

**Novel Antifungal Activity of Geraniol and its Synergistic Effect in
Combination with Fluconazole Against Resistant *Candida albicans***

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ABSTRACT

Fungal infections are more common today than ever before. Increasing rates of invasive procedures, bone marrow transplants, and the use of immunosuppressive agents and broad spectrum antibiotics have resulted in an increasing susceptibility to nosocomial infections, mainly fungal infections. Higher morbidity and mortality rates associated with drug resistance have led to a greater need for the development of new, more effective compounds that support the action of the marketed antifungal agents. In this project, the anti-*Candida* activity of the terpenoid component of the plant essential oil—Geraniol was investigated. This was achieved using minimum inhibitory concentration assays (MIC). Furthermore, the synergistic activity of Geraniol combined with the known azole Fluconazole was also evaluated for increased efficacy against *Candida albicans* in vivo. The susceptibility of the fungus to the combination was measured by the amount of cell growth seen after exposure to the mixture. The data indicate a synergistic activity of the Geraniol-Fluconazole mixture against drug-resistant *C. albicans*. Various mutant strains of *C. albicans*, expressing different levels of resistance were also tested in the study. Checkerboard MIC assays, with serial dilutions of the azole and Geraniol were used to assess synergy.

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INTRODUCTION

Fungal infections caused by *Candida albicans* are one of the most prevalent nosocomial infections. These infections cause significant cases of fatality, and contribute to millions of dollars annually in medical bills associated with prolonged patient stay at hospitals. Known antifungal agents include mainly azoles and polyenes. Azoles have been used to treat *C. albicans* infections such as vaginitis, which is the most common gynecological disorder affecting women, as well as candidiasis, the most highly prevalent infection in patients suffering from HIV. *Candida* infections are usually manifested in individuals with compromised immune systems, such as those suffering from cancer or HIV. In more serious conditions, Amphotericin B—one of the oldest antifungal agents, a polyene—is often used. As with most infectious agents, *C. albicans* may develop resistance to antifungal medications, resulting in recurring infections. Major causes of this resistance include untimely and improper drug administration, high antibiotic intake, or a compromised immune system that allows the fungus to grow and flourish. This problem is the basis of this project, which aims to determine a means of overcoming *C. albicans* resistance to antifungals, specifically the resistance to the azole Fluconazole.

In order to address the question, “How can *Candida albicans* resistance to azole antifungals be overcome?” it was critical to understand some of the common azole resistance mechanisms employed by the fungus. Some molecular mechanisms of azole resistance include the over-expression of the efflux pump and/or over-expression of the ERG11 target enzyme. These resistance mechanisms compromise the effectiveness of azole antifungal drugs. Fungal acquisition of resistance to drugs has resulted in greater numbers of difficult to treat fungal infections thus expanding the need to overcome this problem. Overcoming fungal resistance to

antifungal drugs might involve using enhancing compounds or alternative drugs to treat patients. Previous studies have shown the anti-Candidal effects of essential oils produced by plants (Chami et al., 2005). The active compounds in essential oils are monoterpenoids (terpenes), which are believed to have protective functions in plants. Terpenes act by permeabilizing cellular membranes, and were therefore selected as the class of enhancing compound for this study, exploring possible synergistic effect resulting from their combination with antifungal drugs. We hypothesized that terpenes would both inhibit *Candida* growth themselves and synergistically overcome Fluconazole resistance by *C.albicans* when combined with the azole.

This study investigated the antifungal effects of the terpenes: Carvacrol and Geraniol when combined with the antifungal agents: Fluconazole and Amphotericin B, against several resistant clinical isolates of *C. albicans*. Possible synergies between the terpenes and antifungal drugs were evaluated and used to determine whether such drug combinations could realistically alleviate the *Candida* resistance to antifungal agents in clinical use.

BACKGROUND

Candida albicans

Fungi are a highly successful and diverse group of eukaryotic organisms consisting of approximately 300,000 different species. *C. albicans* is the most common pathogenic fungus affecting humans (Hull et al., 2000). Unlike other fungal pathogens, this dimorphic fungus exists in two different morphological shapes simultaneously. One form is a yeast-like state that is a non-invasive, sugar-fermenting organism. The other is a fungal form that produces very long root-like structures called rhizoids (Shena et al., 2008). This latter rhizoid form is believed to be invasive—a causal agent of opportunistic oral and genital infections in humans. To infect its host tissue, the usual unicellular yeast-like form of *C. albicans* responds to its surrounding environmental signals and changes into the invasive, multicellular filamentous form. This phenomenon defines dimorphism. *C. albicans* does not reproduce sexually. The exterior of the *C. albicans* cell is made up of many types of polysaccharides such as, glucans, chitin, and mannans—mainly giving structure to the cell wall. The cellular membrane structure of *C. albicans* is considered a dynamic organelle, with ergosterol being the main membrane sterol. **Figures 1** and **2** below show *C. albicans* fungi under a microscope in its normal and pathogenic form, respectively.

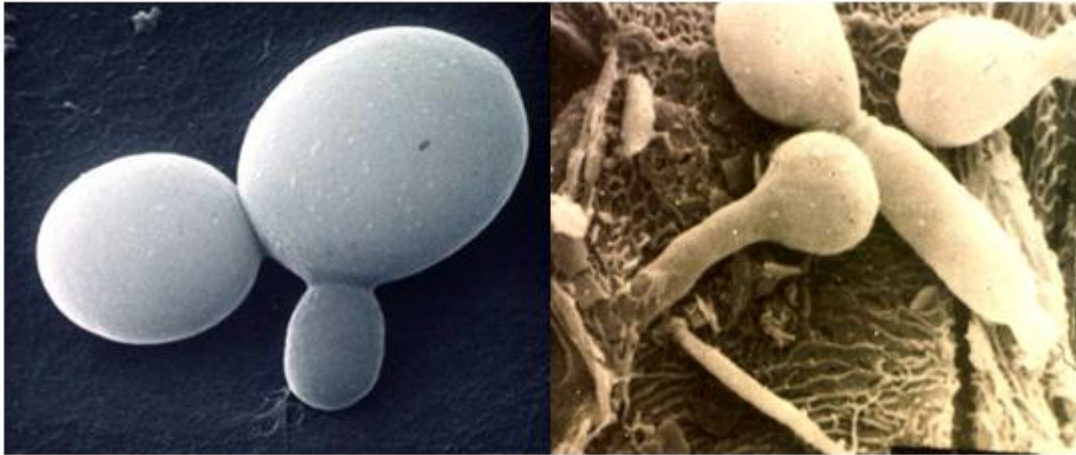


Figure 1: Microscopic image of the cells of the normal yeast-like fungus *C. albicans* (Volk, 1999)

Figure 2: Microscopic image of the cells of pathogenic *C. albicans* with hyphae (Lewis, 2011)

C. albicans is capable of proliferating within the human body where it can lead to infections such as oral thrush, vaginitis, and athlete's feet. This pathogen enters its human or animal host by penetration through a colonized surface. *Candida* contains virulence factors that facilitate its potential to infect an organism. These virulence factors include surface molecules that facilitate *Candida* adherence to other structures. It also contains acid proteases and phospholipids involved in penetration and damage of cell envelopes. Furthermore, *Candida*'s ability to convert into the hyphal form is crucial for pathogenic activity (Shena et al., 2008). Once it is within its host organism, *Candida* proliferates in dark and moist environments, and is most often present in the mouth, feet, vagina, and gastrointestinal tract. *Candida* can also travel through the bloodstream, and can therefore be found anywhere in the body from the brain to the lungs, muscles, or joints.

Like most other microorganisms that live in the intestine, *C. albicans* lives in symbiosis with the other microorganisms present within the host, mostly intestinal bacteria in humans. The fungus lives in about 80% of the human population without causing harmful effects. Because *Candida* has the tendency to proliferate at a high rate, this symbiosis is necessary to maintain proper function of its host's physiological systems—overgrowth of the fungi can cause health complications. The *Candida* to bacteria ratio can be disrupted if the number of “good bacteria” in the host is lowered, through antibiotic administration for instance. Another cause for the disruption of this ratio is an imbalance of sugars relative to vitamins and other key nutrients required to sustain bodily functions. *Candida* feeds on various sugars; therefore an abnormally high amount of sugars in the blood stream can fuel *Candida* growth. Both an irregularly low bacterial population and unusually high blood sugar level will lead to a sprouting of the key diseases associated with an increase in *Candida* population. Furthermore, this uncontrolled *Candida* proliferation is facilitated by defects in its host immune system, such as mucocutaneous wounds and granulocytopenia. The molecular biology of *C. albicans* is chiefly based on the molecular knowledge of the yeast *Saccharomyces cerevisiae*. This is due to the fact that they are similar, and because *C. albicans* genes can be expressed in *Saccharomyces* (Molero et al., 1998).

Diseases Caused by *C. albicans*

Candidiasis or thrush is a fungal infection (mycosis) of any of the *Candida* species (all yeasts), of which *Candida albicans* is the most common. As Cynthia Perkins (2007) writes, “On a daily basis, virtually all physicians are confronted with a positive *Candida* isolate obtained from one or more various anatomical sites”. According to an epidemiological review by Dr.

Hidalgo (2011), there is a 30-55% *Candida* colonization of the oral airway of healthy young adults, and a 40-65% *Candida* colonization of normal fecal flora. Dr. Hidalgo further reports that *Candida* species are the fourth most commonly isolated pathogens from blood cultures. This demonstrates the large *Candida* presence in the microflora of humans, and the danger for *Candida* infection posed to immunocompromised persons.

Vulvovaginal candidiasis (VVC) is the second most common cause of vaginitis in women, and is the most common condition affecting women seeking gynecologic primary care. Nearly 75% of all adult women have had at least one "yeast infection" in their lifetime (CDC, 2012). VVC is characterized by vaginal discharge, burning, and vulvar irritation, and the *Candida* genus is the specific cause for this infection (PubMed Health, 2010). According to an article on VVC by Dr. Samra-Latif (2012), three out of four women experience at least one attack of VVC in their lifetime, backing up the 75% CDC estimate mentioned above. Approximately half of these women will have more than one episode, and a few will have frequent relapses. The United States estimates indicate that about 50% of college-aged women will experience a VVC episode.

Candidiasis infections range from the superficial types (oral thrush, athlete's foot, and vaginitis, **Figures 3 and 4**) to the systemic types which are potentially life-threatening (candidemia). A superficial infection does not penetrate the bloodstream, while systemic infections do. Systemic fungal infections may be caused either by an opportunistic organism that attacks a person with a weakened immune system, or by an invasive organism common in a specific geographic area. Candidiasis is highly prevalent in immunocompromised persons, such as cancer, transplant, and AIDS patients, as well as non-trauma emergency surgery patients. About 90% of HIV patients not treated with highly active antiretroviral therapy develop

oropharyngeal candidiasis (of the oral pathway), and 10% develop esophageal candidiasis (Perkins, 2007). Although mucocutaneous candidiasis rarely causes death in patients with strong immune systems, it can lead to poor oral intake, malnutrition, and early death in patients with advanced immunodeficiency due to HIV infection.



Figure 3: Oral Thrush Caused by *C.albicans*. A close-up of the white tongue caused by oral thrush in affected patient (Marazzi, n.d.).



Figure 4: Clinical manifestations of candidiasis in the toes (Volk, 1999).

However, disseminated candidiasis is associated with much higher mortality rates ranging from 30-40%. According to Dr. Hidalgo (2011), “Systemic candidiasis causes more case fatalities than any other systemic mycosis, [and] investigators reported the enormous economic impact of systemic candidiasis in hospitalized patients [over a decade ago]”. Systemic candidiasis can easily prolong patient hospital stay by a month, which causes greater healthcare costs.

Antifungal Drugs

Fungi are emerging as a major nosocomial agent of infectious disease, and the rate of fungal infections, particularly those caused by *Candida* species has risen significantly in the past few decades. Between 1980 and 1990, there were 30,477 reported fungal infections in the U.S. Data from the National Nosocomial Infections Surveillance System reported an increase in the number of infections/1000 discharge from 2.0 to 3.8 (Beck-Sagué et al., 1993).

Antifungal drugs work by exploiting the differences between mammalian and fungal cells. Both fungi and animals are eukaryotes, thus there is difficulty in designing drugs that specifically target fungi without affecting human cells. In the United States, there are about 10 antifungal drugs approved by the FDA to treat systemic fungal infections. Generally, these drugs fall under four main categories of antifungal agents: polyenes, azoles, echinocandins, and pyrimidines (Dismukes, 2000). Other drugs are available for treating superficial infections. Compared to superficial infections, systemic fungal infections can be life-threatening. Amphotericin B is a major drug in the market against fungal infections. This polyene exerts its pharmacological effects by forming a barrel-stave assembly in fungal membranes (Murata et al., 2009). Amphotericin B specifically interacts with membrane sterols upon forming an ion channel assembly. This accounts for its selectivity against fungi, and in particular the yeast *C. albicans*, which has a sterol membrane containing ergosterol (Baginski et al., 1997). Amphotericin B is a polyene macrolide antifungal agent that has been the major drug choice for the treatment of systemic fungal infections for over 40 years. Even though there are newer antifungal agents such as the azoles, Amphotericin B remains a popular antifungal choice (Gallis et al., 1990). Although Amphotericin B is highly effective against fungal infections, its dose-dependent toxicity characterized by fever, chills, vomiting and nausea limits its usefulness (Lemke et al., 2005).

In order to overcome Amphotericin B's detrimental nephrotoxic effects, new pharmaceutical lipid formulations have been developed. These lipid formulations are Amphotericin B lipid complex, Amphotericin B cholesteryl sulfate, and liposomal Amphotericin B. These formulations have the advantage over conventional Amphotericin B as they better deliver the drug to the tissues, and have milder or less acute side effects (Dismukes, 2000). However, they are expensive. Amphotericin B is often combined with other antifungal drugs to tackle fungal infections.

The next set of antifungal agents: azoles have provided more options for the treatment of fungal infections. The availability of azole antifungal agents in recent decades has created a significant therapeutic advance for treating fungal infections. Azoles are made up of nitrogen containing five-membered organic rings. They are divided into two classes: the imidazoles—clotrimazole, miconazole, ketoconazole—and the triazoles—Itraconazole and fluconazole—(Baron, 1996). There is a wide variety of these agents in the form of ointments, creams, and powders to treat cutaneous dermatophyte and some yeast infections (Bodey, 1992).

The principle mechanism of action of azole antifungal agents is to selectively inhibit Lanosterol 14 α -demethylase, a fungal cytochrome P-450 enzyme encoded by the *ERG11* gene. These enzymes are present in most living cells including fungi and yeasts, and they catalyze the conversion of lanosterol to ergosterol, which is a major component of the fungal cell membrane. Thus, azoles inhibit the production of ergosterol. Furthermore, they might also affect fatty acids of the cell membrane, interfering with transport across the membrane, inhibiting the catalase systems and decreasing fungal adherence (Bodey, 1992). A schematic visualization of the azole action is shown in **Figure 5**.

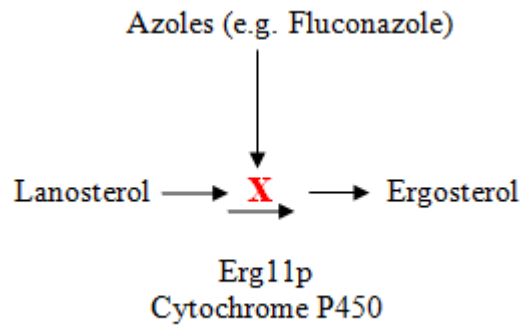


Figure 5: Mechanism of Azole Antifungal Activity.

As the figure above shows, the Azoles are effective because they selectively inhibit fungal enzymes (lanosterol 14 α -demethylase) over mammalian ones, inhibiting ergosterol synthesis. Azoles can tackle a broad spectrum of fungal pathogens including *Candida* species, and their effectiveness is exceeded only by Amphotericin B. Compared to Amphotericin B, their ease of administration as well as their lower toxicity levels makes the oral azoles: ketoconazole, itraconazole, and fluconazole, a preferred substitute (Como et al., 1994). Fluconazole is specifically preferred because it is rapidly absorbed, crosses the blood-brain barrier, and is minimally metabolized in the liver, with the bulk of the active ingredient excreted in urine (Como et al., 1994). However, side effects of the azoles may include liver toxicity over a long period of time, nausea, and vomiting. Miconazole, the first azole in the market was withdrawn because of its toxicity and limited activity (Bodey, 1992).

Ketoconazole can be administered both orally and topically, and is potent against a range of infections. Some of these infections are caused by fungi such as *H. capsulatum* and *B. dermatitidis*, for which ketoconazole is often used in non-immunocompromised patients. Ketoconazole is not indicated for treatment of yeast systemic infections. The triazoles,

fluconazole and itraconazole, have become the standard for the azoles, and have replaced Amphotericin B for managing certain forms of the systemic mycoses. Fluconazole is now routinely used to treat candidiasis in non-neutropenic hosts, and is gaining acceptance for use in cryptococcosis and selected forms of coccidioidomycosis. Itraconazole has proven to be effective for histoplasmosis, blastomycosis, sporotrichosis, coccidioidomycosis, consolidation treatment for cryptococcosis, and certain forms of aspergillosis. Fluconazole can be administered either orally, or intravenously.

The fungal cell wall is an attractive target for echinocandins. It inhibits β 1,3-glucan synthase. Fungi have cell walls, but mammals do not. Furthermore, the enzyme is active outside the cell, so the drug does not need to enter the cell. Additional advantages of echinocandins include low toxicity and rapid fungicidal activity against most isolates of *Candida* spp. The first licensed echinocandin product was caspofungin acetate (Cancidas; Merck), and others include micafungin (Fujisawa).

Among the pyrimidines, flucytosine is the only one approved for antifungal use. However, this water soluble pyrimidine can only work on a small range of species such as *Candida*, *Cryptococcus neoformans*, and some molds (Dismukes, 2000). Furthermore, it is toxic, and develops resistance readily when used. It is therefore used in combination with Amp B for treatment of certain fungal infections (Francis et al., 1992). Flucytosine is converted to 5-fluorouracil (5-FU) by the enzyme cytosine deaminase in certain fungi. This in turn is converted to 5-fluoro-2'-deoxyuridylic acid (FdUMP), which inhibits thymidylate synthase, inhibiting DNA synthesis (Waldorf et al., 1983). A summary of the mechanism by which the different classes of antifungal drugs in clinical use exhibit their effect on the *C. albicans* cells is shown in **Figure 6**.

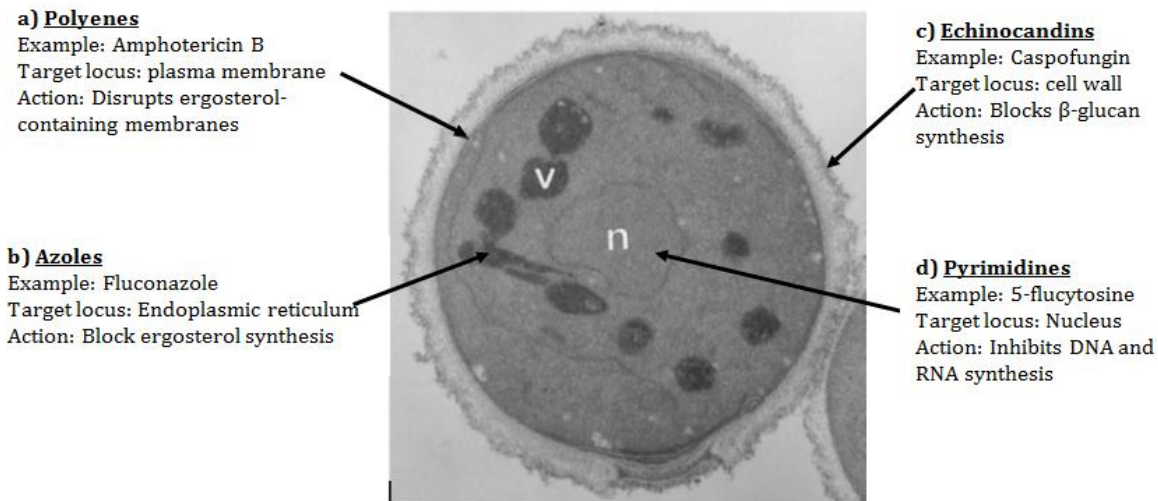


Figure 6: An image of a fungal cell under the electron microscope. The major classes of antifungal drugs in clinical use and their effects on the fungal cell are highlighted. Polyenes (a), e.g. Amphotericin B, bind to ergosterol membrane, forming pores across it and disrupting it within cell membranes. Echinocandins, such as caspofungin, blocks β - (1, 3)-D-glucan synthase, and thus disrupt cell-wall integrity (b). Azoles, such as fluconazole, inhibit Erg11, blocking ergosterol synthesis in the endoplasmic reticulum(c). This causes the accumulation of a toxic sterol intermediate, causing cell membrane stress. Pyrimidines such as 5-flucytosine inhibits DNA and RNA synthesis (d) (Aaron P. Mitchell, 2007)

Fungal Drug Resistance

Drug resistance is complex, but may be termed as the persistence of a disease despite treatment with the appropriate drug. The last couple of decades have witnessed a transition in medicine, especially the use of new types of toxic agents to control and kill unwanted disease agents including fungi. Unfortunately, biological evolution and natural selection facilitate the emergence of drug resistant pathogenic microorganisms. Antimicrobial agents almost completely kill the entire pathogenic population, leaving survivors to evolve resistance (Taylor et al., 1996).

Opportunistic fungal infections are a threatening problem causing significant morbidity and mortality, especially for those with weakened or compromised immune systems such as AIDS and cancer patients (Kanafan et al., 2008). *Candida* infections have increased dramatically since the onset of the AIDS epidemic, and so has *Candida* resistance. With only a

hand full of antifungal drugs on the market, and the increasing occurrence of fungal infections, the emergence of drug resistance in fungal pathogens compromising the effectiveness of the drugs presents a serious health issue. For this reason, the evaluation of the susceptibility of a specific pathogen to a drug is important. Drug resistance by a pathogen is typically measured using a minimum inhibitory concentration (MIC) assay (Reyes et al., 2000). MIC assays determine the minimum concentration of drug required to show a visual effect on pathogen growth. The effect on growth can also be determined by quantitating the amount of drug that provides a specific percentage drop in infection relative to WT, such as 50% (MIC₅₀).

Microbiological antifungal resistance is either primary (intrinsic) or secondary (acquired). Primary resistance can occur naturally among some fungi types without prior exposure to the drug. Secondary resistance is acquired when susceptible fungi strains are exposed to the antifungal agent, and this is generally dependent on gene mutations. Fluconazole resistance among *C. albicans* is an example of a secondary resistance (Pfaller, 2012).

Clinical resistance occurs when the pathogen is inhibited by an antimicrobial drug concentration that is greater than could be safely achieved with normal dosing. These failures could be due to a combination of factors from the pathogen, the host, or the drug itself (Kanafan et al., 2008). Generally, resistance is said to be present when the infection is persistent, despite the use of the usual concentrations of the agent with normal dosage schedules, and/or when the pathogen shows MIC's that fall within the range of resistance strains.

As explained above, antifungal agents combat the pathogens through different mechanisms. The azole antifungals inhibit the enzyme that makes ergosterol, which is the major component of the fungal cell wall (Bodey, 1992). As a result, ergosterol content in the cell membrane is depleted, leading to improper functioning of the membrane, which leads to fungal

cell death. The mechanism of resistance of fungi to azoles has been well characterized, however resistance for the other classes of antifungal drugs is still being investigated.

The main known mechanisms of resistance to azoles in *Candida* species are: 1) active efflux pump development, 2) mutation of the ERG11 gene, 3) overexpression of the target enzyme, and 4) the development of bypass pathways. Active efflux pump development results in decreased drug concentrations at the site of action. Efflux pumps are encoded in the *Candida* species by two transporter families: the CDR genes of the ATP binding cassette super-family, and the MDR genes of the major facilitators' class. Up-regulation of efflux pumps (MDR1, CDR1, CDR2) encoded by either gene results in *Candida* resistance to azoles. CDR gene overexpression confers resistance to nearly all azoles; however MDR encoded efflux pumps usually select for fluconazole specifically (Kanafan et al., 2008).

The second form of antifungal resistance encountered is one facilitated through mutations in the ERG11 gene that encodes the target enzyme. This target enzyme, lanosterol C14 α -demethylase, prevents or reduces binding of azoles to the enzymatic site (Löffler et al., 1997).

The third resistance mechanism is overexpression of the altered Lanosterol 14 α -demethylase target enzyme. This may lead to targets not binding well with the azole drugs and inhibiting ergosterol synthesis. However, minimal up-regulation of altered target enzymes has been observed to date, and so this mechanism contributes little to the resistance observed.

The final known mechanism of azole resistance in *Candida* species involves the development of bypass pathways. This negates the membrane-disruptive effects of azole drugs. This mechanism has been linked with mutation of the *ERG3* gene in certain resistant strains of *Candida* (Kelly et al., 1997).

Polyenes like amphotericin B and its lipid formulations act by forming a barrel-stave assembly in fungal membranes in close association with ergosterol. This leads to porin channel formation, and the loss of transmembrane potential and impaired cell functioning (Murata et al., 2009). Resistance breakpoints for polyenes have not yet been determined. An MIC of ≥ 1.0 $\mu\text{g/mL}$ is widely used to indicate resistance to Amphotericin B. Mutations in the *ERG3* gene involved in ergosterol synthesis lead to a buildup of other sterols in the fungal membrane. Polyene resistant strains of *Candida* have relatively low ergosterol content, compared to sensitive strains (Dick et al., 1980).

Resistance of clinical isolates of *C. albicans* to the echinocandin drug caspofungin is slowly developing. It is related to mutations in short conserved regions in the FKS1 gene. The most prominent changes occur at the serine 645 position in Fks1p with substitutions of proline, tyrosine, and phenylalanine. An allele-specific real-time PCR molecular-beacon assay has been developed for rapid identification of drug resistance by targeting FKS1 mutations. Mutations altering serine 645 were reliably identified in both heterozygous and homozygous states.

Pyrimidines such as flucytosine inhibit cellular DNA and RNA synthesis by inhibiting the enzyme thymidylate synthase (Waldorf et al., 1983). Some yeast strains are intrinsically resistant to this drug because of decreased cellular uptake of the drug due to a mutation in cytosine permease. However, acquired resistance can also occur through defects in flucytosine metabolism caused by mutations in cytosine deaminase or uracil phosphoribosyl transferase. However, resistance to flucytosine is readily developed by fungi, prompting clinicians to use flucytosine only in combination with other antifungal agents, mainly Amphotericin B (Francis et al., 1992). A summary of the various resistance mechanisms applied by the different drug classes is shown in **Figure 7**.

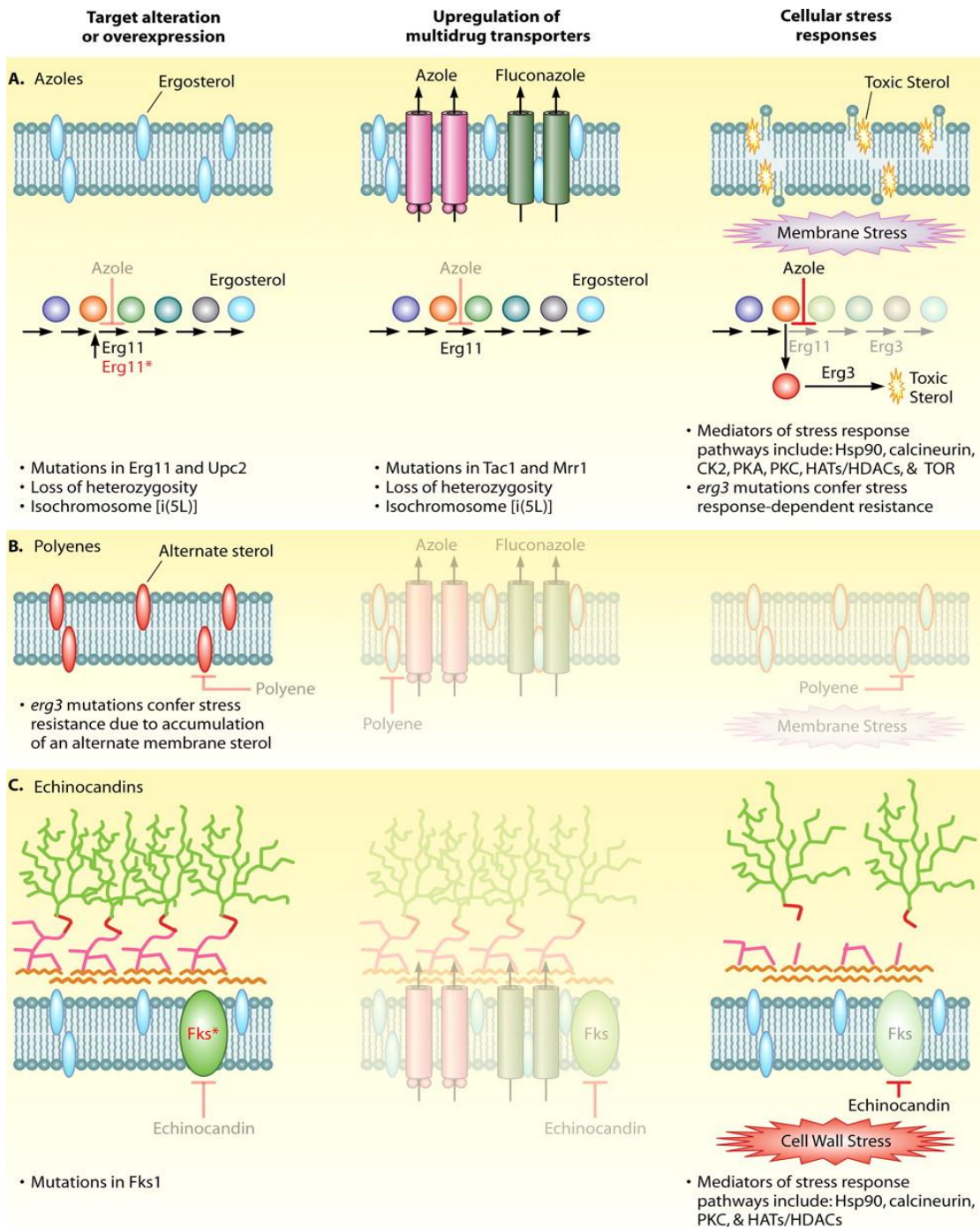


Figure 7: Drug resistance mechanisms in *C. albicans*. (A) *C. albicans* develops resistance to the azoles through several mechanisms including the upregulation and or alteration of the target *Erg11*; the increased expression of the efflux pump—*Cdr1*, *Cdr2*, or *Mdr1* (fluconazole specific); or through cellular stress responses. (B) *C. albicans* resistance to the polyenes is infrequent, but developed by loss-of-function mutations in *ERG3*, blocking the production of ergosterol and the formation of the drug-lipid complex, thereby preventing osmotic cellular lysis. Alteration in drug transporters and cellular stress responses do not contribute much to polyenes resistance. (C) Resistance to the echinocandins, mainly through mutations in two distinct hot-spot regions in *FKS1*, encoding the catalytic subunit of (1,3)- β -d-glucan synthase, has been observed. The induction of cellular stress responses is important for echinocandin resistance. Bright images show key mechanisms for a given class of drug (Cowen, 2008)

Terpenes

Antifungals make up a small but important group of drugs in the market. This is largely due to the growing incidence of failure in the treatment of fatal fungal infections, mostly due to resistance and or opportunistic infections in immunocompromised patients. This has resulted in the need for newer, more effective fungal agents or enhancers for the treatment of affected patients. The history of drug discovery shows that many of the most successful therapeutics come from natural sources like plants (Bevan et al., 1995). Plant essential oil extracts, in addition to defending plants against predators, pathogens, and competitors, also have antiseptic qualities that have been recognized since antiquity. Attempts to characterize these properties in the laboratory date back to the early 1900s (Hoffmann and Evans, 1911). These volatile oils are generally obtained from non-woody plant material by distillation methods that typically include steam or hydro distillation. Plant essential oils are principally made up of terpenoids and a variety of low molecular weight hydrocarbons. Although they usually occur as complex mixtures, their activity can primarily be accounted for in terms of their major monoterpenoid components (Cox et al., 2001). Terpenes are primary chemicals responsible for the medicinal, culinary and fragrant uses of aromatic and medicinal plants.

The antifungal effect of plant essential oils and their promise to enhance the activity of antifungal drugs already in the market has been established. Several studies including the one performed by Chami et al. (2005) titled “Study of anti-*Candida* activity of carvacrol and eugenol *in vitro* and *in vivo*” have proven the antifungal activity of terpenes. In this particular study, the anti-*Candida* activity of eugenol and carvacrol was tested against *C.albicans* in immunosuppressed rats. The fungicidal effect of these terpenes significantly reduced the fungi growth.

Research into the antimicrobial actions of terpenes suggests that they destroy and damage the cellular membrane. They move into the cell membrane by diffusion, increasing the plasma membrane permeability (Andrews et al., 1980). Another study performed by Salvador Uribe et al. (1985) titled “Effects of P-Pinene on Yeast Membrane Functions” suggests inhibition of respiration with glucose or ethanol as the substrate. The inhibition depended on the ratio of the terpene to the amount of yeast cells; for a fixed concentration of terpene, inhibition decreased as the amount of yeast cells increased. The terpene also inhibited the pumping of protons and K^+ transport. The studies on isolated mitochondria showed a series of effects, starting with the loss of respiratory control and de-energization of the organelles. This was followed by an inhibition of respiration at higher concentrations of the terpene. The effect on respiration could be localized to the cytochrome b region of the electron transport chain. No effect could be detected on the activity of ATPase (Uribe et al., 1985).

PROJECT PURPOSE

The goal of this investigation was to test for potential synergy between a known antifungal drug and essential plant oils: terpenes. To do this, the Minimal Inhibitory Concentration (MIC) of antifungal agents and terpenes against a series of sensitive and resistant *Candida* strains was determined. The synergistic effect of Geraniol on fluconazole activity was quantified for five drug-resistant *Candida* isolates. The identification and quantification of the extent of synergy, as well as the understanding of the means by which resistance was overcome will facilitate the development of novel therapies against resistant *Candida* strains. On a larger scale, such therapies offer the potential of eliminating the problem of recurrent fungal infections.

METHODS AND MATERIALS

Candida Strains

Seven *Candida* strains were used in this study. Strains 1, 3, 14, 17, 4568, were a gift from Professor Theodore White, and strains 10261, and 51611 were from ATCC. These strains are mutated to show different levels of resistance to antifungals. The yeast strains were grown in Yeast Peptone Dextrose (YPD) Broth and were stored at -70°C in 20% glycerol until tested. For each experiment, the yeasts were grown from frozen culture in YPD broth for 16 h at 37°C for inoculum preparation, and diluted in saline to a cell density of 10^5 /ml.

Antifungal Drugs

The antifungal activities of the essential oils Geranoil and Carvacrol were evaluated. Geranoil and Carvacrol were purchased from Penta Chemicals. Known antifungals used in the study were Fluconazole (from TCI America), and Amphotericin B (from Sigma).

Essential Oils and Chemicals

Powders of fluconazole and amphotericin B were dissolved in 100 % dimethyl sulphoxide (DMSO) from Sigma at 1000 mg/ml and 100 mg/ml, respectively, and stored at -20°C. Individual aliquots of each solution were used. Based on the concentration needed, the appropriate aliquot was dissolved in the calculated volume of YPD. The calculations took into account the percent of DMSO present in the final solution before application to the yeast cells. The maximum amount of DMSO allowed was 5% DMSO—this ensured that the solvent did not contribute to the killing/inhibition of growth seen. The essential oil components Geranoil and

Carvacrol were kept at room temperature throughout the experimental period, and appropriate volumes were diluted in YPD before MIC and Checkerboard MICs were performed.

Minimal Inhibitory Concentration (MIC) Assay

MIC testing was completed according to the National Committee for Clinical Laboratory Standards (NCCLS) approved standard M27-A for the reference method for broth dilution antifungal susceptibility testing of yeast. To test the MIC of antifungal drug, different concentrations of the dissolved drug in DMSO were further diluted in YPD to prepare the required starting concentration. The assay was performed in 96-well flat bottom microtiter plates. 180 ul YPD growth media was pipetted into all the 96-wells aseptically, followed by 20 ul of the antifungal agent (azole, polyenes or essential oil) into the first well of each column. Using a multichannel pipette, the media and drug were mixed in the first row thoroughly by pipetting up and down multiple times. Then 100 ul of the mixture was transferred to the next row of wells, and repeated until row H, the last 100 ul of mixture was discarded so that all wells contained 100 ul (see **Figure 8**). *Candida* dilutions resulting in $\sim 10^5$ cells/ml population in YPD for each of the strains tested were made, and a 100 ul volume of the assigned candida strain was added corresponding to each well/column. Absorbance readings were taken at time 0 using a microplate spectrophotometer (Tecan) at 550 nm, and plates were incubated for about 24 hours at $\sim 37^\circ\text{C}$. Absorbance readings were taken at the end of this time to determine turbidity of the well contents. Also, the plates were scored microscopically to compare with the spectrophotometric readings.

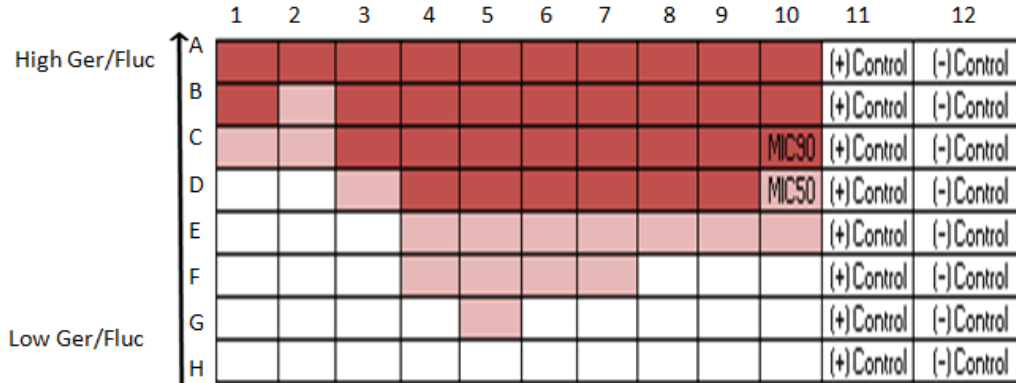


Figure 8: Setup of the MIC Plate. Strains are allocated to each column (1-10) of the plate. Fluconazole or Geraniol is added to well A of each column. This initial concentration is halved with each subsequent row until H. Column 11 is a positive control containing each *C. albicans* strain in separate wells with no drug added. Column 12 is a negative control and does not contain *C. albicans* or drug in any of its wells.

Checkerboard Microtiter Assay

To characterize and quantify the antifungal activity of Fluconazole, Geraniol, and the drug combinations over a range of concentrations, these compounds were tested in a Checkerboard microtiter plate format (96-well polystyrene plates) (**Figure 9**). The experiments were performed according to NCCLS approved standard M27-A. The MICs obtained from the NCCLS standard method performed above were used as basis for the concentration of drugs to start with. The stock solution of Fluconazole (in 100% DMSO) and Geraniol were used to prepare the desirable concentration of each by further dilution in YPD medium (generally 20X > MIC calculated). The dilution was such that the amount of DMSO was $\leq 5\%$ by volume, as DMSO might have a toxic activity against *C. albicans*. Necessary *Candida* dilutions resulting in $\sim 10^5$ cells/ml population in YPD for each of the strains tested was performed. To perform the assay, 100 μ l of YPD was added to each well, followed by 100 μ l 20X MIC of fluconazole to

Rows A- H of Column 1. Next, 100 ul of the YPD-fluconazole was transferred from Column 1 to Column 2, from Column 2 to Column 3, and so forth. Care was taken for 100 ul to be dispensed from Column 11, and for this volume to not be added to Column 12. Geraniol dilutions were performed assuming the weight of the oil was equal to its volume. Following the addition of Fluconazole, 100 ul of Geraniol at 20X MIC was added to Row A of Columns 1-12. The contents of these wells were vigorously mixed by pipetting up and down, after which 100 ul of the contents were transferred from Row A to B for Columns 1-12, Row B to C and so on. The contents of Row G were not transferred into Row H, rather they were disposed of.

The Checkerboard MIC endpoint was defined as the lowest Fluconazole and Geraniol concentrations at which a prominent decrease in turbidity was observed. This corresponded to a growth inhibition of $\geq 90\%$ when the viable counts were compared to those of the growth control. The interaction of each combination tested was characterized by calculated the fractional inhibitory concentrations (FICs) of each drug tested. The sum of the FICs, the so-called FIX index, was calculated on the basis of the MIC endpoint (MIC_{90} and MIC_{50}). As a standard recommended by the American Society for Microbiology, synergistic, indifferent, and antagonistic interactions were determined to be FIX values of ≤ 0.5 , $0.5 - 4.0$ and > 4.0 , respectively. Each experiment was performed in duplicate.

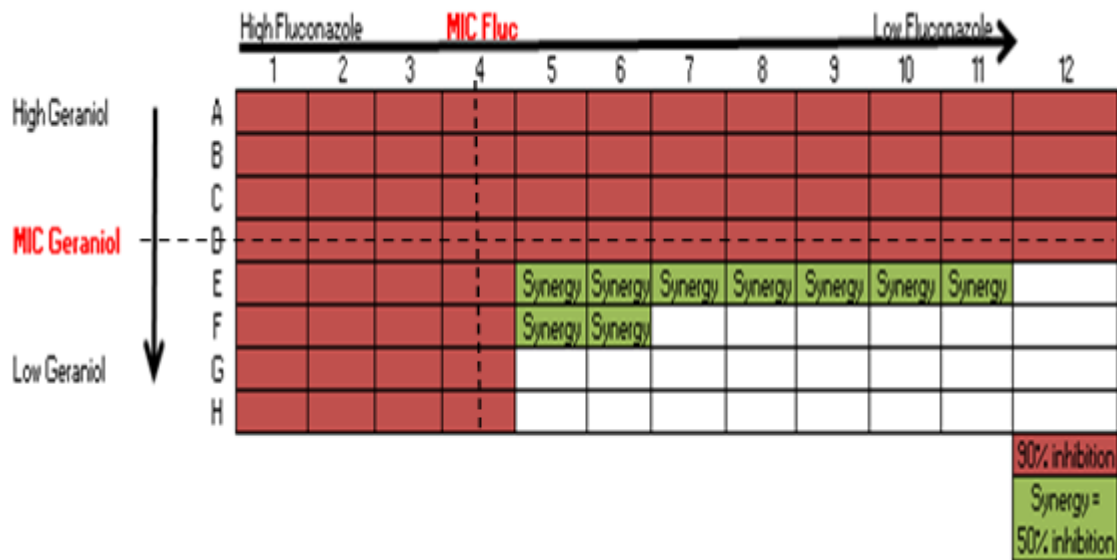


Figure 9: Design of the Checkerboard Assay. Fluconazole is added to all the wells in Column 1 and its concentration halved across the plate as depicted by the arrow. No Fluconazole is added to Column 12. Geraniol is added to all the wells in Row A and its concentration is halved down the plate as depicted by the arrow. No Geraniol is added to Row H. One *Candida* strain is then added to all the wells of the plate. This setup renders well 12H as the positive control because it does not contain drug. The MICs of both drugs are therefore calculated based off of this control.

RESULTS

Quantification of Antifungal Activity of Azoles and Polyenes

The initial stage of this project characterized two known anti-fungal compounds against the seven acquired *Candida* strains. The antifungal activity of fluconazole, an azole, and Amphotericin B, a polyene, were investigated using MIC assays. Fluconazole and amphotericin B are known to have fungistatic activity on *Candida* strains, and this activity was quantified for the seven *Candida* isolates. The independent anti-*Candida* activity of each drug was analyzed and reported as Minimal Inhibitory Concentration (MIC) values (**Table I**). Two fold dilutions of Fluconazole and Amphotericin B were performed serially in the MIC assays with a reproducibility of ± 1 well between the replicates. A mean of the MIC of the strains from two experiments is shown in the table. The data show that strains 4568 and 51611 are the least resistant to Fluconazole, while strains 51611, 14, 4568, and 10261 are the least resistant to Amphotericin B.

Table 1: MICs of Antifungal Agents Against *Candida* Strains.

Strain	Fluconazole (mg/ml)			Amphotericin B (mg/ml)	
	MIC90	MIC50	Resistance	MIC90	MIC50
1	>5.000	>5.000	+++++	10.000	0.500
3	>5.000	5.000	++++	10.000	0.500
14	>5.000	0.625	++++	-	-
17	5.000	0.625	+++	0.500	0.250
4568	1.250	0.625	++	0.500	0.250
10261	>5.000	2.500	+++	10.000	1.000
51611	0.156	0.039	-	-	-

MICs were determined by a micro-dilution method. The MIC was defined as the lowest concentration of the antifungal drug that inhibited the proliferation of *Candida* after a 24 hr incubation in 37°C. Experiments were run in duplicate. — not determined.

The growth inhibition curves (**Figure 10**) show the susceptibility of the *Candida* isolates to varying Fluconazole concentrations. It is clear that the percentage of *C. albicans* growth decreased with increasing Fluconazole concentration. To compare the extent of inhibition, one can either analyze the slope of each individual curve (the steeper the slope, the greater the percent inhibition) or one can determine the MIC₅₀ for each strain. The figure shows that strains 51611, 4568, 17, and 14 have the lowest MIC₅₀ values against Fluconazole. Strains 1 and 3 have the greatest MIC₅₀s.

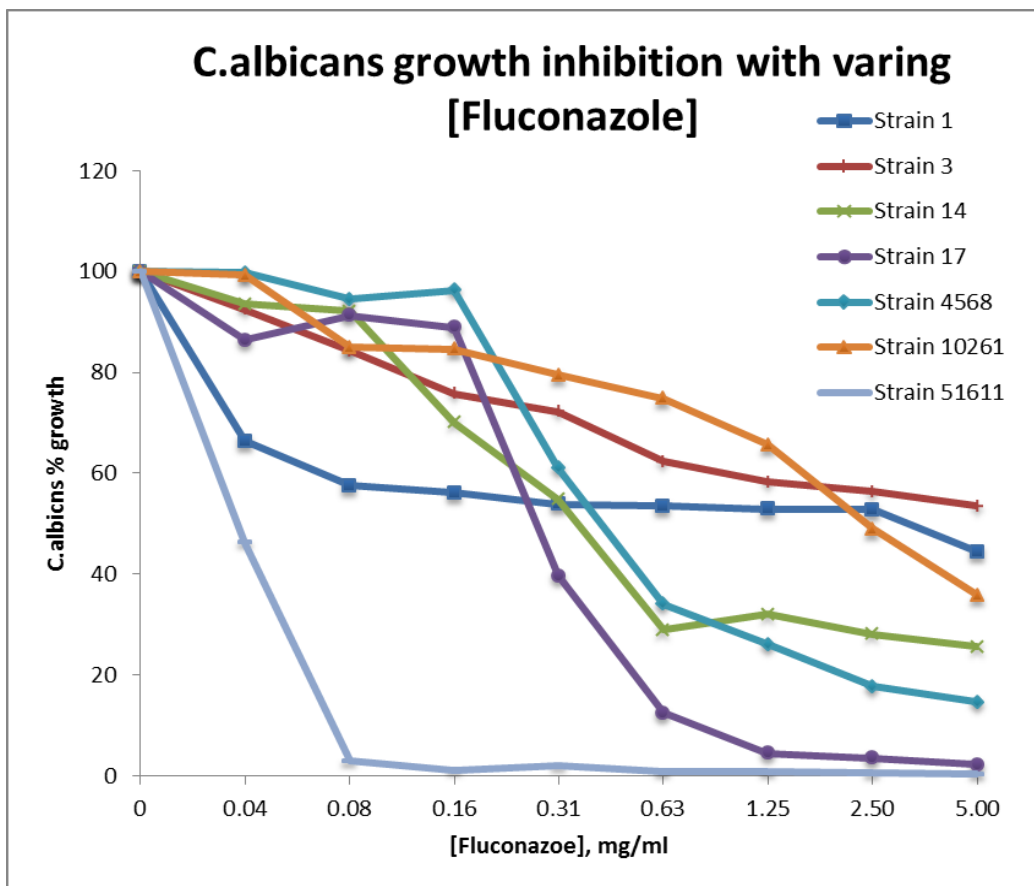


Figure 10: A line plot of *C. albicans* strains' % growth with increasing Fluconazole concentration

Quantification of Antifungal Activity of Plant Essential Oils: Terpenes

The antifungal activity of the terpenes, Geraniol and Carvacrol, were investigated and quantified using MIC assays. As with the antifungals, two-fold dilutions of Geraniol and Carvacrol were performed serially in the MIC assays with a reproducibility of ± 1 well between the replicates. A mean of the independent anti-*Candida* activity of each terpene from two experiments was analyzed and reported as Minimal Inhibitory Concentration (MIC) values in **Table 2**. Both Geraniol and Carvacrol inhibited growth of the *Candida* strains. Geraniol showed MICs for all seven strains ranging from 0.5—2 mg/ml, while Carvacrol showed MICs for the five strains tested ranging from <0.125—1 mg/ml. These MIC values represent the minimum concentration of drug required to inhibit growth of the microbes, and the data suggests that Carvacrol is more potent against the resistant *Candida* isolates than Geraniol.

Table 2: MICs of terpenes against *Candida* Strains

Strain	Geraniol (mg/ml)		Carvacrol (mg/ml)	
	MIC90	MIC50	MIC90	MIC50
1	1	1<MIC50<0.5	0.25	<0.125
3	1	0.825	0.25	<0.125
14	1	0.5	1	0.25
17	0.5	0.5	1	0.25
4568	1	1	0.25	<0.125
10261	1	1<MIC50<0.5	—	—
51611	2	2<MIC50<1	—	—

MICs were determined by a micro-dilution method. The MIC was defined as the lowest concentration of the antifungal agent that noticeably inhibited the proliferation of *Candida* after 24 h incubation in 37°C. Experiments were run in duplicate. —, not determined

The growth inhibition curves in **Figure 11** show the susceptibility of the *Candida* isolates to varying Geraniol concentration. It is clear from the combined line plot that the percentage of *C. albicans* growth decreased with increasing Geraniol concentration. The extent of inhibition was measured by the slope of each individual curve and takes into account the degree by which the starting population of the strain was reduced. The data suggests that Strain 17 is slightly more sensitive followed by 4568 as these strains have the largest gradients. Strain 51611 is slightly more sensitive followed by 4568 as these strains have the largest gradients. Strain 51611 (light blue curve), the control for Fluconazole, is notably the most resistant to Geraniol as it shows the greatest MIC₅₀ value. The graph of Figure 11 allows a comparison of the sensitivity of the different strains to varying Geraniol concentrations along a wider range of concentrations, and correlates with the MIC data reported in Table 2.

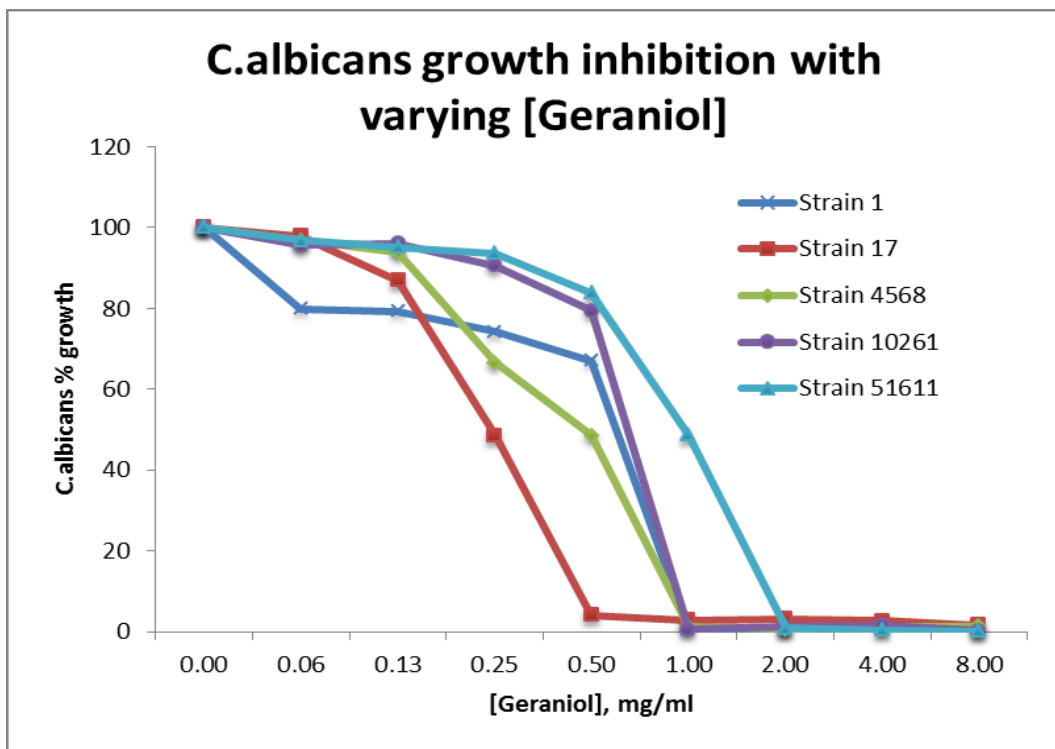
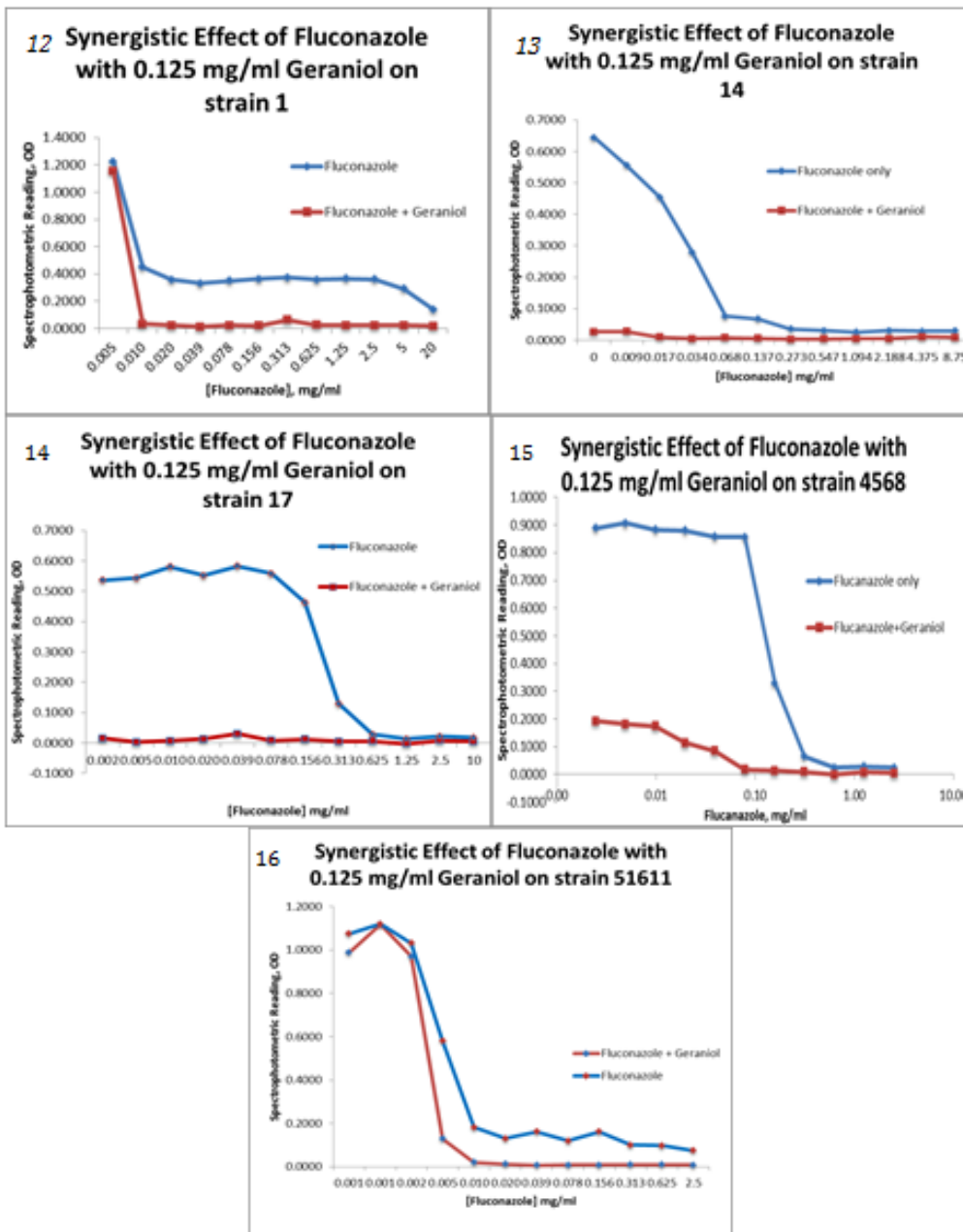


Figure 11: Plot of % *C. albicans* strains' % growth with increasing Geraniol concentrations.

Synergistic Effect of Drugs In Combination Against Resistant *C. albicans*

Once the susceptibilities of the resistant strains to Fluconazole and Geraniol were established, investigations into the potential synergistic effect of Geraniol on Fluconazole were conducted. Synergy was investigated using a Checkerboard micro-dilution assay referenced in the Methods and Materials. This assay facilitated the study of the effect of different concentrations of Geraniol and Fluconazole together on *Candida* growth. Microscopic and spectrophotometric analysis of the plates showed that the extent of turbidity in the wells of the 96 well plates positively correlated with the degree of effectiveness of the combined antifungal agent and terpene.

Figures 12-16 below show the effect of combining Fluconazole and Geraniol on growth of each of the five tested *Candida* strains. The spectrophotometric reading is reported for Fluconazole concentrations ranging from 0.002-2.50 mg/ml after a twenty-four hour incubation period at a sub-inhibitory Geraniol concentration of 0.125 mg/ml. The plots clearly show that the turbidity of the wells treated with a combination of both compounds is low, and the combination of drugs had lower spectrophotometric readings than the wells that were only treated with Fluconazole. The turbidity of the well correlates proportionally with yeast growth.



Figures 12-16: Synergistic effect of combining Fluconazole with Geraniol on spectrophotometric readings, for all five tested strains.

A summary of the synergy assays based on MIC₅₀ values are given in **Table 3** below.

The data is a mean of two experiments per strain. The Fractional Inhibitory Concentration (FIC) value was the quotient of the MIC of the drugs in combination, and the MIC of the drug

individually. The Fractional Inhibitory Concentration Index (FIX) was the sum of the FIC values of Geraniol and Fluconazole for a given *Candida* strain. Synergy was determined using the guidelines set by the Department of Microbiology. FIX values less than or equal to 0.5 were determined to represent synergies induced by the drug combination, FIX values between 0.5 and 4 were determined to represent indifference, and FIX values above 4.0 were determined to represent antagonism in the drug combinations. The results show that based on MIC₅₀ values, synergy was achieved for all five strains tested. The MIC values for all the fluconazole + geraniol combinations were much lower than the MIC values for the drugs individually.

Table 3: Synergy of Fluconazole and Geraniol Against Candida Strains Based on MIC₅₀.

Candida Strain	MIC ₅₀ (ug/ml) of:		FIX	Result
	Fluc with Geraniol	Fluc		
1	<4.88	>5000	0.001	Synergy
14	56	625	0.090	Synergy
17	156	625	0.250	Synergy
4568	156	625	0.250	Synergy
51611	2.44	39	0.062	Synergy

Fractional Inhibitory Concentration Index (FIX) is equal to (MIC of drug in combination/MIC of drug alone)

DISCUSSION

Quantification of Antifungal Activity of Azoles and Polyenes

Azoles and Polyenes are known to inhibit *Candida* growth via interactions with the ergosterol metabolic pathway, and by forming stacking barrels with ergosterol containing membranes, respectively. The results reported in **Table 1** highlight the employment of different resistance mechanisms by the *C. albicans* strains used in this study. The major *C. albicans* drug resistance mechanisms reported in previous studies are: mutation in the ERG11 gene, overexpression of the mutated gene, and overexpression of the efflux pump. Mutation and/or overexpression of the ERG11 gene alter the structure of Lanosterol 14 α -demethylase, the substrate to which azoles bind, thereby rendering the azole ineffective. Overexpression of the efflux pump causes the drug to be ejected out of the cytosol, resulting in a similar consequence as with the previous two mechanisms.

All six resistant *Candida* strains tested in this project showed growth inhibition with increased Fluconazole concentrations in **Figure 10**. Strain 4568 was observed to be the most sensitive to Fluconazole, yet the most resistant to Amphotericin B. This suggests that Strain 4568 is susceptible to azole blocking of ergosterol synthesis, yet that it is able to overcome the polyene's disruption of the cells' ergosterol membrane. This resistance mechanism might be manifested via a loss of function mutation in the ERG3 gene which leads to blocking the production of ergosterol and the formation of a drug-lipid complex (Kelly et al., 1997).

Strains 4568, 17, and 14 showed intermediate resistance to Fluconazole, and likely employ a different pathway of resistance other than ERG11 mutation or overexpression. Strains 1, 3 and 102611 are clearly the most resistant to Fluconazole, and likely employ different resistance mechanisms.

Quantification of Antifungal Activity of Plant Essential Oils: Terpenes

Plant essential oils are documented to have antimicrobial action. Research suggests that terpenes destroy and damage the cellular membrane, moving into the cell membrane by diffusion (Andrews et al., 1980). The results reported in **Table 2** show the antifungal activity of both Geraniol and Carvacrol, pointing to the latter to be more effective at inhibiting the growth of the tested isolates. Even though the strains are resistant to Fluconazole, the terpenes were able to uniformly inhibit their growth at low concentrations of over a range of 0.5—2 mg/ml for Geraniol, and <0.125—1 mg/ml, when Carvacrol was used. This shows that the azole resistance mechanisms in these isolates were ineffective at overcoming the antifungal activities of these terpenes.

A trend of growth inhibition across the five *Candida* strains with increased Geraniol concentrations was seen in **Figure 11**. This demonstrates the activity of Geraniol alone on *C. albicans*.

Interactions Between Fluconazole and Geraniol

The data in **Figures 12-16** clearly show greater inhibition of *Candida* growth by the Fluconazole-Geraniol drug combination when compared with just Fluconazole alone. The figures demonstrate that ineffective levels of fluconazole alone become active in combination with sub-MIC levels of Geraniol (0.125 mg/ml), substantially inhibiting resistant *C. albicans* growth. The Fluconazole only curve across the five strains shows a high initial OD reading due to higher growth levels at ineffective Fluconazole levels. These OD readings decrease with increasing Fluconazole concentrations to equal to and above MIC levels. The Fluconazole-Geraniol data-set for every strain shows a much lower initial OD for sub-MIC Fluconazole and

sub-MIC Geraniol concentration in combination. In summary, the sub-MIC drug combination yielded greater killing of the four resistant strains as well as the sensitive strain.

The synergy results reported in **Table 3** report FIX values of below 0.5 for all five tested strains, which demonstrates that the investigations were successful in proving that Geraniol has the potential to enhance the antifungal activity of Fluconazole against drug-resistant *C. albicans*.

Reliability of Results

Due to inevitable pipetting variations between operators, the MIC determined for the *Candida* isolates varied with an error of about +/- one well on the 96-well plate. Also, the average of two experiments was taken for each MIC value reported in **Tables 2-3** as well as **Figures 10-16**. Looking at the bigger picture, this variability does not cause a significant difference in fungistatic activity of the terpene, or in the FIX value calculated. As such, the MIC data and synergy results reported allow us to conclude Geraniol has the potential to enhance the antifungal activity of Fluconazole against drug-resistant *C. albicans*.

Future Implications

Further studies should be conducted using different antifungal drugs and terpenes. The enhancing potential of Geraniol on other azoles like Ketoconazole, as well as polyenes like Amphotericin B should be investigated. A deterring factor in the clinical use of Amphotericin B is the fact that it is toxic in high concentrations. The potential for Geraniol to reduce the MIC value of Amphotericin B should therefore be explored with the goal of rendering Amphotericin B potent at sub-toxic levels.

The results reported in this study show that Carvacrol is a more potent terpene than Geraniol. Further studies should therefore be conducted into the effect of Carvacrol on other anti-fungal drugs with the hope that it can reduce the MIC values to lesser ones than those reported for Fluconazole-Geraniol combinations.

Investigations should also be performed to conduct efficient loading of the antifungal-terpene drug combinations into micro-particles or liposomes. These studies would facilitate more effective drug delivery to targeted tissues, such as macrophages. In addition, the next step of this study should involve testing the drug-combinations *in vivo*, in mice. A crucial part of this step will involve monitoring the drug toxicity levels *in vivo*, as well as determining that targeted delivery is achieved. This can be done by attaching a fluorescent compound, such as fluorescein to the drug and monitoring the organs of the mouse to which delivery is achieved. The time required achieving delivery using different delivery vehicles such as glucan microparticles and liposomes could also be scored to determine the most effective delivery mechanism.

The grander impact of these results involves the use of antifungal-terpene drug combinations to yield greater potency against fungal infections. The increased potency of the drug combinations could result in the elimination of the problem of the reoccurrence of *Candida* infections due to resistant strains, and could save resources currently used by hospitals and patients to battle resistant *Candida* infections.

CONCLUSIONS

Our results demonstrate synergy between Fluconazole, an antifungal drug, and Geraniol, a monoterpenoid component of an essential plant oil. The fact that essential plant oils lack harmful compounds makes Geraniol a good enhancer for antifungal activity. This demonstrated relationship should be investigated for additional antifungals and essential plant oils. The drug combinations should be loaded in suitable drug delivery vehicles to facilitate targeted delivery to tissues and their effect tested *in vivo* in order to monitor toxic effects and tissue specificity. On a grander scale, the demonstrated synergy offers the potential of significantly reducing the issues with recurrent *Candida* infections due to resistant strains.

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