

COMPARATIVE ANALYSIS OF ANTIFUNGAL ACTIVITY OF NATURAL
REMEDIES VERSUS MICONAZOLE NITRATE SALT AGAINST
CANDIDA ALBICANS

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Abstract

Several natural inhibitors and miconazole nitrate salt were evaluated for their antifungal activity against *Candida albicans*. Spectrophotometer analysis was used to determine the growth rates of *C. albicans* in the presence of specific inhibitors. Garlic, grapefruit seed extract, tea tree oil, and probiotics were investigated for their potential anti-*Candida* activity at different concentrations. Miconazole nitrate salt suppressed the growth rates of *C. albicans* to $< 0.02 \text{ hr}^{-1}$ at concentrations above $100 \mu\text{g mL}^{-1}$. Of the natural inhibitors investigated, grapefruit seed extract exhibited the highest anti-*Candida* activity at concentrations of $100 - 120 \mu\text{g mL}^{-1}$, lowering the growth rate to below 0.02 hr^{-1} .

1. Introduction

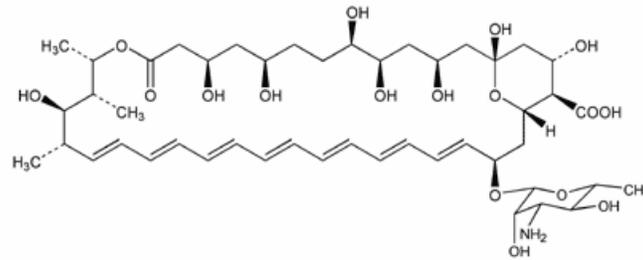
Candida albicans is a yeast-like fungus that can be found to naturally colonize the skin, buccal mucosa, intestinal tract, and vagina mucosa. Classified as an opportunistic pathogen, changes in physiology may allow *C. albicans* to cause infection. *C. albicans* is the primary causative agent of candidiasis, the most common form of mycotic infection (Wilson et al., 1970). Risk factors that may increase the incidence of *Candida* infection include compromised immunity, hormonal imbalances, use of broad spectrum antibiotics, use of oral contraceptives, pregnancy, metabolic and nutritional disorders, and poor oral hygiene (Nader-Djalal et al., 1998).

More than 50% of women over 25 have had at least one episode of vulvovaginal candidiasis (Ringdahl, 2000). Pregnancy, contraception, and other causes of hormonal imbalance may affect the normal acidic environment of the vagina, increasing susceptibility to infection. Diabetes and diets rich in carbohydrates may also contribute to chronic yeast infections (de Leon et al., 2002).

Infections caused by *C. albicans* in immunocompetent individuals may include oral thrush, vulvar rash, vaginitis, conjunctivitis, endophthalmitis, diaper rash, and infections of the nail, rectum, and other skin folds. Infections in immunocompromised individuals include invasive systemic illnesses such as myocarditis, hepatosplenic abscesses, pulmonary infection, central nervous system infection, and chronic disease (Nader-Djalal et al.).

There are three classes of systemic antimycotic agents: polyenes, flucytosine, and ergosterol biosynthesis inhibitors (Merck et al., 1989). Amphotericin B is a polyene

antimycotic that has been the standard antifungal treatment for the past 40 years (Keele et al., 2001).

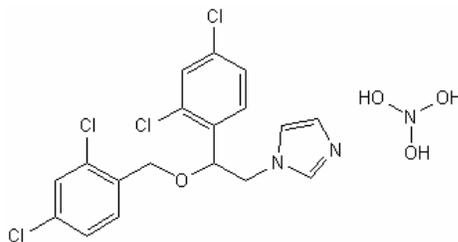


amphotericin B

Amphotericin B inhibits fungal activity by binding to ergosterol, the primary sterol of the fungal cell membrane, disrupting the osmotic integrity of the fungal membrane and resulting in leakage of intracellular potassium, magnesium, sugars, and metabolites and causing cellular death (Terrell et al., 1992). Amphotericin B is administered intravenously and exhibits low rates of resistance and true fungicidal activity. However, Amphotericin B is used cautiously due to its toxicity and possible side effects (Richter et al., 2004). Recent developments in lipid formulations of Amphotericin B have been useful in reducing its toxicity (i.e., ABLC, ABCD, L-AMB, Liposomal Nystatin) (Anderson et al., 1995).

The development of azole antifungals quickly took precedent as an easily accessible,

Miconazole Nitrate



less toxic form of treatment. Azole antifungal agents inhibit the synthesis of ergosterol by inhibiting the cytochrome P450 responsible for 14-alpha-demethylation (Merck et al., 1989). This class of ergosterol biosynthesis inhibitors is lower in toxicity but only exhibits fungistatic properties (Richter et al., 2004).

Although intravenous polyene antibiotics and oral and topical azole therapies are proven to be effective in treating *Candida* infections, adverse effects to drug use can be a major deterrent. Oral antifungals are not recommended during pregnancy and breast-feeding due to potential adverse effects. While prescribed antifungals may be necessary in the treatment of invasive or chronic infections, natural remedies may offer a safer alternative for less severe infections. Alternative forms of treatment may also address the growing problem with the development of drug resistance. Previous studies have shown that over-the-counter treatments may promote azole resistance in *Candida* spp (Cross et al., 2000).

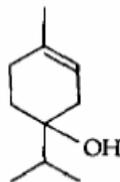
The recent craze in natural health and wellness has also contributed to the growing interest in commercially available naturopathic remedies. Although many herbal products have proclaimed to have antifungal properties, few scientific studies have compared the efficacy of natural antifungal agents. Garlic, grapefruit seed extract, tea tree oil, and probiotics were tested in this experiment for possible antimycotic activity.

Garlic has been used throughout history as a safe and natural remedy for a variety of ailments such as snake bites, parasitic infections, abdominal pains, rheumatism, and hemorrhoids. Studies have shown garlic extract to exhibit a range of antimicrobial affects including the ability to inhibit *C. albicans* (Ankri et al., 1999). Allicin, the active

antifungal ingredient found in freshly crushed garlic, has also been suggested to have immunostimulatory effects, making it a good candidate for prophylactic use.

Grapefruit (*Citrus paradisi*) seed extract is derived from the pulp and seeds of grapefruit. GSE products are commonly used as naturopathic remedies, supplements, disinfectant and sanitizing agents as well as preservatives in the food and cosmetics industry. GSE is commercially available at many health food stores, with claims of having beneficial health properties including the treatment of yeast infections. Previous in vitro studies have demonstrated the antimicrobial properties of GSE against a range of gram-positive and gram-negative organisms (Heggors et al., 2002). Although there have been studies of GSE effectively inhibiting bacteria and fungi, studies have shown that the synthetic chemical preservatives added to commercially available GSE enhances antimicrobial and antifungal activity (Woedtke et al., 1999). The most recent study using natural ethanolic extract of grapefruit seeds and pulp found that the extract exhibited antimicrobial efficacy, including the inhibition of *C. albicans* (Cvetnic et al., 2004).

Melaleuca alternifolia (Tea Tree) Oil has been used medicinally in Australia for its antimicrobial and anti-inflammatory properties. It is mostly used in the manufacturing of antiseptic agents, cosmetics, and germicides. Tea tree oil is a clear liquid with mobile consistency and a distinct odor, and has been shown to effectively treat dandruff and oral candidiasis in clinical trials. There have also been studies that report tea tree oil's success



Terpinen-4-ol

in treating mucous membrane infections such as *Trichomonas vaginalis*. The most common antimicrobial compounds found in tea tree oil include terpenen-4-ol, linalool, and alpha-terpineol. These components have been shown to affect the bacterial cell wall and to inhibit glucose-dependent respiration. In-vitro studies demonstrated Terpenen-4-ol, the main component of tea tree oil, to suppress the activity of inflammatory mediator production by monocytes and to have low cytotoxic effects on human fibroblast cells (Halcon et al., 2004, Soderburg et al., 1996).

The ecological balance of the intestine can be favorably altered upon the ingestion of probiotics. Probiotics are micro-organisms that exert health benefits beyond innate general nutrition. The qualifying characteristics defining a probiotic includes: resistance to acid and bile, ability to attach to human intestinal mucosal cells, capability of colonization of the human intestinal tract, ability to produce antimicrobial substances, ability to demonstrate probiotic activity and to survive modern manufacturing processes, and must be of human origin (O'Sullivan et al., 2001). Their role in the prevention and treatment of different gastrointestinal disorders continues to be supported by ongoing research.

Therapeutic activity of probiotics is due to lactic acid production and microbial attachment to enterocytes. Microbial attachment can lead to displacement of intestinal pathogens and cell resistance to viral attack. The presence of lactic acid alters the composition of the flora, which are also active against pathogens. Additional benefits include mucosal micronutrient production and elimination of toxins. Consumption of live-cultured *L. acidophilus* through supplements or yogurt has been recommended to decrease episodes of bacterial vaginosis (Tao et al., 1997). It is unknown whether *L.*

acidophilus and other probiotics have similar effects on *C. albicans*. The probiotics used in this experiment include *Bifidobacterium lactis*, *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, and *Streptococcus thermophilus*. The probiotics were grown for 24 and 48 hours and then centrifuged to remove the cells. The supernatant of the probiotics were tested for possible antifungal properties.

The objective of this study was to determine the ability of specific concentrations of natural remedies in comparison to azole antifungals in the inhibition *C. albicans*. Spectrophotometric methods were used to observe the effects of potential antifungals on the growth rate of *C. albicans* over 24 hours. The antifungal activity of Amphotericin B was also determined as a known standard antimycotic to evaluate the potential antifungals against.

2. Materials and Methods

2.1. Antifungal Agents

Amphotericin B (MP Biomedicals, Inc., Solon, OH), Miconazole Nitrate Salt (Sigma Chemical Co., St. Louis, MO), Grapefruit Seed Extract (NutriBiotic, Lakeport, CA), Tea Tree Oil (Thursday Plantation Health Ltd., Ballina, Australia), *iFlora* Probiotics (Sedona Labs, Inc., Cottonwood, AZ), Garlic (local grocery market).

2.2. Test Isolates

Candida albicans was obtained from the Department of Microbiology, California Polytechnic State University, San Luis Obispo, CA. *C. albicans* was subcultured and isolated on Sabouraud Dextrose Agar and Broth. Cultures were inoculated from isolated colonies into Sabouraud Dextrose Broth and grown aerobically at 37°C. Growth was monitored by spectrophotometer readings for approximately 24 hours until stationary phase.

2.2. Preparation of standard solutions

2.2.1. Amphotericin B

1 ±.1 mg of Amphotericin B was accurately weighed using an analytical balance and dissolved in 50µL of Dimethyl Sulfoxide (Sigma Chemical Co., St. Louis, MO). A stock solution was prepared using deionized water to dilute to a final concentration of 1 mg mL⁻¹, then stored at 4°C until use. Further dilutions were prepared using deionized water to obtain a range of 0.04-5 µg mL⁻¹. Growth controls were conducted using dimethyl sulfoxide to verify the lack of an inhibitory affect on *C. albicans*.

2.2.2. Miconazole Nitrate Salt

1 ±.1 mg of Miconazole Nitrate Salt was quantitatively weighed using an analytical balance and dissolved in 100 µL of DMSO. A stock solution of 1 mg mL⁻¹ was prepared in deionized water and stored at -4°C until use. Further dilutions were prepared from the stock solution using deionized water to obtain a range of 10-120 µg mL⁻¹.

2.2.3. Grapefruit Seed Extract

Approximately 10 mg (1 drop) of Grapefruit Seed Extract, *liquid concentrate*, was diluted in 10 mL of deionized water and stored at -4°C. Dilutions were prepared in deionized water to obtain a range from 10-120 µg mL⁻¹.

2.2.4. Tea Tree Oil

100 µL of tea tree oil (87.6 mg) was dissolved in 900 µL of dimethyl sulfoxide. The tea tree oil was further diluted in deionized water to obtain a stock concentration of 1 mg mL⁻¹. Dilutions were made to obtain a range of 10-120 µg mL⁻¹.

2.2.5. Probiotics

One capsule of iFlora yeast/*Candida* control formula containing 15 billion viable cells of six strains of probiotics was cultured in approximately 5 milliliters of Trypticase Soy Broth and incubated at 37°C in an automated shaker. One culture was incubated for 24 hours and one for 48 hours. The cultures were then centrifuged through a filter at 13000 rpm for 3 minutes. Dilutions were made using deionized water to obtain a range of 10-120 µg mL⁻¹.

2.2.6. Garlic Juice

Cloves of garlic were peeled and pressed through a garlic presser into 8 layers of cheesecloth then squeezed to extract garlic juice. The liquid was then centrifuged with a filter at 13000 rpm for 3 minutes. The liquid was then weighed and a stock concentration of 1mg mL^{-1} was prepared using deionized water. Further dilutions were made to obtain a range of $10\text{-}120\ \mu\text{g mL}^{-1}$

2.3. Growth Rate Studies

C. albicans was subcultured from isolated colonies on Sabouraud dextrose agar plates and suspended in Sabouraud dextrose broth. The culture was grown aerobically in an automated shaker set at 37°C for approximately 24 hours, until stationary phase. $20\ \mu\text{L}$ of *C. albicans* and $30\ \mu\text{L}$ of inhibitor (concentration adjusted for a total volume of 200) was added to $150\ \mu\text{L}$ of Sabouraud dextrose agar in a microtiter plate. $30\ \mu\text{L}$ of deionized water was used for the controls. Spectramax software was used to measure the optical density and monitor growth over a period of 24 hours. Conditions for absorbance readings were set at 700nm at 37°C . The growth rate of *C. albicans* and each concentration of inhibitor were performed in triplicates.

2.4. Analysis

Optical density was plotted for each concentration against time (hours). The optical density of the of each triplicate was averaged and plotted. The growth rate is equivalent to the slope of $\log(\text{Optical Density})$ versus time during the exponential phase. A plot of the average growth rate versus concentration was constructed for each inhibitor. The standard deviation for the growth rate at each concentration was also graphed.

3. Results

3.1. Growth Rate Studies

Each of the antifungal agents exhibited a different pattern of activity. The control growth curves showed growth rates ranging from 0.0681-0.1059. Growth Rates above .06 were considered minimally effective at inhibiting growth; concentrations producing growth rates above .08 were considered non-inhibitory. Concentrations producing growth rates of ≤ 0.02 were considered inhibitory.

3.2. Amphotericin B

Concentrations above the literature MIC concentration (MIC = 0.13 $\mu\text{g/mL}$) exhibited full inhibitory effects after the initial 2 hours. SubMIC concentrations were slightly inhibitory, though not fungicidal. A concentration of 1.0 $\mu\text{g mL}^{-1}$ of amphotericin B was suppressive for the first 20 hours. Growth rates gradually increased for dilutions 0.50 - 0.04 $\mu\text{g mL}^{-1}$. Concentrations of 0.1 - 0.04 $\mu\text{g mL}^{-1}$ were non-inhibitory, producing growth rates similar to that of the controls.

3.3. Miconazole Nitrate Salt

Miconazole Nitrate Salt completely inhibited growth after two hours at concentrations of 110-120 $\mu\text{g mL}^{-1}$. A concentration of 100 $\mu\text{g mL}^{-1}$ reached a stagnant growth rate after 5-7 hours. Growth Rates gradually increased for concentrations 90 - 10 $\mu\text{g mL}^{-1}$. Concentrations 10 - 30 were non-inhibitory and exhibited full growth rates similar to the control growth rates.

3.4. Grapefruit Seed Extract

Grapefruit Seed Extract exhibited inhibition at concentrations 90-120 $\mu\text{g mL}^{-1}$. At 120 $\mu\text{g mL}^{-1}$, GSE exhibited inhibition on the growth rate of *C. albicans* after the

initial 2 hours. Concentrations 80-110 $\mu\text{g mL}^{-1}$ exhibited inhibition within the first 3-5 hours. A concentration of 70 $\mu\text{g mL}^{-1}$ GSE was suppressive for the first 16 hours. Concentrations 40-60 $\mu\text{g mL}^{-1}$ were minimally inhibitory, and growth rates were concentration-dependent. Concentrations 10-30 $\mu\text{g mL}^{-1}$ were non-inhibitory, exhibiting growth rates similar to the controls.

3.5. *Tea Tree Oil*

Tea tree oil did not exhibit concentration-dependent inhibitory patterns. Concentrations 10- 110 $\mu\text{g mL}^{-1}$, showed a variation of growth rates (.0921-.1229 hr^{-1}) within the range of the control growth rates.

3.6. *Probiotics supernatant*

The supernatant from the 24 and 48 hour probiotic cultures did not exhibit any inhibition on the growth rate of *C. albicans*. The growth rates ranged from 0.1029 - 0.1215 hr^{-1} for the 24 hour culture, and 0.1020 - 0.1254 hr^{-1} for the 48 hour culture, within the range of the control growth rates.

3.7. *Garlic Juice*

The concentrations of 10-120 $\mu\text{g mL}^{-1}$ of Garlic juice did not show any inhibitory patterns on the growth rate of *C. albicans*. The growth rates ranged from 0.0924 - 0.1215 hr^{-1} with no significant correlation to concentration and within the range of the control growth rates.

Fig. 1A. Average growth curves of *C. albicans* with 0.04 - 5.0 $\mu\text{g mL}^{-1}$ of amphotericin B over 24 hours.

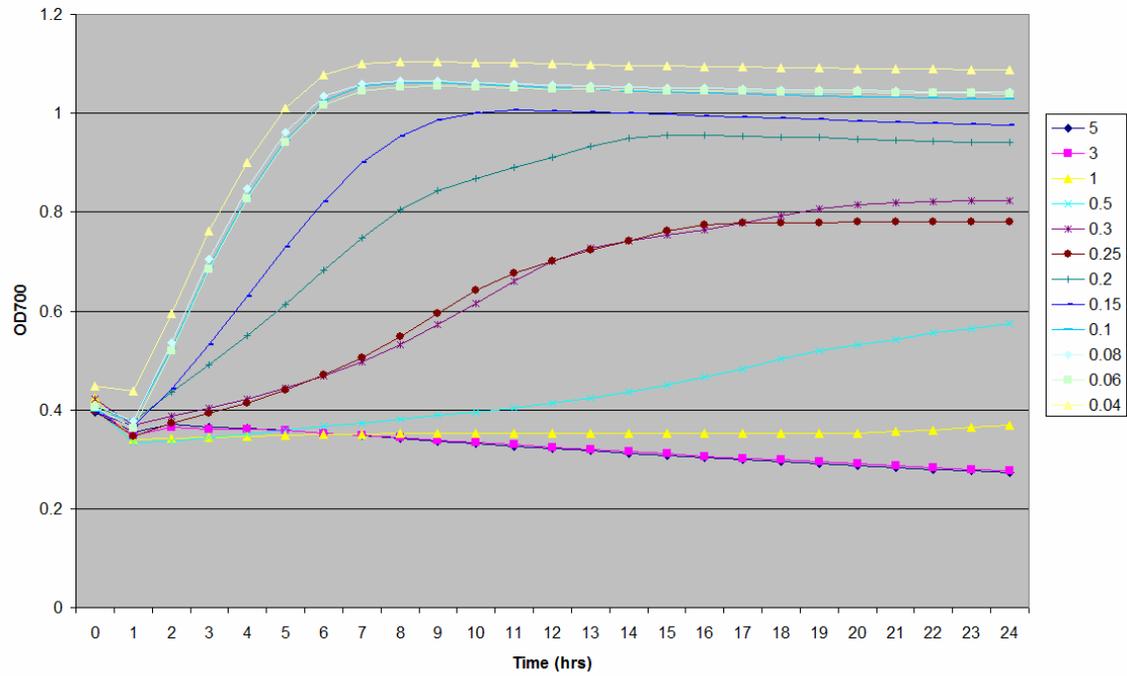


Fig. 1B. Average growth rates of *C. albicans* with 0.04 - 5.0 $\mu\text{g mL}^{-1}$ of amphotericin B.

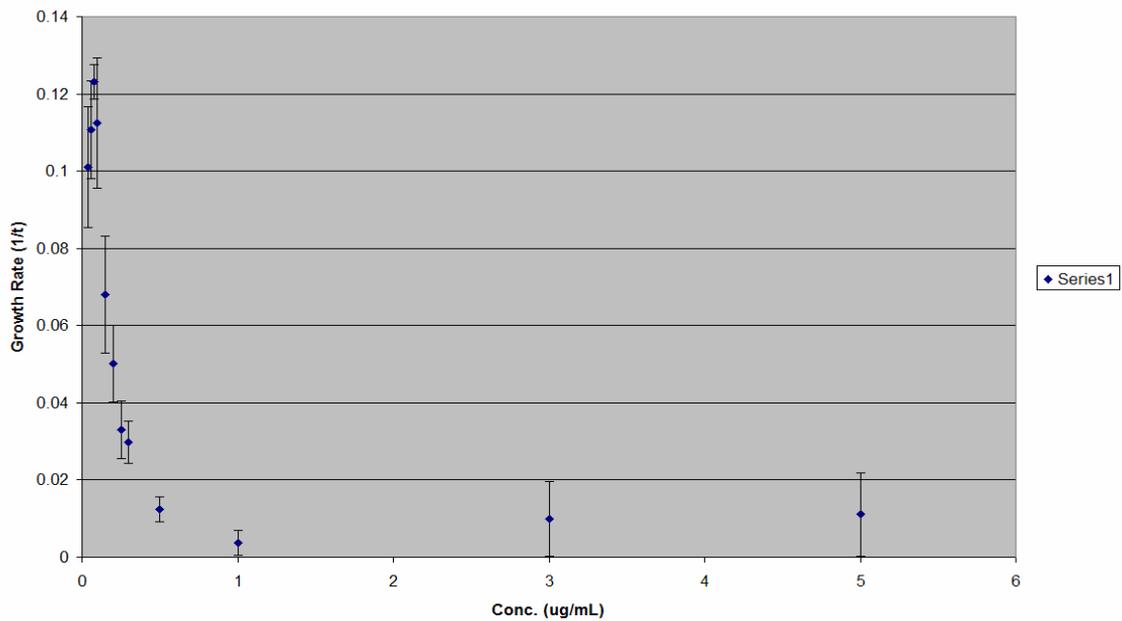


Fig. 2A. Average growth curves of *C. albicans* with 10 - 120 $\mu\text{g mL}^{-1}$ of miconazole nitrate salt over 24 hours.

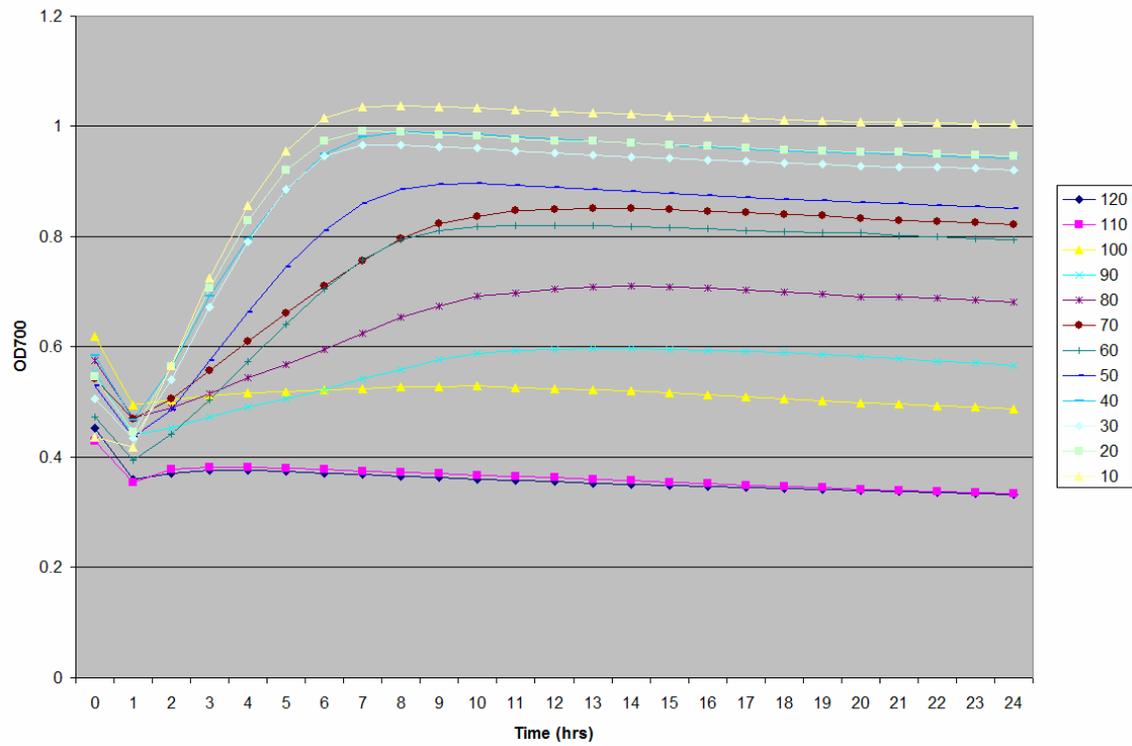


Fig. 2B. Average growth rates of *C. albicans* with 10 - 120 $\mu\text{g mL}^{-1}$ of miconazole nitrate salt.

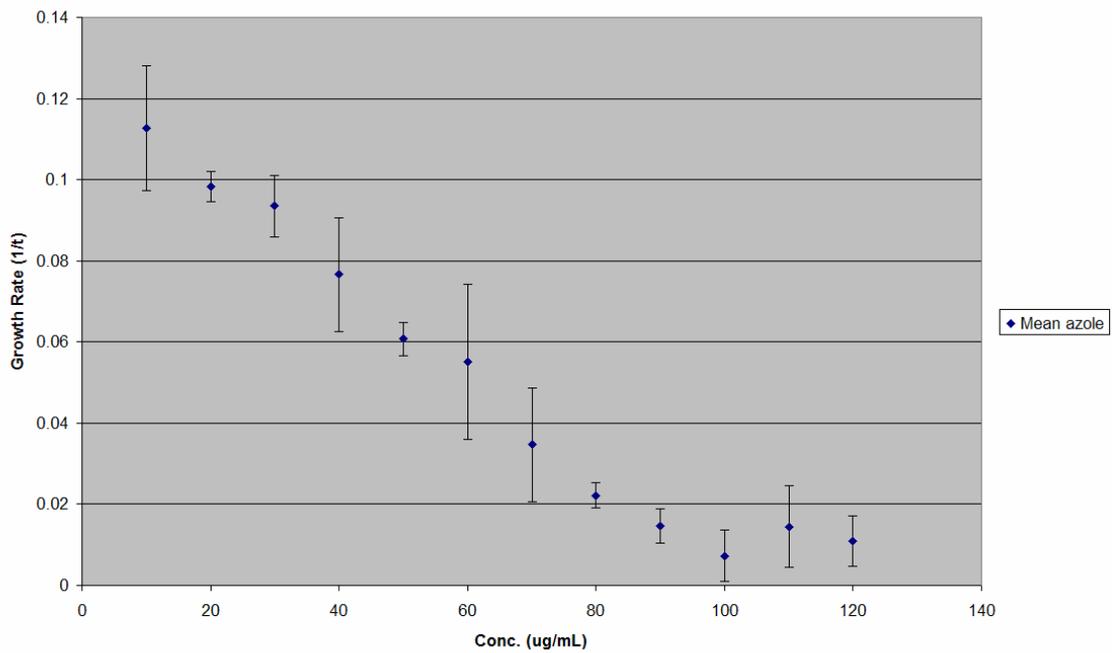


Fig. 3A. Average growth curves of *C. albicans* with 10 - 120 $\mu\text{g mL}^{-1}$ of GSE over 24 hours.

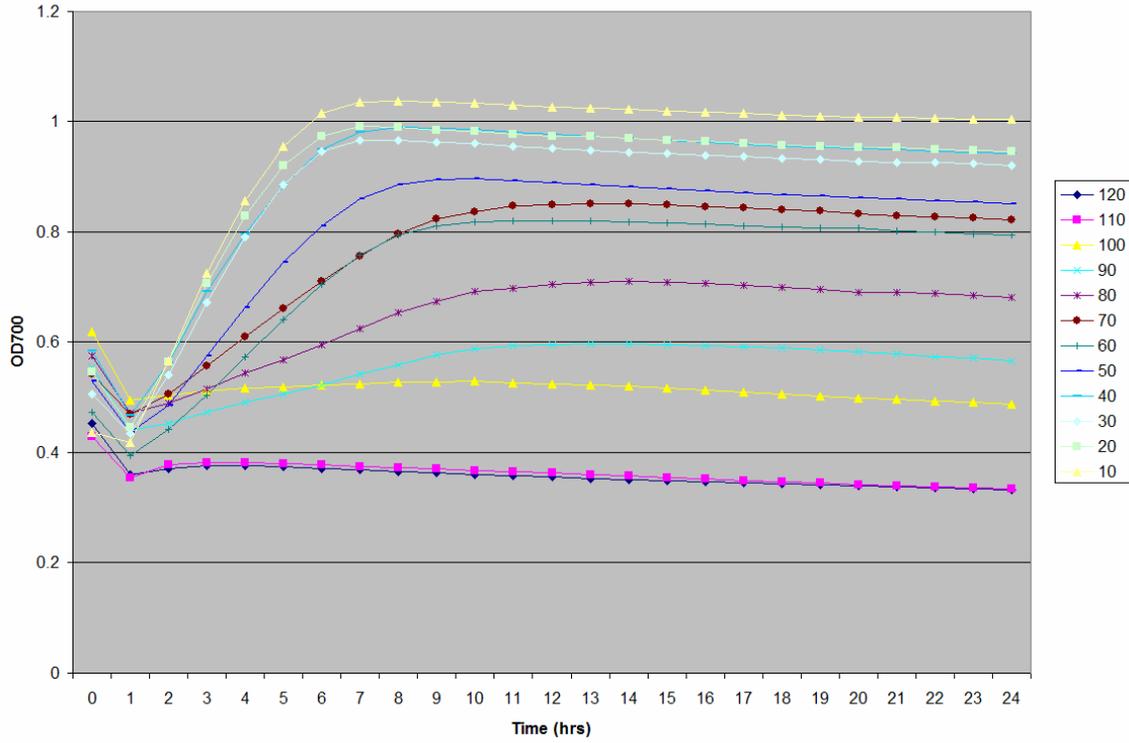


Fig. 3B. Average growth curves of *C. albicans* with 10 - 120 $\mu\text{g mL}^{-1}$ of GSE.

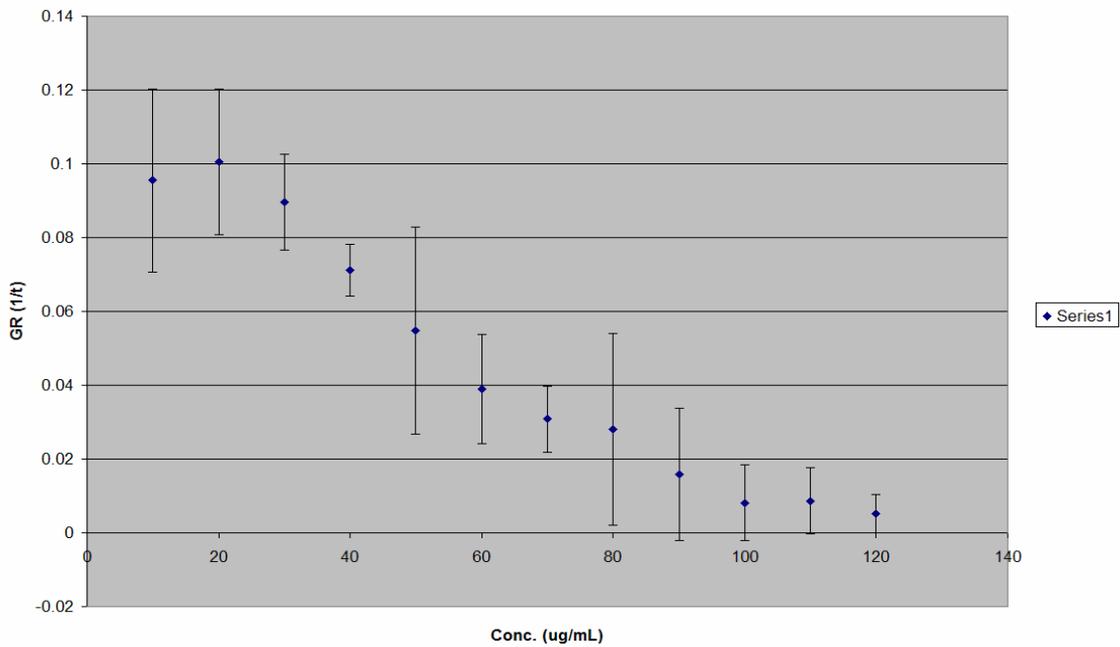


Fig. 4A. Average growth curves of *C. albicans* with 10 - 120 $\mu\text{g mL}^{-1}$ of tea tree oil over 24 hours.

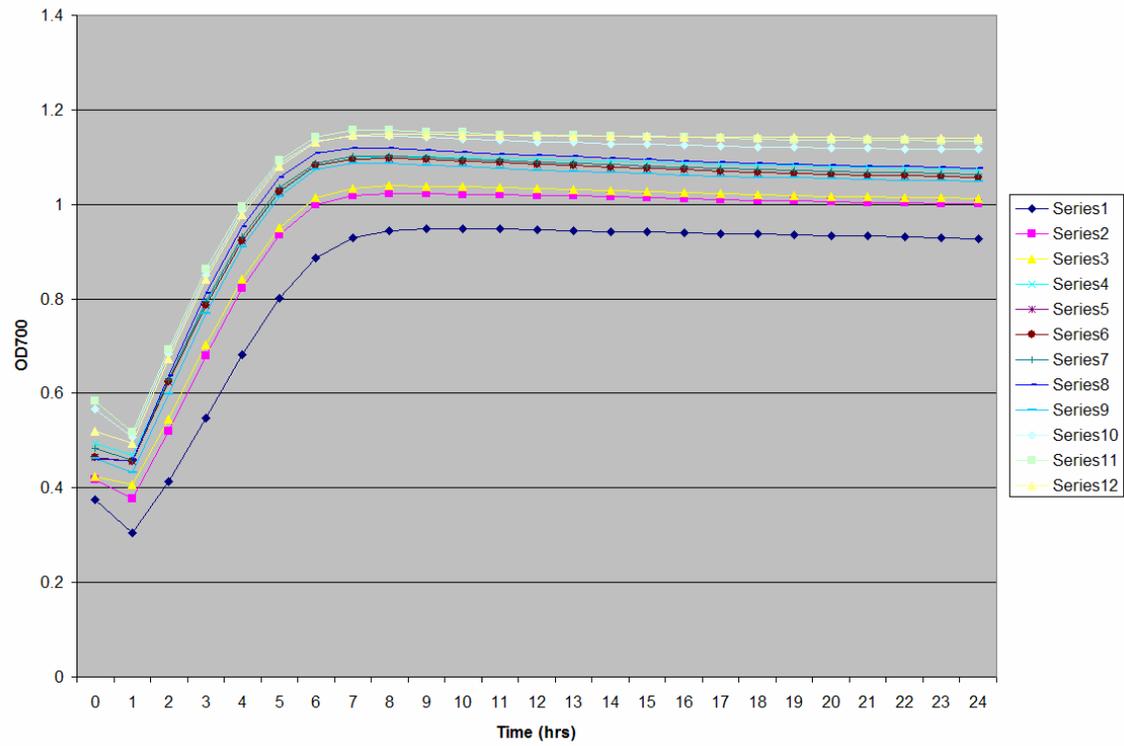


Fig. 4B. Average growth rates of *C. albicans* with 10 - 120 $\mu\text{g mL}^{-1}$ of tea tree oil.

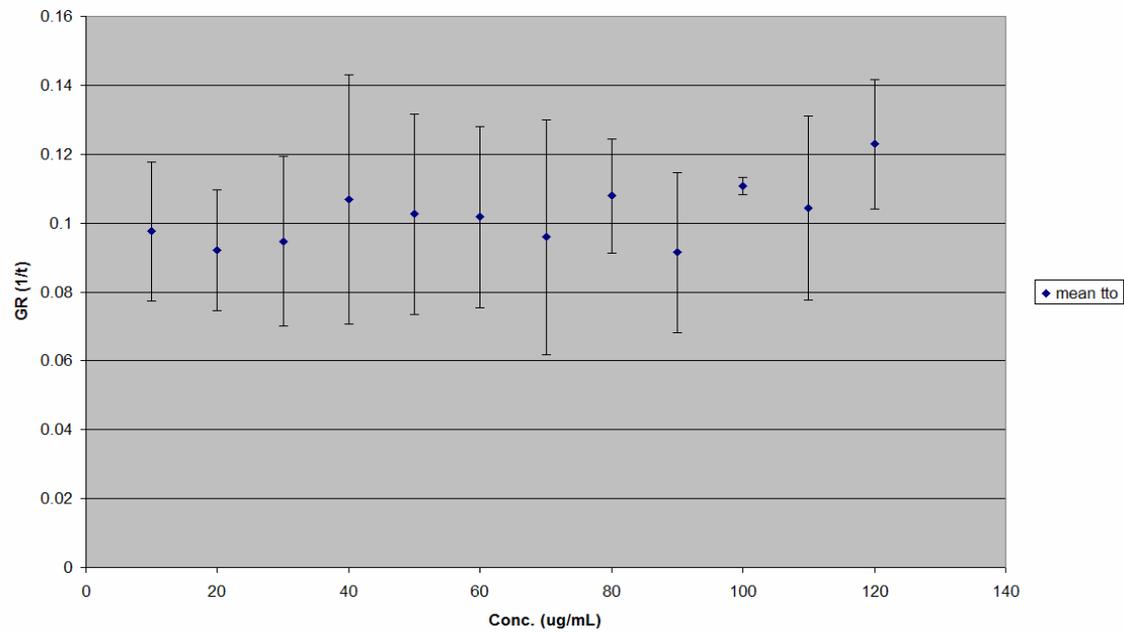


Fig. 5A. Average growth curves of *C. albicans* with 10 - 120 $\mu\text{g mL}^{-1}$ of 24 hour probiotic supernatant over 24 hours.

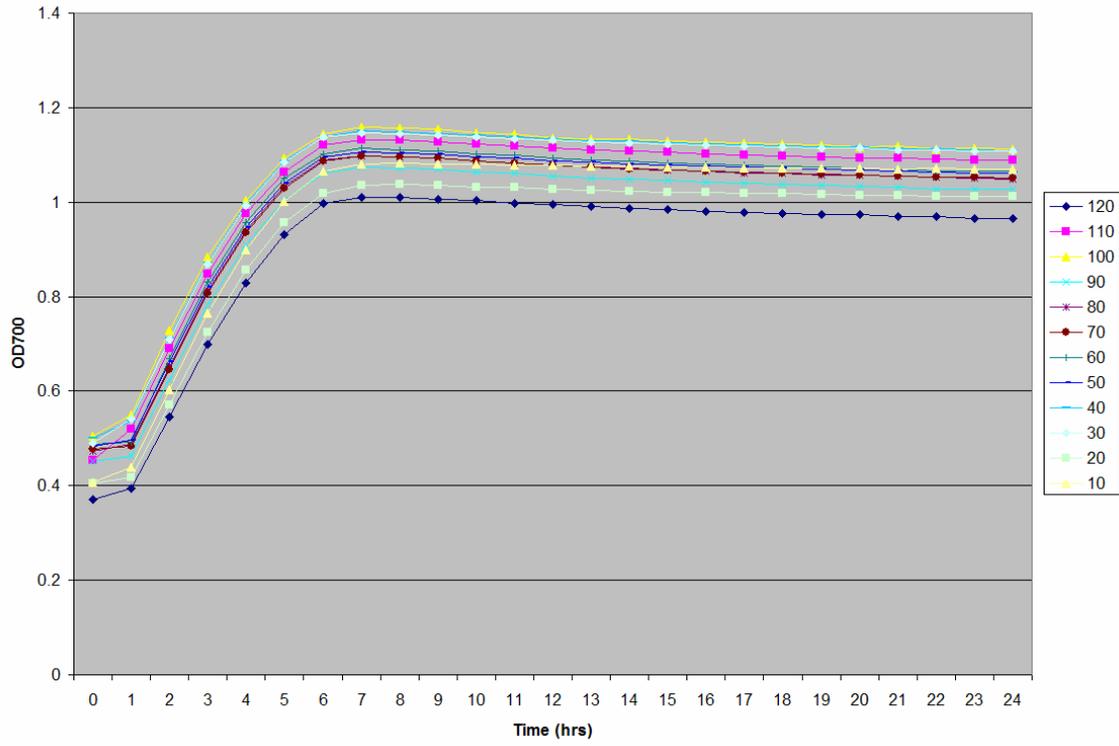


Fig. 5B. Average growth rates of *C. albicans* with 10 - 120 $\mu\text{g mL}^{-1}$ of 24 hour probiotic supernatant.

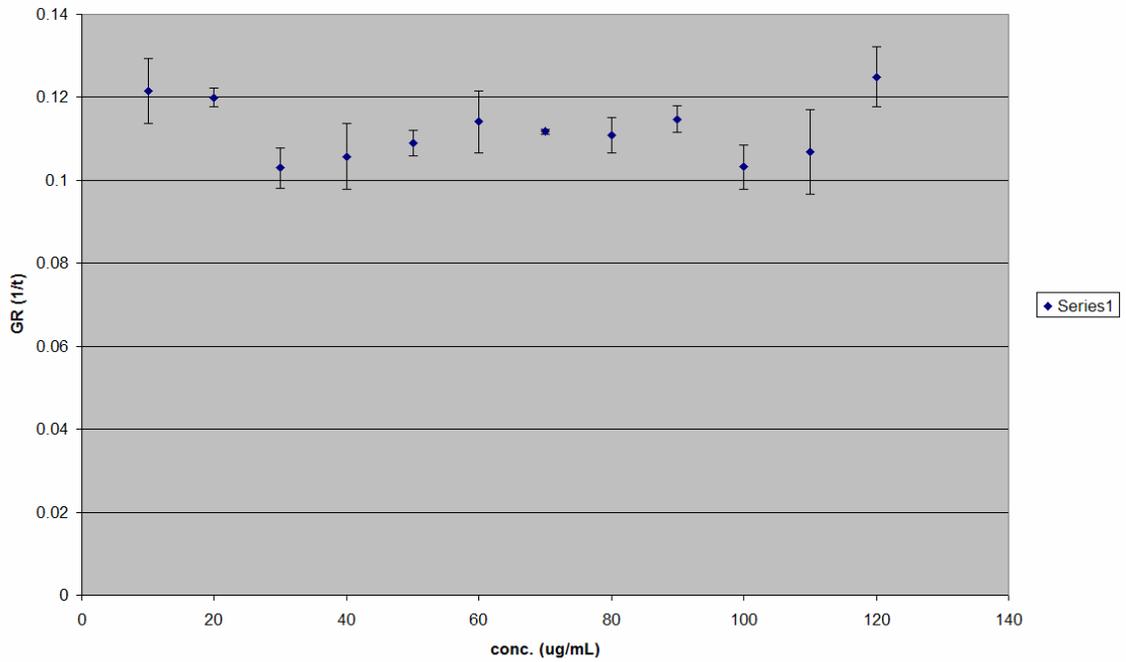


Fig. 6A. Average growth curves of *C. albicans* with 10 - 120 $\mu\text{g mL}^{-1}$ of 48 hour probiotic supernatant over 24 hours.

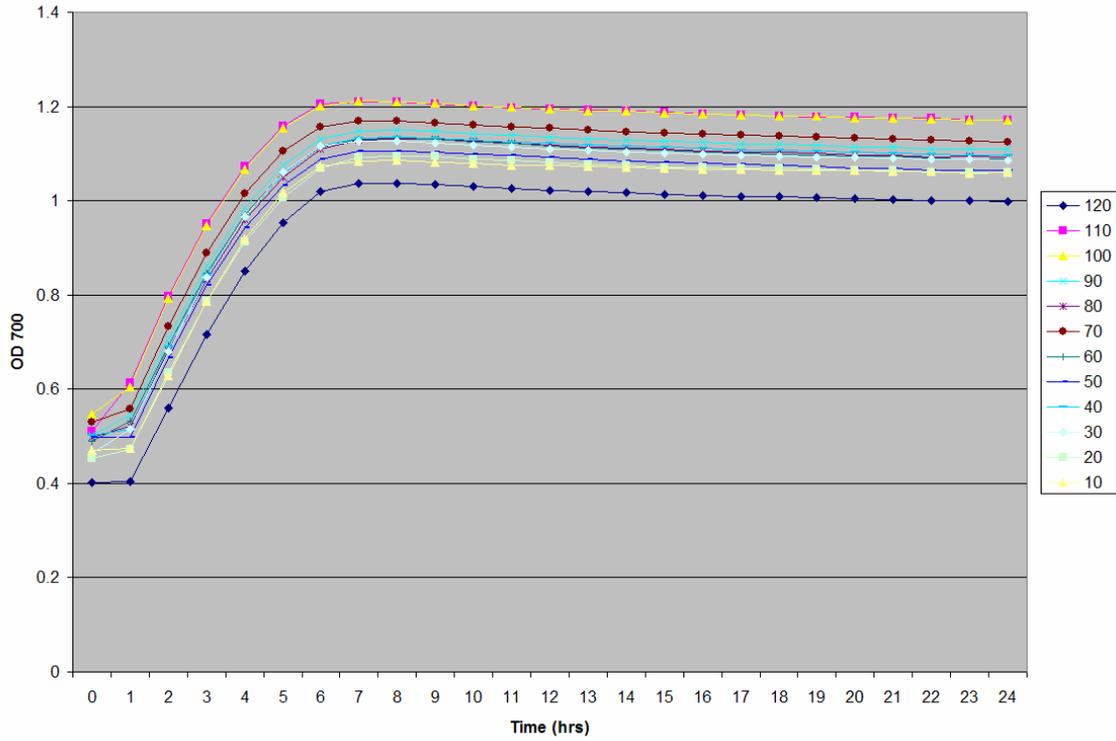


Fig. 6B. Average growth curves of *C. albicans* with 10 - 120 $\mu\text{g mL}^{-1}$ of 48 hour probiotic supernatant.

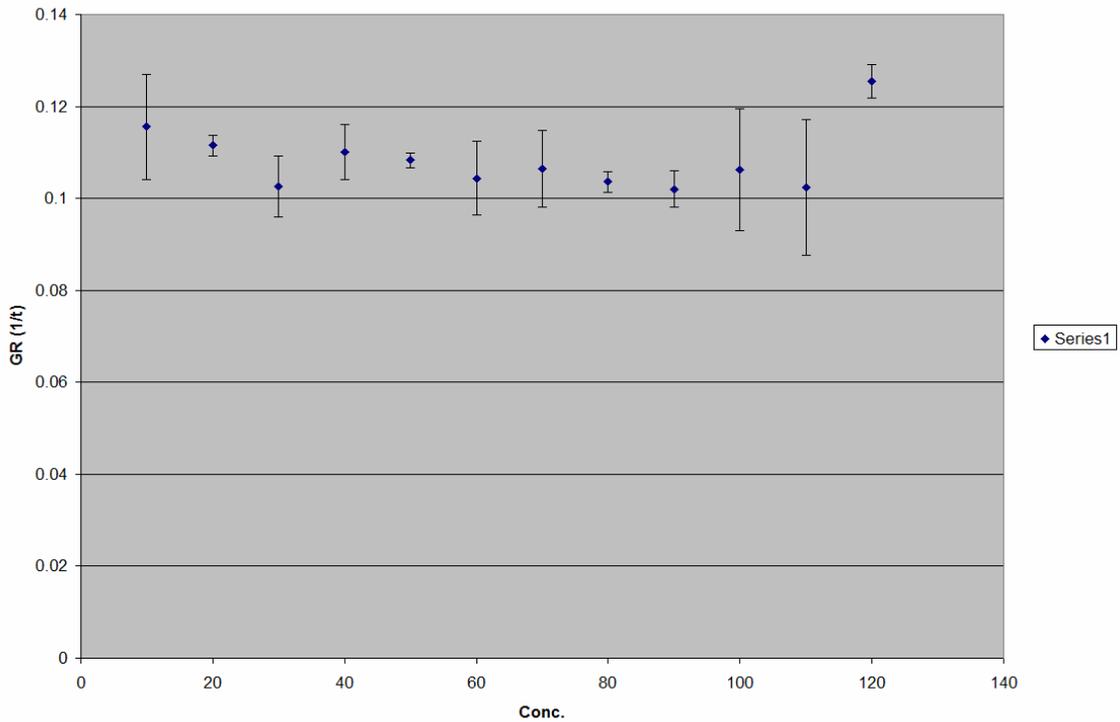


Fig. 7A. Average growth curves of *C. albicans* with 10 - 120 $\mu\text{g mL}^{-1}$ of garlic juice over 24 hours.

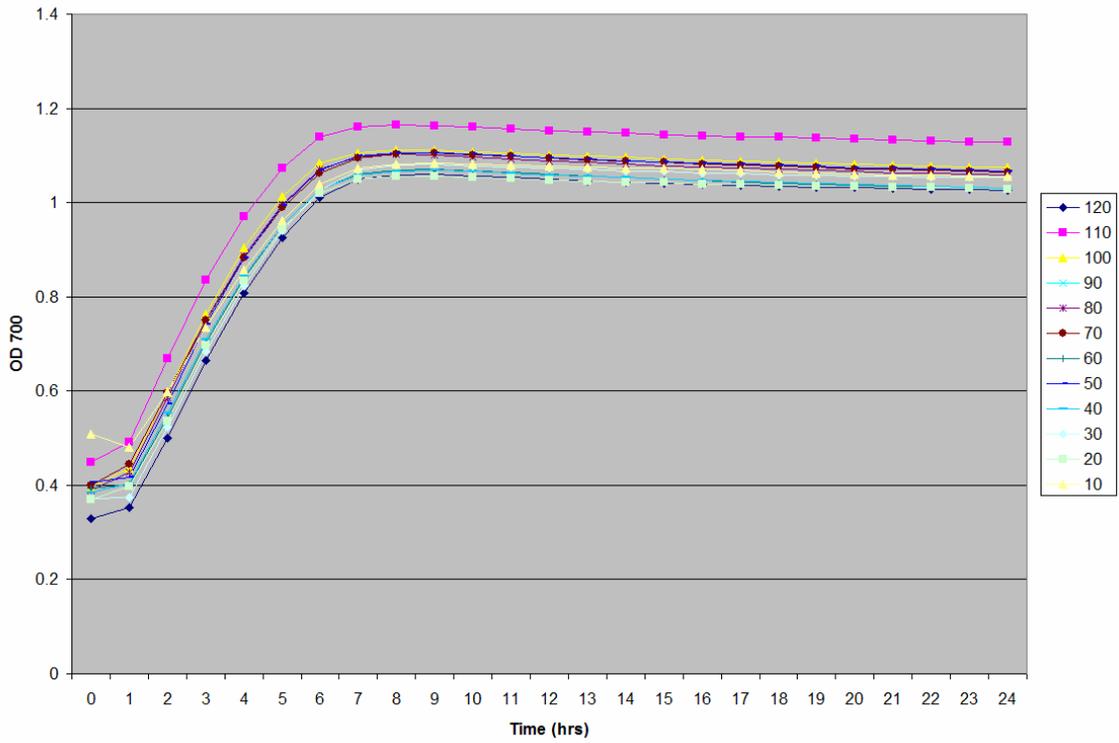
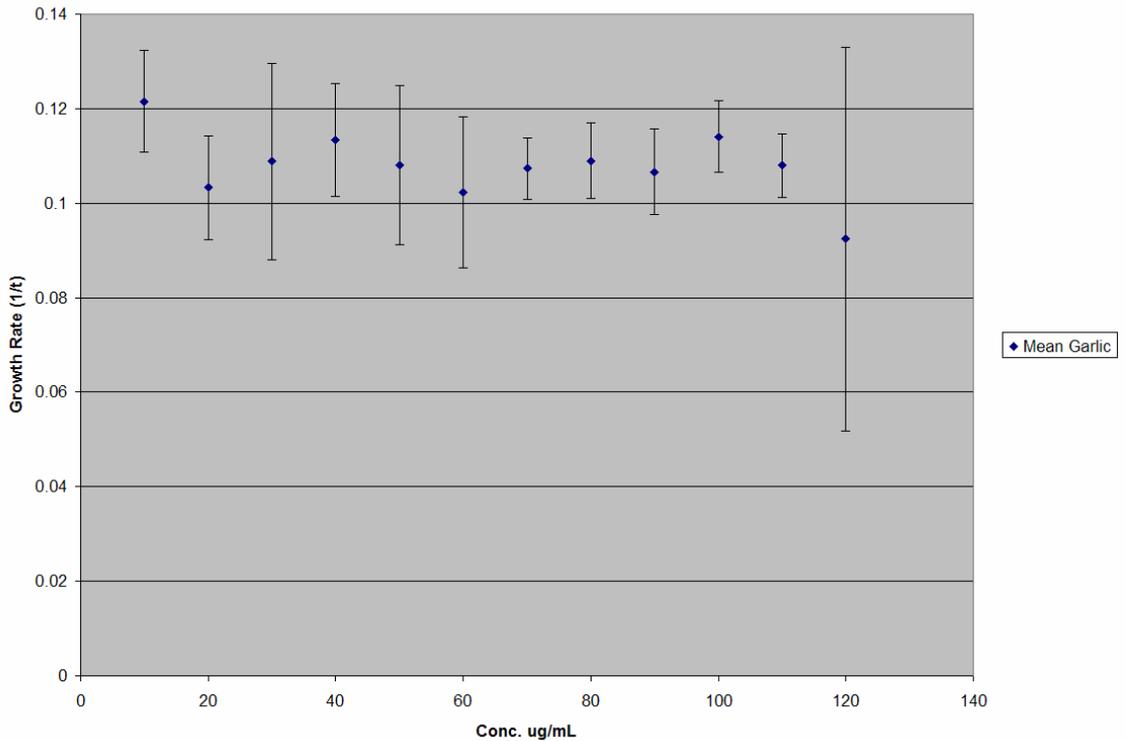


Fig. 7B. Average growth rates of *C. albicans* with 10 - 120 $\mu\text{g mL}^{-1}$ of garlic juice.



4. Discussion

Inhibitory activity of grapefruit seed extract, tea tree oil, garlic, and probiotic supernatant were tested and compared against the anti-*Candida* activity of miconazole nitrate salt. Amphotericin B, the control antifungal agent used in this experiment, exhibited the highest inhibitory activity against *C. albicans*, inhibiting the growth rate to $< 0.02 \text{ hr}^{-1}$ at concentrations as low as $0.50 \mu\text{g mL}^{-1}$ (Fig. 1A, 1B). However, Azole antifungal agents continue to be the predominant drug of choice against superficial *Candida* infections (Elewski, 1998), due to its efficacy and low toxicity in comparison to amphotericin B.

The inhibitory activity of Miconazole Nitrate Salt occurred within the concentration range of $100\text{-}120 \mu\text{g mL}^{-1}$, suppressing the growth rates to $< 0.02 \text{ hr}^{-1}$ (Fig. 2A, 2B). The only natural remedy in this study to exhibit inhibitory activity against *C. albicans* along the same magnitude as miconazole nitrate salt was grapefruit seed extract. GSE exhibited inhibitory activity at concentrations of $100 - 120 \mu\text{g mL}^{-1}$, lowering the growth rate to below 0.02 hr^{-1} . GSE is used in the food industry for its antioxidant and preservation qualities and is considered to have no toxicological significance (Bentivegna et al., 2002).

Tea tree oil, garlic, and probiotic supernatant did not exhibit inhibitory activity on the growth rate of *C. albicans* within the concentration range of $10 - 120 \mu\text{g mL}^{-1}$ (Fig. 4B, 5B, 6B, 7B). Tea tree oil and garlic have been shown to have anti-*Candida* activity at higher concentrations, indicating that they may be useful in the topical treatment of superficial candida infections (Hammer et al., 1998). These natural antimycotic

remedies are likely to not be as effective as azole antifungal agents, and may require higher doses to produce similar results.

The antifungal properties of Probiotics have been suggested to be due to its lactic acid production and microbial attachment to enterocytes. Although the probiotic supernatant and garlic juice did not show any inhibitory activity against *C. albicans* in this study, further studies may evaluate possible antifungal activity at higher concentrations. Due to the limitations of in vitro studies and the spectrophotometer method used in evaluating growth rate, the full potential antifungal activity of probiotics could not be tested. Further studies should be conducted to evaluate the role of the colonization of normal flora and probiotics in prohibiting the overgrowth of potential harmful organisms such as *Candida*. Further clinical studies would also provide a more accurate evaluation of the effectiveness of the natural antifungal agents in comparison to azole antifungal agents.

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