NT
449	16	64	NT
451	8	16	256
637	128	>256	>256
1251	<32	128	256
KTZ (control)	<2	2	2
AmB (control)	2	16	NT

MIC values determined at 48-hour growth inhibition as compared to DMSO control. MFC values determined via fungicidal plate test after 96 hours of incubation. Values are expressed as concentration (μg/mL). NT = not tested.

Characteristics of *Allium* Genus

Allium is one of the largest genera including more than 800 species (Sobolewska et al., 2016). The genus *Allium* is characterized by the presence of organosulfur compounds, which contributes to the plant's distinctive aroma and potent biological activity (Block, 1992). The botanical name *Allium* derives from Celtic and means pungent (Block, 1992).

Plants of the genus, such as *Allium cepa* (onion) and *Allium sativum* (garlic) have been used for thousands of years in folk medicine (Block, 1992). *Allium sativum* and *Allium cepa*, which originated in central Asia, are among some of the oldest plants to be cultivated, pre-dating written history (Block, 1985). The Ebers Papyrus is an Egyptian medical scroll that dates back to around 1600 BCE (Block, 1985). It contains more than 800 therapeutic formulas and mentions garlic in 22 formulas as an effective remedy for heart problems, headache, bites, worms, and tumors (Block, 1985). Garlic and onions were so widely used in Egypt that tombs were engraved with their images to ensure pharaohs' meals were well-seasoned in the afterlife (Block, 1985). Garlic and onion were also recommended by renowned Greek physician Hippocrates and Roman

naturalist Pliny the Elder for numerous therapeutic uses (Block, 1985). Dioscorides was an ancient Greek physician for the Roman Army and author of *De Materia Medica*, a 5-volume encyclopedia about herbal medicine and related substances (Block, 1985). Dioscorides prescribed garlic as a purgative for helminthic infections (Block, 1985). In India, garlic was included in lotions as an antiseptic for wounds and ulcers (Block, 1985). In China, onion tea was concocted for fever, headache, cholera, and dysentery (Block, 1985). Overall, garlic and onion were widely utilized for their medicinal properties throughout various parts of the world.

Today, extracts of garlic and onion have been found to be antibacterial (Hughes and Lawson, 1991), antifungal (Shams-Ghahfarokhi et al., 2006), anthelminthic (Ayaz et al., 2008), anti-cancer/antitumor (Nicastro et al., 2015), antioxidant (Lazarevic et al., 2011), antihypertensive (Lazarevic et al., 2011), and antithrombotic (Block, 1985). Non-volatile flavor precursors, alk(en)yl-L-cysteine sulfoxides are converted into alk(en)yl thiosulfinates by the enzyme alliinase via enzymatic hydrolysis when plant tissue is disrupted (Lazarevic et al., 2011). The medicinal properties of fresh garlic and, to a lesser extent, onion are due to allicin, the most prevalent alk(en)yl thiosulfinate compound produced after crushing the bulbs (Block, 1985; Nicastro et al., 2015). Allicin is unstable and further breaks down to ajoene and other sulfide compounds (Nicastro et al., 2015).

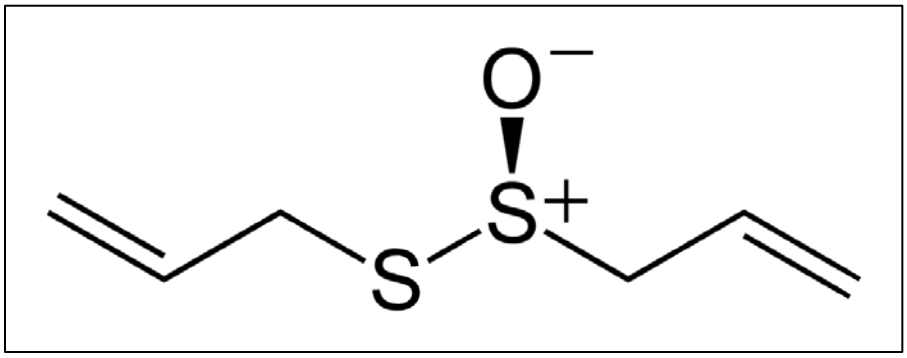


Figure 18. Structure of Allicin. The presence of sulfur in the organic compound is unique and contributes to the both the odor and potent antimicrobial properties of *Allium* genus (Lazarevic et al., 2011). The presence of the allyl group is also characteristic of organosulfur compounds in the *Allium* genus. Image obtained from Wikimedia Commons with permission to reuse.

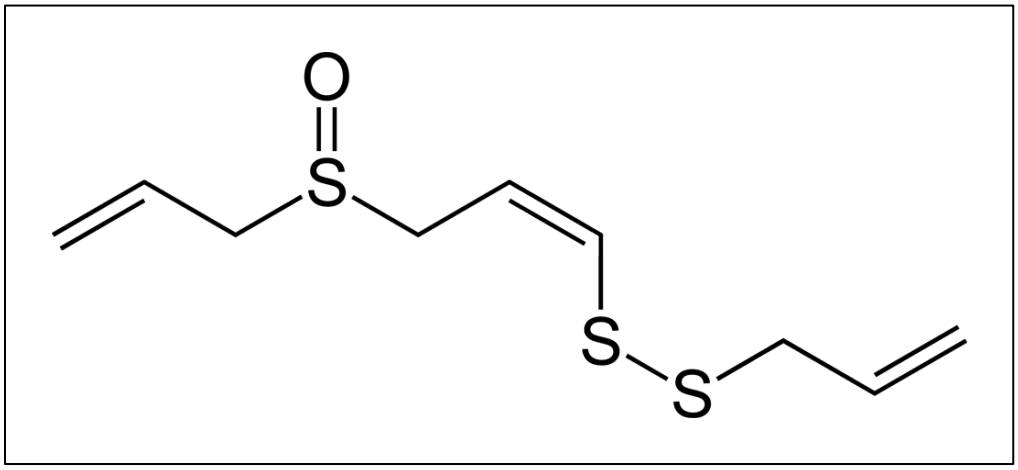


Figure 19. Structure of Cis-Ajoene. Ajoene is formed from the decomposition of Allicin and also contributes to the plant's antimicrobial and antithrombotic properties (Block, 1985). Cis-ajoene is more potent than trans-ajoene (Block, 1985). Again, note the presence of the allyl groups, characteristic of organosulfur compounds found in plants in the *Allium* genus. Image obtained from Wikimedia Commons with permission to reuse.

Other medicinal compounds found in the genus *Allium* include polyphenols, such as flavonoids, fructans, and antioxidative enzymes, as well as sapogenins and steroidal saponins that are more stable to cooking and exhibit antimicrobial properties (Lanzotti, 2012; Sobolewska et al., 2016). Steroidal saponins can be broken into three groups based on structure: spirostanols, furostanols, and open-chain saponins (Sobolewska et al., 2016). Although the type of steroidal saponins present in *Allium amethystinum* have not been elucidated to date, a furostanol saponin was found in the bulbs of *Allium sphaerocephalon*, a closely-related species (Mimaki et al., 1996). It should be noted however, that saponin content is relatively low compared to organosulfur compounds and saponins with a spirostanol skeleton exhibit higher antifungal activity than furostanols (Sobolewska et al., 2016). This data provides evidence that any steroidal saponins present in extract 1251 may not be the main active constituent against *Malassezia furfur*, although more research and chemical analysis is needed.

Characteristics of *Allium amethystinum*

Allium amethystinum (Amaryllidaceae) is a wild-growing, herbaceous, and perennial plant more commonly known as Red Mohican (Kew, 2018). It is found throughout the Mediterranean in Albania, Bulgaria, Greece, and Italy (Kew, 2018), and the specimen tested in this study was collected in Southern Italy during the summer of 2017. While the inflorescence showed antifungal activity, botanical extracts made from different parts of the plant – including the stems, roots, and bulbs – did not exhibit any activity. The *Allium* genus is well known for its antifungal activity due to the presence of sulfuric compounds in the chemical structures (Shams-Ghahfarokhi et al., 2006). Previous studies observed anti-*Malassezia* activity for both *Allium cepa* (onion) and *Allium sativum* (garlic), likely due to the active compounds allicin and/or

ajoene (Gitanjali et al., 2014; Hughes and Lawson, 1991; Kaur et al., 2016; Shams-Ghahfarokhi et al., 2006). In the present study only extract 1251 in the *Allium* genus exhibited antifungal activity at 128 μg/mL, providing evidence for a unique and novel active compound, or presence of allicin and/or ajoene in high concentrations in the inflorescence as compared to *Allium cepa*. Previous studies have demonstrated that wild-growing plants of the *Allium* genus contain higher amounts of aroma precursors (cysteine sulfoxides) and may be a more potent source of biologically active compounds (Lazarevic et al., 2011).

Allium sphaerocephalon is a close relative to *Allium amethystinum* and exhibits both genotypic and phenotypic similarity (Lazarevic et al., 2011). *A. sphaerocephalon* is an herbaceous, perennial plant found throughout Europe in rocky slopes, sandy ground, vineyards, and shrubby habitats (Lazarevic et al., 2011). The whole plant is used as a condiment and onion substitute, and is reported as being consumed in Ukraine, Italy, Spain, and Siberia (Lazarevic et al., 2011). Folk medicinal remedies include using the sap and plant parts as an insect repellant (Lazarevic et al., 2011).

There also several studies analyzing the phytochemical content of *A. sphaerocephalon* to discover active constituents. One study assessing cysteine sulfoxide content in several species in the genus *Allium* revealed that *A. sphaerocephalon* bulbs contained 42% alliin, the compound that is converted into allicin by alliinase, as compared to approximately 10% alliin content in *Allium sativum* bulbs (Krest et al., 2000). This data supports other research determining that wild-growing plants in the genus *Allium* contain higher organosulfur content and may be more biologically active (Lazarevic et al., 2011). Other research has focused on biological activity of steroidal saponins and elucidated presence of a furostanol saponin with six sugars in the bulbs of *A. sphaerocephalon*, although steroidal saponin content is much lower than organosulfur

compounds (Mimaki et al., 1996). To date, only one study has tested the antimicrobial effects of *A. sphaerocephalon* essential oil extracted from the inflorescence (Lazarevic et al., 2011). Chemical composition of the oil was determined with gas chromatography and mass spectrometry and revealed sulfur compounds (16.5%) and non-sulfur compounds (75.1%) including monterpenoids, hydrocarbons, and sesquiterpenoids such as fatty acid derivatives and elemane, of which Shyobunol was found to be the main oil constituent (15.3%). In terms of antimicrobial activity, *Pseudomonas aeruginosa* (MBC 2.5 mg/mL) and *Aspergillus niger* (MFC 0.63 mg/mL) were the most susceptible. Although a different species, this data provides promising evidence that *A. amethystinum* may contain similar active compounds in the inflorescence that contribute to its anti-*Malassezia* activity.

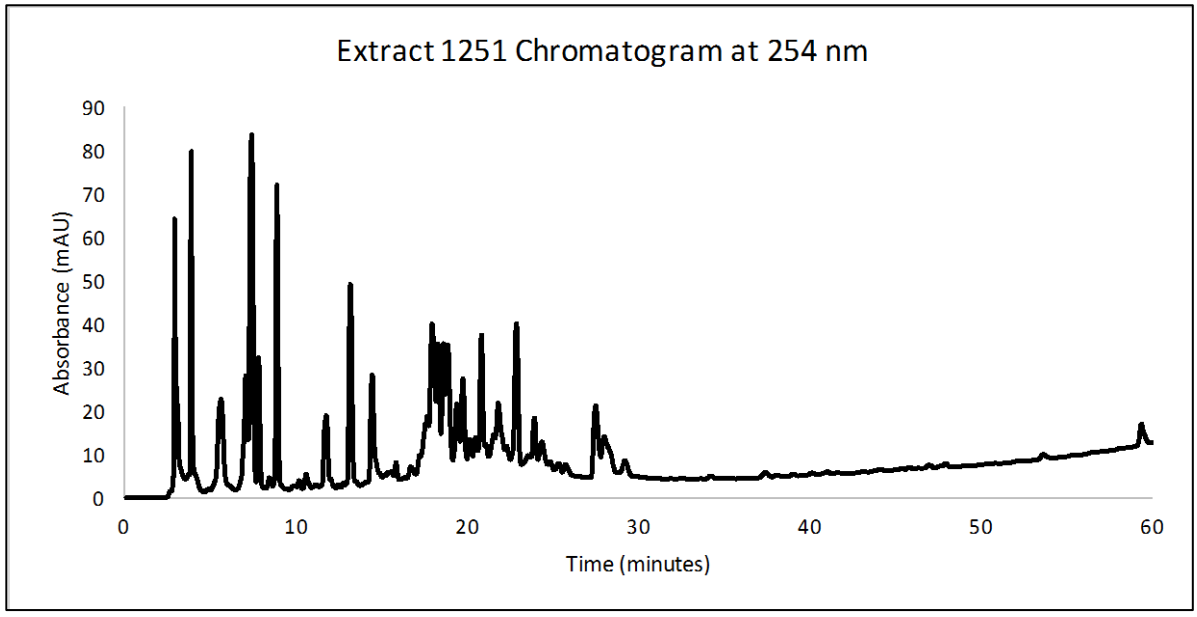


Figure 20. HPLC Results of *Allium amethystinum* at 254 nm. Quantification of extract 1251 with high performance liquid chromatography. Most compounds eluted early in the analysis as designated by high absorbance before 30 minutes. This data signifies considerable presence of hydrophilic compounds such as glycosides and slightly polar compounds. The increase in absorbance around 60 minutes indicates presence of highly nonpolar compounds such as waxes and oils with hydrocarbon tails, however these compounds are not as prevalent in crude extract 1251.

Figure 21. Image of *Allium amethystinum* (Red Mohican). Note the rich red-purple color of the inflorescence, the plant part that showed Anti-*Malassezia* activity in the present study. Image obtained with permission from Luc Coakaerts on Flickr.com.

Figure 22. Image of *Allium sphaerocephalon* (Round-headed Leek). Striking phenotypic similarity to *Allium amethystinum*. *Allium sphaerocephalon* is also found throughout Europe and Northern Africa (Kew, 2018). Image obtained with permission to reuse from Wikimedia Commons.

Limitations

There are a few limitations to this study. First, we chose to use YPD broth microdilution method, but a different culture method (such as RPMI) may have resulted in varied effectiveness of extracts that demonstrated antifungal activity in the present study. Moving forward, it is pertinent to develop a standardized CLSI methodology for *Malassezia* testing to ensure accurate and reproducible therapeutic breakpoints for both standard antifungal treatment and botanical extract testing.

Secondly, *Malassezia furfur* was the only species tested in the present study, although other species such as *M. globosa*, *M. restricta*, and *M. sympodialis* seem to also be implicated in several chronic skin disorders. *M. furfur* was chosen for the initial QNPL screen because it has robust and fast growth on LNA plates, only taking 3 days as opposed to 7 days with *M. globosa* and *M. restricta*. Further, *M. furfur* cultured in YPD broth only needs to be standardized to an absorbance value of 0.430 as compared to 1.0 for *M. globosa* and *M. restricta* requiring less time and also a lower quantitative cell count. As specified earlier, high cell counts required for assay susceptibility testing with *Malassezia* spp. can result in clumping and skew OD results.

Lastly, in previous in vitro studies testing *M. furfur*, *M. globosa*, *M. restricta*, and *M. sympodialis*, an extract that exhibited antifungal activity against *M. furfur* typically had the same MIC for the other *Malassezia* species or a lower MIC, demonstrating similar mechanisms of activity across species (Brodin et al., 2007; Mariappan et al., 2013; Mishra et al., 2016; Onlom et al., 2014). This also provides promising evidence that future studies with extract 1251 against *M. globosa* and *M. restricta* should also produce similar MIC values as seen with *M. furfur* in the current study.

Future Directions

The present study only conducted growth inhibition experiments against *M. furfur*; several studies, however, have demonstrated evidence of *Malassezia* species producing a biofilm (Angiolella et al., 2018; Figueredo et al., 2013; Iatta et al., 2014; Sardi et al., 2014; Simonetti et al., 2015). As mentioned earlier, antifungal resistance is still possible with botanical extracts if an insufficient concentration is used or dosing length is incorrect. Future research assessing biofilm inhibition with botanical extracts is warranted. Antibiofilm extracts could prevent virulence factors of *Malassezia* spp. thereby decreasing allergen and inflammatory responses that worsen symptoms in chronic skin disorders, without increasing risk for resistance.

Future research should also be directed towards chemical analysis of extract 1251 to isolate and determine the structure of the active compound. The active compound should be tested against *M. furfur*, *M. globosa*, and *M. restricta* to establish MICs and conclude if antifungal activity is observed at a lower concentration than current treatments available (Ketoconazole).

Conclusions

Overall, out of the entire QNPL screen of almost 1000 extracts, five extracts (31, 55, 66, 637, 1251) and three pure compounds (448, 449, 451) exhibited antifungal activity against *M. furfur*. Of the pure compounds, 451 demonstrated the best growth inhibition with a fungicidal concentration of 256 μg/mL. Of the extracts that were further tested with serial dilution, extract 1251 demonstrated the most potent activity with a fungicidal concentration of 256 μg/mL. The fact that a crude extract demonstrated fungicidal activity at such a low concentration provides promising evidence that the isolated active compound would provide robust antifungal activity at a much lower concentration. To our knowledge, this is the first study reporting antifungal

activity of *Allium amethystinum*. The next major step towards applying these results involves *in vivo* testing with the isolated active compound. As antifungal resistance is a growing concern for *Malassezia* species, it is increasingly important to consider all possible sources of new treatments.

References Cited

(2011). XTT Cell Proliferation Assay Kit Instruction Manual. In ATCC (ATCC), pp. 1-6.

(2017a). Atopic dermatitis (eczema) Causes - Mayo Clinic (Mayo Clinic: Mayo Clinic).

(2017b). Bragg Organic Apple Cider Vinegar (Bragg: Bragg).

(2017c). Types of Eczema (National Eczema: National Eczema), pp. An Overview of the Different Types of Eczema.

Abbasi, S., Kamalinejad, M., Babaie, D., Shams, S., Sadr, Z., Gheysari, M., Askari, V.R., and Rakhshandeh, H. (2017). A new topical treatment of atopic dermatitis in pediatric patients based on Ficus carica L. (Fig): A randomized, placebo-controlled clinical trial. Complementary Therapies in Medicine *35*, 85-91.

Akaza, N., Akamatsu, H., Takeoka, S., Mizutani, H., Nakata, S., and Matsunaga, K. (2012). Increased hydrophobicity in Malassezia species correlates with increased proinflammatory cytokine expression in human keratinocytes. Medical Mycology *50*, 802-810.

Al-Sweih, N., Ahmad, S., Joseph, L., Khan, S., and Khan, Z. (2014). Malassezia pachydermatis fungemia in a preterm neonate resistant to fluconazole and flucytosine. Medical Mycology Case Reports *5*, 9-11.

Al-Waili, N.S. (2003). Topical application of natural honey, beeswax and olive oil mixture for atopic dermatitis or psoriasis: partially controlled, single-blinded study. Complementary Therapies in Medicine *11*, 226-234.

Angiolella, L., Leone, C., Rojas, F., Mussin, J., de Los Angeles Sosa, M., and Giusiano, G. (2018). Biofilm, adherence, and hydrophobicity as virulence factors in Malassezia furfur. Medical Mycology *56*, 110-116.

Arellano, F.M., Wentworth, C.E., Arana, A., Fernandez, C., and Paul, C.F. (2007). Risk of lymphoma following exposure to calcineurin inhibitors and topical steroids in patients with atopic dermatitis. Journal of Investigative Dermatology *127*, 808-816.

Arumugam, A., and Dhailappan, A. (2012). Fatty acid composition and antidermatophytic and antidiarrheal activity of Nelumbo nucifera seed oil. International Journal of Pharmacy and Pharmaceutical Sciences *4*, 769-775.

Ashcroft, D.M., Dimmock, P., Garside, R., Stein, K., and Williams, H.C. (2005). Efficacy and tolerability of topical pimecrolimus and tacrolimus in the treatment of atopic dermatitis: meta-analysis of randomised controlled trials. BMJ *330*, 1-9.

Ayaz, E., Turel, I., Gul, A., and Yilmaz, O. (2008). Evaluation of the anthelmentic activity of garlic (Allium sativum) in mice naturally infected with Aspiculuris tetraptera. Recent Patents on Anti-infective Drug Discovery *3*, 149-152.

Azwanida, N. (2015). A Review on the Extraction Methods Use in Medicinal Plants, Principle, Strength and Limitation. Medicinal & Aromatic Plants *4*, 1-6.

Back, O., Scheynius, A., and Johansson, S.G. (1995). Ketoconazole in atopic dermatitis: therapeutic response is correlated with decrease in serum IgE. Archives of Dermatological Research *287*, 448-451.

Balakrishnan, K.P., Narayanaswamy, N., Mathews, S., and Gurung, K. (2011). Evaluation Of Some Medicinal Plants For Their Dandruff Control Properties. International Journal of Pharma and Bio Sciences *2*, 38-45.

Balkis, M.M., Leidich, S.D., Mukherjee, P.K., and Ghannoum, M.A. (2012). Mechanisms of Fungal Resistance. Drugs *62*, 1025-1040.

Baroni, A., Orlando, M., Donnarumma, G., Farro, P., Iovene, M.R., Tufano, M.A., and Buommino, E. (2006). Toll-like receptor 2 (TLR2) mediates intracellular signalling in human keratinocytes in response to Malassezia furfur. Archives of Dermatological Research *297*, 280-288.

Bassett, R., Burton, G., Robson, D., and Hepworth, G. (2004). Efficacy of an acetic acid/boric acid ear cleaning solution for treatment and prophylaxis of Malassezia sp. otitis externa. Australian Veterinary Practitioner *34*, 79-82.

Bernard, P., and Pesando, D. (1989). Antibacterial and Antifungal Activity of Extracts from the Rhizomes of the Mediterranean Seagrass Posidonia oceanica (L.) Delile. Botanica Marina *32*, 85-88.

Biabiany, M., Roumy, V., Hennebelle, T., François, N., Sendid, B., Pottier, M., Aliouat, E.M., Rouaud, I., Lohézic-Le Dévéhat, F., Joseph, H., *et al.* (2012). Antifungal Activity of 10 Guadeloupean Plants. Phytotherapy Research *27*, 1640-1645.

Block, E. (1985). The Chemistry of Garlic and Onions. Scientific American *252*, 114-119.

Block, E. (1992). The Organosulfur Chemistry of the Genus Allium – Implications for the Organic Chemistry of Sulfur. Angewandte Chemie International Edition *31*, 1135-1178.

Borda, L.J., and Wikramanayake, T.C. (2015). Seborrheic Dermatitis and Dandruff: A Comprehensive Review. Journal of Clinical and Investigative Dermatology *3*, 1-22.

Borelli, D., Jacobs, P.H., and Nall, L. (1991). Tinea versicolor: epidemiologic, clinical, and therapeutic aspects. Journal of the American Academy of Dermatology *25*, 300-305.

Bourgaud, F., Gravot, A., Milesi, S., and Gontier, E. (2001). Production of plant secondary metabolites: a historical perspective. Plant Science *161*, 839-851.

Brodin, K., Alahyar, H., Hedner, T., Sterner, O., and Faergemann, J. (2007). In vitro activity of artemisia abrotanum extracts against Malassezia Spp., Candida albicans and Staphylococcus aureus. Acta Dermato-Venereologica *87*, 540-542.

Brunke, S., and Hube, B. (2006). MfLIP1, a gene encoding an extracellular lipase of the lipid-dependent fungus Malassezia furfur. Microbiology *152*, 547-554.

Carmo, E.S., Pereira Fde, O., Cavalcante, N.M., Gayoso, C.W., and Lima Ede, O. (2013). Treatment of pityriasis versicolor with topical application of essential oil of Cymbopogon citratus (DC) Stapf - therapeutic pilot study*. Anais Brasileiros de Dermatologia *88*, 381-385.

Cheung, Y., Lee, S., Hui, M., and Luk, T. (2014). Effect of pH on fungal growth: problems with using vinegar (5% acetic acid) in treating superficial fungal infections. Hong Kong Journal of Dermatology and Venereology *22*, 57-64.

Choi, J., Kim, W., Park, J., and Cheong, H. (2016). The Beneficial Effects of Extract of Pinus densiflora Needles on Skin Health. Microbiology and Biotechnology Letters *44*, 208-217.

Damodaran, S., and Venkataraman, S. (1994). A study on the therapeutic efficacy of Cassia alata, Linn. leaf extract against Pityriasis versicolor. Journal of Ethnopharmacology *42*, 19-23.

Dessinioti, C., and Katsambas, A. (2013). Seborrheic dermatitis: Etiology, risk factors, and treatments: Facts and controversies. Clinics in Dermatology *31*, 343-351.

Dhanalakshmi, P., Jaya Prakash Priya, A., Sagadevan, E., Sudha Lakshmi, Y., Manimaran, A., Sindu, S., and Arumugam, P. (2013). Evaluation of inhibitory effect of Vernonia cinerea L. leaf extracts on different fungal species (PDF Download Available). International Journal of Pharmacy and Pharmaceutical Sciences *5*, 414-416.

Didehdar, M., Mehbod, A.S.A., Eslamirad, Z., Mosayebi, M., Hajihossein, R., Ghorbanzade, B., and Khazaei, M.R. (2014). Identification of Malassezia Species Isolated from Patients with Pityriasis Versicolor Using PCR-RFLP Method in Markazi Province, Central Iran. Iranian Journal of Public Health *43*, 682-686.

Diehl, C., and Ferrari, A. (2013). Efficacy of topical 4% Quassia amara gel in facial seborrheic dermatitis:a randomized, double-blind, comparative study. Journal of Drugs in Dermatology *12*, 312-315.

Eberlein-Konig, B., Schafer, T., Huss-Marp, J., Darsow, U., Mohrenschlager, M., Herbert, O., Abeck, D., Kramer, U., Behrendt, H., and Ring, J. (2000). Skin surface pH, stratum corneum hydration, trans-epidermal water loss and skin roughness related to atopic eczema and skin dryness in a population of primary school children. Acta Dermato-Venereologica *80*, 188-191.

Ebiloma, G.U., Igoli, J.O., Katsoulis, E., Donachie, A.M., Eze, A., Gray, A.I., and de Koning, H.P. (2017). Bioassay-guided isolation of active principles from Nigerian medicinal plants identifies new trypanocides with low toxicity and no cross-resistance to diamidines and arsenicals. Journal of Ethnopharmacology *202*, 256-264.

Evangelista, M.T., Abad-Casintahan, F., and Lopez-Villafuerte, L. (2014). The effect of topical virgin coconut oil on SCORAD index, transepidermal water loss, and skin capacitance in mild to moderate pediatric atopic dermatitis: a randomized, double-blind, clinical trial. International Journal of Dermatology *53*, 100-108.

Farnsworth, N.R., Akerele, O., Bingel, A.S., Soejarto, D.D., and Guo, Z. (1985). Medicinal plants in therapy. Bulletin of the World Health Organization *63*, 965-981.

Figueredo, L.A., Cafarchia, C., and Otranto, D. (2013). Antifungal susceptibility of Malassezia pachydermatis biofilm. Medical Mycology *51*, 863-867.

Filip, R., Davicino, R., and Anesini, C. (2010). Antifungal activity of the aqueous extract of Ilex paraguariensis against Malassezia furfur. Phytotherapy Research *24*, 715-719.

Fingleton, J., Helm, C., Tofield, C., Weatherall, M., and Beasley, R. (2014). A randomised controlled trial of topical Kanuka honey for the treatment of eczema. Journal of the Royal Society of Medicine *5*, 1-3.

Galvan, I.J., Mir-Rashed, N., Jessulat, M., Atanya, M., Golshani, A., Durst, T., Petit, P., Amiguet, V.T., Boekhout, T., Summerbell, R., *et al.* (2008). Antifungal and antioxidant activities of the phytomedicine pipsissewa, Chimaphila umbellata. Phytochemistry *69*, 738-746.

Gao, Z., Perez-Perez, G.I., Chen, Y., and Blaser, M.J. (2010). Quantitation of Major Human Cutaneous Bacterial and Fungal Populations▽. Journal of Clinical Microbiology *48*, 3575-3581.

García Rodríguez, L.A., Duque, A., Castellsague, J., Pérez-Gutthann, S., and Stricker, B.H.C. (1999). A cohort study on the risk of acute liver injury among users of ketoconazole and other antifungal drugs. British Journal of Clinical Pharmacology *48*, 847-852.

Ghannoum, M.A., and Rice, L.B. (1999). Antifungal Agents: Mode of Action, Mechanisms of Resistance, and Correlation of These Mechanisms with Bacterial Resistance. Clinical Microbiology Reviews *12*, 501-517.

Gitanjali, D., Prajakta, P., Swati, B., Kiran, E., and Rajendra, B. (2014). Antimalassezia Activity of Medicated Antidandruff Shampoo Formulated with Microwave Dried Garlic Powder with Improved Allicin Stability. The Natural Products Journal *4*, 23-32.

Glatz, M., Bosshard, P.P., Hoetzenecker, W., and Schmid-Grendelmeier, P. (2015). The Role of Malassezia spp. in Atopic Dermatitis. Journal of Clinical Medicine *4*, 1217-1228.

Goh, C.L., Wong, J.S., and Giam, Y.C. (1997). Skin colonization of Staphylococcus aureus in atopic dermatitis patients seen at the National Skin Centre, Singapore. International Journal of Dermatology *36*, 653-657.

Grice, E.A., and Segre, J.A. (2011). The Skin Microbiome. Nature Reviews Microbiology *9*, 244-253.

Gunduz, K., Inanir, I., and Sacar, H. (2005). Efficacy of terbinafine 1% cream on seborrhoeic dermatitis. The Journal of Dermatology *32*, 22-25.

Gupta, A., Bluhm, R., and Summerbell, R. (2002). Pityriasis versicolor. Journal of the European Academy of Dermatology and Venereology *16*, 19-33.

Gupta, A., Kohli, Y., Li, A., Faergemann, J., and Summerbell, R. (2000). In vitro susceptibility of the seven Malassezia species to ketoconazole, voriconazole, itraconazole and terbinafine. British Journal of Dermatology *142*, 758-765.

Gupta, A.K., Batra, R., Bluhm, R., Boekhout, T., and Dawson, T.L. (2004a). Skin diseases associated with Malassezia species. Journal of the American Academy of Dermatology *51*, 785-798.

Gupta, A.K., Kohli, Y., Summerbell, R.C., and Faergemann, J. (2001). Quantitative culture of Malassezia species from different body sites of individuals with or without dermatoses. Medical Mycology *39*, 243-251.

Gupta, A.K., Nicol, K., and Batra, R. (2004b). Role of Antifungal Agents in the Treatment of Seborrheic Dermatitis. American Journal of Clinical Dermatology *5*, 417-422.

Gurib-Fakim, A. (2006). Medicinal plants: traditions of yesterday and drugs of tomorrow. Molecular Aspects of Medicine *27*, 1-93.

Hanifin, J.M., Ling, M.R., Langley, R., Breneman, D., and Rafal, E. (2001). Tacrolimus ointment for the treatment of atopic dermatitis in adult patients: part I, efficacy. Journal of the American Academy of Dermatology *44*, S28-38.

Hanifin, J.M., and Reed, M.L. (2007). A population-based survey of eczema prevalence in the United States. Dermatitis *18*, 82-91.

Harada, K., Saito, M., Sugita, T., and Tsuboi, R. (2015). Malassezia species and their associated skin diseases. The Journal of Dermatology *42*, 250-257.

Hazen, K.C., Mandell, G., Coleman, E., and Wu, G. (2000). Influence of fluconazole at subinhibitory concentrations on cell surface hydrophobicity and phagocytosis of Candida albicans. FEMS Microbiol Lett *183*, 89-94.

Heng, M.C., Henderson, C.L., Barker, D.C., and Haberfelde, G. (1990). Correlation of Pityosporum ovale density with clinical severity of seborrheic dermatitis as assessed by a simplified technique. Journal of the American Academy of Dermatology *23*, 82-86.

Huang, J.T., Abrams, M., Tlougan, B., Rademaker, A., and Paller, A.S. (2009). Treatment of Staphylococcus aureus colonization in atopic dermatitis decreases disease severity. Pediatrics *123*, e808-814.

Hughes, B.G., and Lawson, L.D. (1991). Antimicrobial effects of Allium sativum L. (garlic), Allium ampeloprasum L. (elephant garlic), and Allium cepa L. (onion), garlic compounds and commercial garlic supplement products. Phytotherapy Research *5*, 154-158.

Hui, R.L., Lide, W., Chan, J., Schottinger, J., Yoshinaga, M., and Millares, M. (2009). Association between exposure to topical tacrolimus or pimecrolimus and cancers. Annals of Pharmacotherapy *43*, 1956-1963.

Iatta, R., Figueredo, L.A., Montagna, M.T., Otranto, D., and Cafarchia, C. (2014). In vitro antifungal susceptibility of Malassezia furfur from bloodstream infections. Journal of Medical Microbiology *63*, 1467-1473.

Kaga, M., Sugita, T., Nishikawa, A., Wada, Y., Hiruma, M., and Ikeda, S. (2009). Molecular analysis of the cutaneous Malassezia microbiota from the skin of patients with atopic dermatitis of different severities. Mycoses *54*, e24-e28.

Kaur, S.J., Bhakti, M., and Thankamani, M. (2016). Antimycotic activity of Noni juice against seborrhoeic dermatitis. International Journal of Pharma and Bio Sciences *7*, 403-407.

Kew (2018). World Checklist of Selected Plant Families (WCSP) (Royal Botanic Garden Kew Science).

Kim, H.K., Chang, H.K., Baek, S.Y., Chung, J.O., Rha, C.S., Kim, S.Y., Kim, B.J., and Kim, M.N. (2012). Treatment of Atopic Dermatitis Associated with Malassezia sympodialis by Green Tea Extracts Bath Therapy: A Pilot Study. Mycobiology *40*, 124-128.

Kim, Y.R., Kim, J.H., Shin, H.J., Choe, Y.B., Ahn, K.J., and Lee, Y.W. (2014). Clinical Evaluation of a New-Formula Shampoo for Scalp Seborrheic Dermatitis Containing Extract of Rosa centifolia Petals and Epigallocatechin Gallate: A Randomized, Double-Blind, Controlled Study. Annals of Dermatology *26*, 733-738.

Krest, I., Glodek, J., and Keusgen, M. (2000). Cysteine sulfoxides and alliinase activity of some Allium species. Journal of Agricultural and Food Chemistry *48*, 3753-3760.

Krishnamoorthy, J.R., Ranganathan, S., Shankar, S.G., and Ranjith, M.S. (2006). Dano: A herbal solution for dandruff. African Journal of Biotechnology *5*, 960-962.

Kuhn, D.M., Balkis, M., Chandra, J., Mukherjee, P.K., and Ghannoum, M.A. (2003). Uses and Limitations of the XTT Assay in Studies of Candida Growth and Metabolism. Journal of Clinical Microbiology *41*, 506-508.

Lalitha, S.G., Ramachandrappa, L.T., Krishnamurthy, S., Basavaraju, J., and Thirumale, S. (2016). Insignificant antifungal activity of plant extracts on Malassezia furfur. Journal of Pharmaceutical Negative Results *7*, 16-20.

Lanzotti, V. (2012). Bioactive polar natural compounds from garlic and onions. Phytochemistry Reviews *11*, 179-196.

Lazarevic, J.S., Ethordevic, A.S., Zlatkovic, B.K., Radulovic, N.S., and Palic, R.M. (2011). Chemical composition and antioxidant and antimicrobial activities of essential oil of Allium sphaerocephalon L. subsp. sphaerocephalon (Liliaceae) inflorescences. Journal of the Science of Food and Agriculture *91*, 322-329.

Lee, J., Jung, E., Park, B., Jung, K., Park, J., Kim, K., Kim, K.H., and Park, D. (2005). Evaluation of the anti-inflammatory and atopic dermatitis-mitigating effects of BSASM, a multicompound preparation. Journal of Ethnopharmacology *96*, 211-219.

Lee, J.H., and Lee, J.S. (2010). Chemical Composition and Antifungal Activity of Plant Essential Oils against Malassezia furfur. Korean Journal of Microbiology and Biotechnology *38*, 315-321.

Levin, N.A. (2009). Beyond Spaghetti and Meatballs: Skin Diseases Associated with the Malassezia Yeasts. Dermatology Nursing *21*, 7-13.

Lintu, P., Savolainen, J., Kortekangas-Savolainen, O., and Kalimo, K. (2001). Systemic ketoconazole is an effective treatment of atopic dermatitis with IgE-mediated hypersensitivity to yeasts. Allergy *56*, 512-517.

Loeffler, J., and Stevens, D.A. (2003). Antifungal Drug Resistance. Clinical Infectious Diseases *36*, S31-41.

Lone, A.H., Ahmad, T., Anwar, M., and Sofi, G. (2012). Clinical efficacy and safety of a pharmacopial polyherbal Unani formulation in pityriasis versicolor: a comparative randomized single-blind study. The Journal of Alternative and Complementary Medicine *18*, 978-982.

Lopez-Garcia, B., Lee, P.H., and Gallo, R.L. (2006). Expression and potential function of cathelicidin antimicrobial peptides in dermatophytosis and tinea versicolor. Journal of Antimicrobial Chemotherapy *57*, 877-882.

Mahmoud, Y.A.G., Metwally, M.A., Mubarak, H.H., and Zewawy, N.E. (2015). Treatment of tinea versicolor caused by Malassezia furfur with dill seed extract: An experimental study (PDF Download Available). International Journal of Pharmacy and Pharmaceutical Sciences *7*, 1-7.

Margolis, D.J., Hoffstad, O., and Bilker, W. (2007). Lack of association between exposure to topical calcineurin inhibitors and skin cancer in adults. Dermatology *214*, 289-295.

Mariappan, P.M., Sabesan, G., Koilpillai, B., Janakiraman, S., and Sharma, N.K. (2013). Chemical characterisation and antifungal activity of methanolic extract of Cinnamomum verum J. Presl bark against Malassezia spp. Pharmacognosy Journal *5*, 197-204.

Matousek, J.L., Campbell, K.L., Kakoma, I., Solter, P.F., and Schaeffer, D.J. (2003). Evaluation of the effect of pH on in vitro growth of Malassezia pachydermatis. The Canadian Journal of Veterinary Research *67*, 56-59.

Meshkinpour, A., Sun, J., and Weinstein, G. (2003). An open pilot study using tacrolimus ointment in the treatment of seborrheic dermatitis. Journal of the American Academy of Dermatology *49*, 145-147.

Mills, K.H. (2011). TLR-dependent T cell activation in autoimmunity. Nature Reviews Immunology *11*, 807-822.

Mimaki, Y., Satour, T., Kuroda, M., Kameyama, A., Sashida, Y., Li, H.-y., and Harada, N. (1996). A New Furostanol Saponin with Six Sugars from the Bulbs of Allium sphaerosephalon Structural Elucidation by Modern NMR Techniques. Chemistry Letters *25*, 431-432.

Mishra, R.K., Mishra, V., Pandey, A., Tiwari, A.K., Pandey, H., Sharma, S., Pandey, A.C., and Dikshit, A. (2016). Exploration of anti-Malassezia potential of Nyctanthes arbor-tristis L. and their application to combat the infection caused by Mala s1 a novel allergen. BMC Complement Altern Med *16*, 1-14.

Naik, S., Bouladoux, N., Wilhelm, C., Molloy, M.J., Salcedo, R., Kastenmuller, W., Deming, C., Quinones, M., Koo, L., Conlan, S., *et al.* (2012). Compartmentalized control of skin immunity by resident commensals. Science *337*, 1115-1119.

Nakabayashi, A., Sei, Y., and Guillot, J. (2000). Identification of Malassezia species isolated from patients with seborrhoeic dermatitis, atopic dermatitis, pityriasis versicolor and normal subjects. Medical Mycology *38*, 337-341.

Nenoff, P., Haustein, U.F., and Brandt, W. (1996). Antifungal activity of the essential oil of Melaleuca alternifolia (tea tree oil) against pathogenic fungi in vitro. Skin Pharmacology *9*, 388-394.

Nguyen, M.T., Hanzelmann, D., Hartner, T., Peschel, A., and Gotz, F. (2015). Skin-Specific Unsaturated Fatty Acids Boost the Staphylococcus aureus Innate Immune Response. Infection and Immunity *84*, 205-215.

Nicastro, H.L., Ross, S.A., and Milner, J.A. (2015). Garlic and onions: Their cancer prevention properties. Cancer Prevention Research *8*, 181-189.

Onlom, C., Khanthawong, S., Waranuch, N., and Ingkaninan, K. (2014). In vitro anti-Malassezia activity and potential use in anti-dandruff formulation of Asparagus racemosus. International Journal of Cosmetic Science *36*, 74-78.

Oyelami, O.A., Onayemi, O., Oladimeji, F.A., Ogundaini, A.O., Olugbade, T.A., and Onawunmi, G.O. (2003). Clinical evaluation of Acalypha ointment in the treatment of superficial fungal skin diseases. Phytotherapy Research *17*, 555-557.

Paller, A., Eichenfield, L.F., Leung, D.Y., Stewart, D., and Appell, M. (2001). A 12-week study of tacrolimus ointment for the treatment of atopic dermatitis in pediatric patients. Journal of the American Academy of Dermatology *44*, S47-57.

Pandey, A., Mishra, R.K., Tiwari, A.K., Kumar, A., Bajaj, A.K., and Dikshit, A. (2013). Management of cosmetic embarrassment caused by Malassezia spp. with fruticose lichen Cladia using phylogenetic approach. BioMed Research International *2013*, 1-8.

Pednekar, P.A., and Raman, B. (2013a). Antimicrobial and antioxidant potential with ftir analysis of Ampelocissus Latifolia (Roxb.) planch. leaves. Asian Journal of Pharmaceutical and Clinical Research *6*, 1-6.

Pednekar, P.A., and Raman, B. (2013b). Assessment of Semecarpus Anacardium (Linn. F.) leaf methanolic extract for their antibacterial, antifungal and antioxidant activity. International Journal of Pharmacy and Pharmaceutical Sciences *5*, 1-5.

Pietta, P.-G. (2000). Flavonoids as antioxidants. Journal of Natural Products *63*, 1035-1042.

Plotkin, L.I., Mathov, I., Squiquera, L., and Leoni, J. (1998). Arachidonic Acid Released from Epithelial Cells by Malassezia furfur Phospholipase A_2: A Potential Pathophysiologic Mechanism. Mycologia *90*, 163-169.

Pooja, A., Arun, N., and Maninder, K. (2013). Screening of plant essential oils for antifungal activity against Malassezia furfur. International Journal of Pharmacy and Pharmaceutical Sciences *5*, 1-3.

Quave, C.L., Lyles, J.T., Kavanaugh, J.S., Nelson, K., Parlet, C.P., Crosby, H.A., Heilmann, K.P., and 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*, 1-32.

Quave, C.L., and Pieroni, A. (2015). A reservoir of ethnobotanical knowledge informs resilient food security and health strategies in the Balkans. Nature Plants *1*, 1-6.

Rad, F., Aala, F., Reshadmanesh, N., and Yaghmaie, R. (2008). Randomized comparative clinical trial of Artemisia sieberi 5% lotion and clotrimazole 1% lotion for the treatment of pityriasis versicolor. Indian Journal of Dermatology *53*, 115-118.

Rad, F., Nik-Khoo, B., Yaghmaee, R., and Gharibi, F. (2014). Terbinafin 1% Cream and Ketoconazole 2% Cream in the Treatment of Pityriasis Versicolor: A randomized comparative clinical trial. Pakistan Journal of Medical Sciences *30*, 1273-1276.

Regupathi, T., and Chitra, K. (2015). Antidandruff activity of Eclipta alba (l.) Hassk. and Lippia nodiflora linn. International Journal of Research in Pharmaceutical Sciences *6*, 185-188.

Rhimi, W., Salem, I.B., Immediato, D., Saidi, M., Boulila, A., and Cafarchia, C. (2017). Chemical Composition, Antibacterial and Antifungal Activities of Crude Dittrichia viscosa (L.) Greuter Leaf Extracts. Molecules *22*, 942-955.

Ricci, G., Patrizi, A., Giannetti, A., Dondi, A., Bendandi, B., and Masi, M. (2010). Does improvement management of atopic dermatitis influence the appearance of respiratory allergic diseases? A follow-up study. Clinical and Molecular Allergy *8*, 8-20.

Rincón, S., Cepero de García, M.C., and Espinel-Ingroff, A. (2006). A Modified Christensen's Urea and CLSI Broth Microdilution Method for Testing Susceptibilities of Six Malassezia Species to Voriconazole, Itraconazole, and Ketoconazole. Journal of Clinical Microbiology *44*, 3429-3431.

Rojas, F.D., Sosa Mde, L., Fernandez, M.S., Cattana, M.E., Cordoba, S.B., and Giusiano, G.E. (2014). Antifungal susceptibility of Malassezia furfur, Malassezia sympodialis, and Malassezia globosa to azole drugs and amphotericin B evaluated using a broth microdilution method. Med Mycol *52*, 641-646.

Rukayadi, Y., and Hwang, J.K. (2007). In vitro anti-Malassezia activity of xanthorrhizol isolated from Curcuma xanthorrhiza Roxb. Letters in Applied Microbiology *44*, 126-130.

Ruzicka, T., Simmet, T., Peskar, B.A., and Ring, J. (1986). Skin levels of arachidonic acid-derived inflammatory mediators and histamine in atopic dermatitis and psoriasis. Journal of Investigative Dermatology *86*, 105-108.

Ryu, S., Choi, S.Y., Acharya, S., Chun, Y.J., Gurley, C., Park, Y., Armstrong, C.A., Song, P.I., and Kim, B.J. (2011). Antimicrobial and anti-inflammatory effects of Cecropin A(1-8)-Magainin2(1-12) hybrid peptide analog p5 against Malassezia furfur infection in human keratinocytes. Journal of Investigative Dermatology *131*, 1677-1683.

Saeedi, M., Morteza-Semnani, K., and Ghoreishi, M.R. (2003). The treatment of atopic dermatitis with licorice gel. Journal of Dermatological Treatment *14*, 153-157.

Sardi, J.D.C.O., Pitangui, N.D.S., Rodriguez-Arellanes, G., Taylor, M.L., Fusco-Almeida, A.M., and Mendes-Giannini, M.J. (2014). Highlights in pathogenic fungal biofilms. Revista Iberoamericana de Micología *31*, 22-29.

Satchell, A.C., Saurajen, A., Bell, C., and Barnetson, R.S. (2002). Treatment of dandruff with 5% tea tree oil shampoo. Journal of the American Academy of Dermatology *47*, 852-855.

Saunders, C.W., Scheynius, A., and Heitman, J. (2012). Malassezia Fungi Are Specialized to Live on Skin and Associated with Dandruff, Eczema, and Other Skin Diseases. PLoS Pathogens *8*, 1-4.

Schempp, C.M., Windeck, T., Hezel, S., and Simon, J.C. (2003). Topical treatment of atopic dermatitis with St. John's wort cream--a randomized, placebo controlled, double blind half-side comparison. Phytomedicine *10*, 31-37.

Schneeweiss, S., Doherty, M., Zhu, S., Funch, D., Schlienger, R.G., Fernandez-Vidaurre, C., and Seeger, J.D. (2009). Topical treatments with pimecrolimus, tacrolimus and medium- to high-potency corticosteroids, and risk of lymphoma. Dermatology *219*, 7-21.

Schwartz, R.A., Janusz, C.A., and Janniger, C.K. (2006). Seborrheic dermatitis: An Overview. American Family Physician *74*, 125-130.

Shams-Ghahfarokhi, M., Shokoohamiri, M.R., Amirrajab, N., Moghadasi, B., Ghajari, A., Zeini, F., Sadeghi, G., and Razzaghi-Abyaneh, M. (2006). In vitro antifungal activities of Allium cepa, Allium sativum and ketoconazole against some pathogenic yeasts and dermatophytes. Fitoterapia *77*, 321-323.

Shemer, A., Nathansohn, N., Kaplan, B., and Trau, H. (1999). Itraconazole versus ketoconazole in the treatment of tinea versicolor. Journal of Dermatological Treatment *10*, 19-23.

Shibata, N., Saitoh, T., Tadokoro, Y., and Okawa, Y. (2009). The cell wall galactomannan antigen from Malassezia furfur and Malassezia pachydermatis contains beta-1,6-linked linear galactofuranosyl residues and its detection has diagnostic potential. Microbiology *155*, 3420-3429.

Sibi, G., Alam, A., Shah, J., and Razak, M. (2014). Susceptibility pattern of Malassezia species to selected plant extracts and antifungal agents. International Journal of Green Pharamacy *8*, 226-230.

Simonetti, G., Tocci, N., Valletta, A., Brasili, E., D'Auria, F.D., Idoux, A., and Pasqua, G. (2015). In vitro antifungal activity of extracts obtained from Hypericum perforatum adventitious roots cultured in a mist bioreactor against planktonic cells and biofilm of Malassezia furfur. Natural Product Research *30*, 544-550.

Simonetti, G.D.A., Felicia Diodata; Mulinacci, Nadia, Innocenti, M., Antonacci, D., Angiolella, L., Santamaria, A.R., Valletta, A., Donati, L., and Pasqua, G. (2017). Anti-Dermatophyte and Anti-Malassezia Activity of Extracts Rich in Polymeric Flavan-3-ols Obtained from Vitis vinifera Seeds. Phytotherapy Research *31*, 124-131.

Sivasankar, C., Gayathri, S., Bhaskar, J.P., Krishnan, V., and Pandian, S.K. (2017). Evaluation of selected Indian medicinal plants for antagonistic potential against Malassezia spp. and the synergistic effect of embelin in combination with ketoconazole. Microbial Pathogenesis *110*, 66-72.

Sobolewska, D., Michalska, K., Podolak, I., and Grabowska, K. (2016). Steroidal saponins from the genus Allium. Phytochemistry Reviews *15*, 1-35.

Sreelatha, G.L., Babu, U.V., Sharath Kumar, L.M., Soumya, K., and Sharmila, T. (2015). Investigation on biochemical characterisation and In Vitro antifungal efficacy of plant extracts on Malassezia Furfur. International Journal of Pharma and Bio Sciences *6*, 1027-1041.

Stratigos, J.D., Antoniou, C., Katsambas, A., Bohler, K., Fritsch, P., Schmolz, A., Michaelidis, D., and De Beule, K. (1988). Ketoconazole 2% cream versus hydrocortisone 1% cream in the treatment of seborrheic dermatitis. A double-blind comparative study. Journal of the American Academy of Dermatology *19*, 850-853.

Sugita, T., Suto, H., Unno, T., Tsuboi, R., Ogawa, H., Shinoda, T., and Nishikawa, A. (2001). Molecular Analysis of Malassezia Microflora on the Skin of Atopic Dermatitis Patients and Healthy Subjects. Journal of Clinical Microbiology *39*, 3486-3490.

Sugita, T., Tajima, M., Ito, T., Saito, M., Tsuboi, R., and Nishikawa, A. (2005). Antifungal Activities of Tacrolimus and Azole Agents against the Eleven Currently Accepted Malassezia Species. Journal of Clinical Microbiology *43*, 2824-2829.

Sugita, T., Zhang, E., Tanaka, T., Tajima, M., Tsuboi, R., Ishibashi, Y., and Nishikawa, A. (2012). Atopic Dermatitis and Skin Fungal Microorganisms. In Atopic Dermatitis - Disease Etiology and Clinical Management, J. Esparza-Gordillo, ed. (InTech: InTech), pp. 123-140.

Thyssen, J.P., Hamann, C.R., Linneberg, A., Dantoft, T.M., Skov, L., Gislason, G.H., Wu, J.J., and Egeberg, A. (2017). Atopic dermatitis is associated with anxiety, depression, and suicidal ideation, but not with psychiatric hospitalization or suicide. Allergy, 1-7.

Valero, A., Perez-Rodriguez, F., Carrasco, E., Fuentes-Alventosa, J.M., Garcia-Gimeno, R.M., and Zurera, G. (2009). Modelling the growth boundaries of Staphylococcus aureus: Effect of temperature, pH and water activity. International Journal of Food Microbiology *133*, 186-194.

Vandeputte, P., Ferrari, S., and Coste, A.T. (2012). Antifungal Resistance and New Strategies to Control Fungal Infections. International Journal of Microbiology *2012*, 1-26.

Vardy, D., Cohen, A., Tchetov, T., Medvedosky, E., and Biton, A. (1999). A double-blind, placebo-controlled trial of an Aloe vera (A. barbadensis) emulsion in the treatment of seborrheic dermatitis. Journal of Dermatological Treatment *10*, 7-11.

Varsani, M., Graikou, K., Velegraki, A., and Chinou, I. (2017). Phytochemical Analysis and Antimicrobial Activity of Origanum dictamnus Traditional Herbal Tea (decoction). Natural Product Communications *12*, 1801-1804.

Verallo-Rowell, V.M., Dillague, K.M., and Syah-Tjundawan, B.S. (2008). Novel antibacterial and emollient effects of coconut and virgin olive oils in adult atopic dermatitis. Dermatitis *19*, 308-315.

Vollekova, A., Kostalova, D., and Sochorova, R. (2001). Isoquinoline alkaloids from Mahonia aquifolium stem bark are active against Malassezia spp. Folia Microbiologica *46*, 107-111.

Warner, R.R., Schwartz, J.R., Boissy, Y., and Dawson, T.L., Jr. (2001). Dandruff has an altered stratum corneum ultrastructure that is improved with zinc pyrithione shampoo. Journal of the American Academy of Dermatology *45*, 897-903.

Watanabe, S., Koike, A., Kano, R., Nagata, M., Chen, C., Hwang, C.Y., Hasegawa, A., and Kamata, H. (2014). In Vitro Susceptibility of Malassezia pachydermatis Isolates from Canine Skin with Atopic Dermatitis to Ketoconazole and Itraconazole in East Asia. The Journal of Veterinary Medical Science *76*, 579-581.

Weckesser, S., Engel, K., Simon-Haarhaus, B., Wittmer, A., Pelz, K., and Schempp, C.M. (2007). Screening of plant extracts for antimicrobial activity against bacteria and yeasts with dermatological relevance. Phytomedicine *14*, 508-516.

White, T.C., Findley, K., Dawson, T.L., Scheynius, A., Boekhout, T., Cuomo, C.A., Xu, J., and Saunders, C.W. (2014). Fungi on the Skin: Dermatophytes and Malassezia. Cold Spring Harbor Perspectives in Medicine *4*, 1-16.

WHO (2002). WHO Traditional Medicine Strategy 2002-2005, WHO, ed. (Geneva), pp. 1-61.

WHO (2003). WHO guidelines on good agricultural and collection practices [GACP] for medicinal plants, W.H. Organization, ed.

Williams, M.R., and Gallo, R.L. (2015). The Role of the Skin Microbiome in Atopic Dermatitis. Current Allergy & Asthma Reports *15*, 65-75.

Ying, S., Zeng, D.N., Chi, L., Tan, Y., Galzote, C., Cardona, C., Lax, S., Gilbert, J., and Quan, Z.X. (2015). The Influence of Age and Gender on Skin-Associated Microbial Communities in Urban and Rural Human Populations. PLoS One *10*, 1-16.