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Use of Origanum vulgare Essential Oil to Combat Human Pathogenic Yeasts

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A Thesis for the Western Kentucky University Honors College

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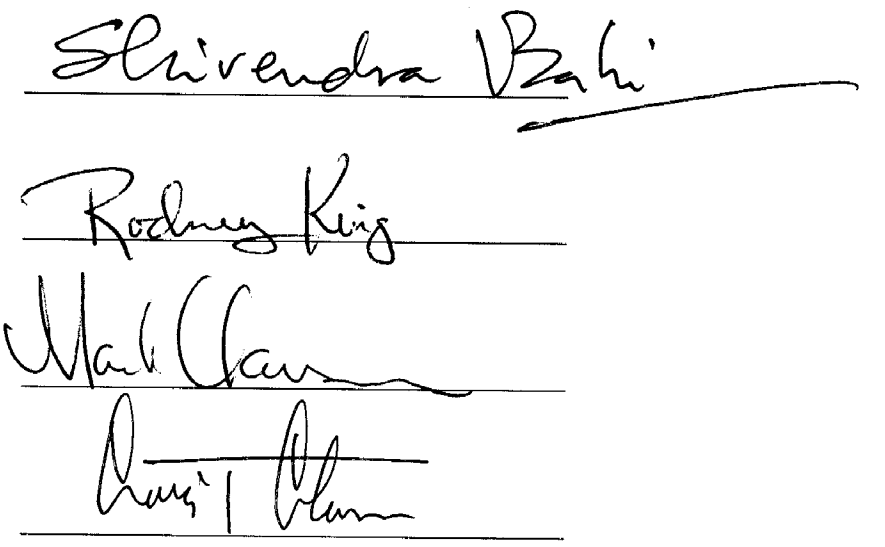
Approved By:



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To my family, friends, and room mates, who put up with thesis-angst, complaining, and endless explanations of just what oregano plants were doing all over the kitchen table: thank you. Without you, I would never have had the determination to finish this project.

ABSTRACT

With the outbreak of autoimmune diseases, pathogenic yeasts have begun to pose a serious medical threat. This threat is compounded as more of these yeasts evolve resistance to existing antifungal drugs. In the ever-widening search for new, effective antifungal treatment, the realm of herbal medicine offers some interesting and potentially valuable alternatives. *Origanum vulgare* is one herb that has demonstrated broad spectrum antimicrobial activity against bacteria and fungi alike. This study undertook to examine the effects of the essential oil of *Origanum vulgare* on *Candida albicans, Cryptococcus neoformans, Rhodotorula rubrum,* and *Cryptococcus albidus*, with the hypothesis that Essential Oil of Oregano (EOO) would prove to be an effective antifungal against these pathogenic yeasts *in vitro*. Upon completion of liquid and solid media growth inhibition testing, it is possible to state that essential oil of oregano exhibits growth inhibitory effects against all four of the tested yeasts at concentrations as low as 200 μg/mL.

INTRODUCTION

The debate over natural, plant-based medicine is one of the hottest issues in the medical field. This debate has intensified over the past several decades as a growing number of studies show that many natural remedies yield positive test results for their supposed medicinal abilities. Digitalis isolated from foxglove and aspirin derived from willow are both examples of widely used natural remedies that can be used to treat heart failure and inflammation/pain, respectively[1, 12, 30]. Taxol is a more recent development. Taxol, or paclitaxel, is a purified version of a compound that was first isolated from the Pacific Yew. It has been shown to be a successful treatment for many different types of cancer[21].

Origanum vulgare, or common oregano, is another herb that is gaining notoriety for its antimicrobial properties. Extensive work has been done to demonstrate the antibacterial action of oregano, and the results depict a broad spectrum of antibacterial effect[2, 6, 11, 23]. Gram negative and Gram positive bacteria—even antibiotic resistant strains—have been shown to exhibit susceptibility to the essential oil of oregano[6]. This wide range of antibacterial effect sets essential oil of oregano apart from many other essential oils, which only show significant bactericidal effects on Gram positive bacteria[6].

The antifungal abilities of oregano have also been addressed—primarily in relation to food borne molds and fungi. The work of Paster at al. clearly shows that essential oil of oregano is able to combat the growth and spore germination of several *Aspergillus* species[24]. Bozin, et al. (2006) conducted another set of experiments that showed the antifungal effects of essential oil of oregano on *Candida albicans,*

Trichophton mentagrophytes, Trichophyton tonsurans, Trichophyton rubrum, Epidermophyton floccosum, and *Microsporum canis*[6]. A few experiments have focused on common yeasts like *Candida albicans* with great success, as demonstrated by Manohar, et al. (2001) and Tampieri, et al. (2005)[22, 31]. In the tests run by Tampieri, et al. (2005), oregano exhibited low minimum inhibitory concentrations that compared favorably with other tested essential oils[31]. Manohar, et al. (2001) showed the fungicidal activity of essential oil of oregano against *C. albicans* both *in vitro* and *in vivo* using mice with systemic candidiasis[22]. The performance of *Origanum vulgare* in these antifungal experiments, and in the Manohar, et al. (2001) *in vivo* test in particular, encouraged further testing of *O. vulgare* for any additional anti-yeast properties it might possess.

The purpose of this project was to expand the existing knowledge of *O. vulgare* activity against human pathogenic yeasts. The yeasts tested (*Cryptococcus neoformans, Rhodotorula rubrum, Candida albicans,* and *Cryptococcus albidus*) represent significant current and emerging threats as human pathogens, especially for immunodeficient patients[13, 14, 16, 19, 28]. Data concerning oregano activity against these yeasts may serve as a starting point for future application of the herb in antiseptic or chemotherapeutic treatment roles.

MATERIALS AND METHODS

Yeast Cultures

Cultures of *C. neoformans* and *C. albicans* were obtained from Presque Island Cultures in Erie, PA. *R. rubrum* and *C. albidus* were obtained from the Western Kentucky University Department of Biology culture collection. Initial culture incubation

was conducted at 37° C for 3 days before cultures were moved to room temperature (25° C). All yeasts were sub-cultured as needed for culture maintenance and inhibition testing.

Culture Media

Initially, all specimens were maintained on DIFCO Sabouraud's Dextrose Agar. They were later cultured on YEPD agar (ATCC medium 1245) for solid media growth inhibition testing, in accordance with literature procedure. YEPD broth (ATCC medium 1245 with no agar) was also used for yeast growth inhibition testing experiments. Media choices were made in accordance with previously published procedures for yeast cultivation, which provided precedent for the usage of both DIFCO Sabourad's Dextrose Agar and YEPD media[10, 13, 14, 31].

Plant Material

Fresh oregano aerial plant material was purchased from Shenandoah Growers in Harrisonburg, VA. All material was shipped overnight from a single source. Upon arrival, stems and leaves were cut into small pieces and air dried for approximately 14 days. Dried material was ground into fine pieces using a GE plant grinder to prepare for oil extraction.

Essential Oil Extraction and Concentration

Dried, powdered oregano (100g) was suspended in Fisher hexanes (>98.5% hexanes) and swirled periodically into a slurry for solvent extraction. After each

suspension, the resulting solution was poured off into a clean flask. The dried, powdered oregano was resuspended a total of three times to complete the solvent extraction. During the process, flasks were covered with aluminum foil in order to prevent contamination and hexane evaporation. Resulting solution of oregano extract in hexane was concentrated by rotary evaporation, where hexane was removed under low pressure at 30° C. Remaining concentrated residue was dissolved in 100% ethanol and again rotary evaporated under low pressure at 35-40 °C. This yielded a second, purer concentrated residue, known as the absolute, which was dissolved in a minimal volume of 100% ethanol, removed from the rotary evaporator, and transferred to a clean, pre-weighed beaker. Ethanol was removed from the absolute via heating in a sand bath. Gravimetric measure was used to determine the mass of absolute remaining.

The extracted absolute was dissolved in 9.6 mL of 100% ethanol to yield a 100 mg/mL concentration of Essential Oil of Oregano (EOO) stock solution. One mL of stock (100 mg/mL) EOO was transferred aseptically to a sterile tube and diluted with 9 mL of 100% ethanol to yield a 10 mg/mL solution of EOO, which was the EOO solution used for the liquid and solid growth inhibition studies.

Liquid Media Inhibition Testing

Growth inhibition of yeast cells in liquid media was tested at concentrations of 200 and 400 μg/mL EOO in culture tubes containing 5 mL of media. Details of tube contents and experimental setup for the liquid media growth inhibition experiment are shown in Tables 1 and 2.

Pre-cultures for *Candida albicans*, *Cryptococcus neoformans*, *Cryptococcus albidus* and *Rhodotorula rubrum* were set up by inoculating 10 mL of YEPD broth with the appropriate yeast strain and incubating for 12-15 hours at 37 °C to ensure that yeast cells were actively dividing[22, 31]. These pre-cultures were then used to inoculate the tubes in the test groups as shown in Table 3.

Tubes were incubated at 37 °C on a rotary shaker. Optical density of cultures was measured at 600 nm. Growth measurements were taken over a period of one week, at ~2 hour intervals during the log phase of growth, followed by ~24 hour intervals after growth rate plateaued. The spectrophotometer was zeroed with sterile YEPD broth. Uninoculated controls of ethanol, 200 μg/mL EOO and 400 μg/mL EOO were monitored (Table 2, Figure 5). Results were then graphed to show change in absorbance over time.

All tests were conducted in triplicate, and each liquid media growth inhibition test was conducted twice for *C. neoformans*, *C. albicans*, and *C. albidus* to verify the effect of EOO for those yeasts. A repetition of the growth inhibition experiment in liquid media was attempted for *R. rubrum*, but the culture did not remain viable long enough to complete the experiment.

Solid Media Inhibition Testing

Solid media growth inhibition tests were conducted using the disk diffusion method [3, 7, 15, 27, 29]. For the solid media inhibition tests, 10 mg/mL EOO solution was aseptically added drop-wise to each 7 mm sterile paper disk. Assuming a consistent drop-size of 0.05 mL per drop, one drop of 10 mg/mL EOO solution yielded 500 μg EOO per disk, while concentrations of 1000 μg and 1500 μg were created by adding two and

three drops of 10 mg/mL EOO, respectively. A control was set up by adding 1 to 3 drops of 90% ethanol to each sterile disk, to ensure that residual ethanol was not responsible for any observed antifungal activity. All sterile disks were then allowed to air-dry in a sterile hood for a minimum of one hour, or until ready for application to the agar plate.

Candida albicans and *Cryptococcus neoformans* were assessed for oregano susceptibility using the disk diffusion method. *Candida albicans* was tested using the disk diffusion method on Sabourad's dextrose agar plates, which were swabbed to create a lawn pattern using sterile swabs holding a small amount of 0.5 McFarland liquid inoculant. 500 μg and 1000 μg disks of EOO were applied to the freshly-inoculated plates, with each plate containing three disks of either 500 μg or 1000 μg EOO; ethanol control disks were also set up in a similar fashion. Plates were then incubated for 3 days at 37 °C, and the zones of inhibition were measured.

Disk diffusion tests for *C. neoformans* were conducted on YEPD agar plates, using a slightly different method of inoculation. Procedural changes concerning media and inoculant strength were made due to difficulties cultivating *C. neoformans*[10]. Plates were inoculated with 1 mL of *C. neoformans* cell suspension standardized to a McFarland number one[7]. The 1 mL inoculum was spread across the plate using an L-rod under sterile conditions. Plates were allowed to dry at room temperature in a sterile hood. EOO was applied to the plates via sterile disks, which held EOO concentrations of 1000 μg, 1500 μg, and 2000 μg as previously discussed. Higher concentrations of EOO were used for *C. neoformans* because the lower 500 μg concentration did not produce an observable antifungal effect. Control plates, containing sterile disks that were prepared

as indicated under the "Essential Oil Extraction and Concentration" section, were also observed.

RESULTS

Liquid Media

During the exponential growth phase of *C. albicans*, the optical density (OD) at 600 nm in both control groups (uninoculated control group and ethanol control group) was comparable to OD value at 600 nm for the two experimental groups for oil of oregano (200 μg/mL EOO and 400 μg/mL EOO) as shown in Figure 1. At 21 hours, in the 200 μg/mL and 400 μg/mL EOO treatment groups, OD at 600 nm began to plateau, followed by a slight decline, while OD at 600 nm in the blank and ethanol control treatment groups continued to rise for approximately 26 more hours.

Growth of *Cryptococcus albidus* was initially inhibited in the 200 and 400 μg/mL EOO test groups (Figure 2). EOO in both cases suppressed the optical density by more than 15% during the exponential growth phase when compared to both the blank and ethanol control treatments. After 139 hours of incubation, the ending OD value for both the 200 μg/mL and the 400 μg/mL EOO groups was more than 40% less than the OD value in the two control groups.

Both control treatments for *Cryptococcus neoformans* began exhibiting exponential growth approximately 9 hours after inoculation and continued for 52 hours (Figure 3). In the 200 μg/mL EOO treatment, however, oil of oregano delayed the exponential growth phase until 85 hours after the initial inoculation. The 400 μg/mL

EOO treatment showed no increase in optical density for the duration of the 120 hour experiment.

Exponential growth for both the control treatments of *Rhodotorula rubrum* began 21 hours after inoculation and continued for approximately 40 hours (Figure 4). The 200 μg/mL EOO group for *R. rubrum* did not begin exponential growth until 85 hours after inoculation. The 400 μg/mL EOO test group for *R. rubrum* did not exhibit any cell growth for the duration of the experiment.

Solid Media

Experiments performed using disk diffusion protocol for *C. albicans* demonstrated that 500 μg EOO inhibited yeast growth. The zone of inhibition was approximately 22 mm across after 185 hours (Table 3; Figure 5a). The 1000 μg of oregano oil inhibited yeast growth to a diameter of 27 mm for 7 days post inoculation (Figure 5b). The ethanol control plate exhibited no inhibition on yeast growth during the experiment (Figure 5c).

For *Cryptococcus neoformans*, yeast growth inhibition in the presence of oil of oregano was observed as well (Table 4). After 216 hours, the 500 μg and 1000 μg concentrations of EOO showed zones of inhibition measuring 12 mm and 14.5 mm, respectively. The higher 1500 μg concentration of EOO inhibited yeast growth in a zone of 16 mm over the same period.

In both the solid media growth inhibitions of *C. albicans* and *C. neoformans*, it was apparent that as the applied concentrations of EOO were increased, the corresponding zones of inhibition increased in diameter.

DISCUSSION

Liquid Media

Oil of oregano did not inhibit the exponential growth phase of *Candida albicans,* although it did appear to shorten the length of time *Candida albicans* spent undergoing rapid growth. These results contrast those seen for *C. neoformans* and *R. rubrum* (Fig. 1). This inability of oil of oregano to inhibit *Candida* growth may be explained by the nature of *Candida albicans*. *C. albicans* is most often found in the human digestive tract, while *C. albidus, C. neoformans,* and *R. rubrum* are environmental yeasts often residing in soil and decaying wood[9, 13, 25, 28]. Also, all tested yeasts were incubated at uniform conditions and a temperature of 37 °C during this experiment. This incubation temperature did not affect the growth of *C. albicans*, which is naturally found in a similar environment, but it may have slowed the growth of *C. albidus, C. neoformans,* and *R. rubrum*, which may be adapted to grow ideally in temperatures below 37 °C. In the liquid media growth inhibition testing, the inoculum size for *C. albicans* was relatively large when compared to those of the other tested yeasts. This also may have had an effect on *Candida albicans*' response to EOO.

It is also important to note the positioning for the growth curves of the 200 and 400 μg/mL EOO test groups on each of the four graphs. The optical density values for the 400 μg/mL EOO group should fall below those observed for the 200 μg/mL EOO group, if greater concentrations of EOO result in increased inhibition of yeast growth. In each of the graphs for the four yeasts, however, the 400 μg/mL EOO group data curve lies above that of the 200 μg/mL EOO group, making it appear that the 200 μg/mL concentration of EOO has greater growth inhibition than the 400 μg/mL EOO. During

the experiment, the elevated initial OD values for the EOO treatment groups indicated that the EOO itself may have been absorbing light at the 600 nm wavelength measured. To test this, blanks containing only growth media and 200 μg/mL or 400 μg/mL EOO were measured spectrophotometrically, with the instrument blanked against the YEPD broth growth media. The EOO present in the media did register significant absorbance values, and the tubes containing the 400 μg/mL EOO displayed a greater optical density than the 200 μg/mL EOO tubes. The spectrophotometric readings from these controls confirmed that the higher concentration of oregano oil in the 400 μg/mL EOO test group was the reason the 400 μg/mL EOO group's growth curve lay above that of the 200 μg/mL EOO group on the graph (Figure 5). Therefore, it is essential to note the optical density changes between two points on the growth curve. It is this change in optical density that reflects the presence of cell growth.

While the 400 μg/mL concentration of EOO did appear to inhibit the growth of *R. rubrum* and *C. neoformans* in our experiment, it did not exert noticeable inhibitory effects on either *C. albicans* or *C. albidus*. Some suppression of the growth curve was observed when compared to control group growth, but complete inhibition did not occur. This suggests that the effects of EOO may be fungistatic in nature, rather than fungicidal. It may also be important to observe the effects of extended incubation at 37 °C on the EOO itself. If the oil is denaturing over the course of the incubation period, this may also help explain the lack of fungicidal activity observed in our experiment.

Solid Media

For *Candida albicans*, the results for disk diffusion testing on solid Sabourad's dextrose agar plates are shown in Table 3. During the 185 hours that the *C. albicans* plates were observed, the ethanol control plate exhibited no inhibitive influence on the growth of *C. albicans* (Figure 5c), so it can be assumed that the inhibition of *C. albicans* growth on the plates containing disks of 500 μg and 1000 μg EOO was due to the essential oil of oregano. The 500 μg EOO group initially prevented yeast growth at approximately the same level as the 1000 μg EOO group—31.0 mm. At 41 hours, a reduction in the initial zone diameter was observed for the 500 μg disks. After 185 hours, the 500 μg zone measured 22 mm across; a 29% decrease from the initial 41 hour measurement.

The 1000 μg EOO inhibition zones were measured 15 hours after testing began and were found to be 32.7 mm in diameter. Unlike the 500 μg EOO group, however, the 1000 μg EOO inhibition zones did not suffer a large drop in diameter, but rather only a slow but steady decline over time until the diameter of the inhibition zones at 185 hours measured 27.0 mm across—a 17.4% decrease from the initial 15 hour measurement.

The results of oregano disk diffusion inhibition of *Cryptococcus neoformans* are shown in Table 4. In the 500 μg EOO test, the inhibition zones decreased from 12.6 mm to 6.2 mm over the test period of nine days. The 1000 μg EOO inhibition zones also decreased, but only by 0.3 mm, and the 1500 μg EOO inhibition zones did not decrease at all during the experimental period.

It may be possible that as the oil of oregano concentrations are applied below minimal inhibitory and fungicidal concentrations, the tested yeast cells can develop

resistance to the presence of oregano extract and continue to grow, despite their initial inhibition. This would explain the decrease in the diameter of the initial zone of inhibition over time. If this is the case, oil of oregano should be employed at a concentration large enough to destroy targeted cells with the first application, to keep the yeast cells from developing resistance to the oregano extract.

Literature Comparison

The results of yeast growth inhibition testing obtained from this experiment using 200 and 400 μg/mL of EOO compare favorably to existing data on the antifungal properties of essential oil of oregano (EOO). Experimental results for *Candida albicans* served to compare the EOO antifungal activity during this experiment with existing literature data, because *C. albicans* has been tested for EOO susceptibility on a number of occasions[6, 22, 31].

The results Bozin, et al. (2006) experiment yielded a minimum inhibitory concentration (MIC) of 200 μg/mL EOO against *C. albicans*. These results were quite remarkable, because the MIC for oregano essential oil was 20 μg/mL, while the MIC for the antifungal drug bifonazole was 200 μg/mL against *C. albicans*. This confirms that EOO is antifungal for *C. albicans*, but it also highlights the idea that of oil of oregano may have the potential to compete with existing antifungal drugs[6].

The tests conducted by Tampieri, et al. (2005) also confirmed the antifungal action of EOO against *C. albicans*. The resulting MIC of EOO needed for *C. albicans* was 500 ppm, which is equivalent to 500 μg/mL[31].

Manohar, et al (2001) showed that the MIC of EOO needed for *C. albicans* was found to be 250 μg/mL. EOO and carvacrol, one of the oil's major constituents, both proved effective in treatment of systemic candidiasis in mice. The concentrations of essential oil of oregano used to treat the systemic candidiasis were examined for toxicity as well, and the results showed that the low concentrations of oregano oil used did not cause mice mortality. Manohar, et al. (2001) also examined the overall effect of essential oil of oregano treatments versus treatments with carvacrol. The results showed that mice that received either the oregano oil or the carvacrol recovered, but those that received treatment with essential oil of oregano had a better overall cosmetic appearance upon recovery[22].

There is clearly some disagreement in the literature as to the exact MIC of EOO needed for *Candida albicans*. This disagreement may be due in part to the variant nature of essential oil extracted from a natural source; two samples of essential oil from two different sources, even of the same species, will not be exactly alike in chemical makeup or action. Different growing and harvesting methods can induce slight differences in the makeup of the resulting essential oil, which results in a spectrum of MIC values, as evidenced and attested to by existing literature[31]. The EOO concentrations tested against *C. albicans* in our experiment (200 μg/mL and 400 μg/mL) did fall within the range of EOO concentrations found to be active against *C. albicans* in the literature[22, 31]. The results obtained in our experiments, however, vary from the results of previous experiments, although there did appear to be some level of oregano extract activity against *C. albicans* based on the suppression of the yeast growth curve (Figure 1).

Less information is found in the literature on the antifungal effects of EOO on *C. neoformans, R. rubrum* and *C. albidus*. A few studies have been done on the effect of EOO on *R. rubrum* and *C. neoformans*, which indicate in some cases that these fungi are susceptible to EOO in some degree, as explored in more detail in this experiment[26, 33]. No literature, however, is currently available to support or augment the results of this experiment regarding *C. albidus*.

Electron microscopy studies by Bennis, et al. (2004) showed that the effects of thymol on *Saccharomyces cerevisiae* were a result of thymol inducing deformities in the envelope of the yeast cells. A similar mechanism may explain the effects of EOO. More specifically, while the surface of simple, undividing yeast cells did show some cracking in the presence of thymol, it was the cells in the process of budding that were most strongly affected. This led Bennis, et al. to the conclusion that the action of thymol was closely related to the exponential growth phase of the tested yeast[5].

Applying the conclusions of Bennis, et al. (2004) to this experiment serves as a possible explanation for the efficacy of EOO against all four of the tested yeasts. In the faster growing yeasts, *C. albicans* and *C. albidus*, EOO was unable to completely halt cell division. This may be because the tested concentrations of EOO were too low to effectively combat the rapid division of these two yeasts in the medium, or because the exponential growth phase in these two yeasts simply happened too quickly for EOO to stop completely. *R. rubrum* and *C. neoformans* took longer to begin their exponential growth phases, thereby allowing EOO enough time to affect their budding cells, resulting either in the complete inhibition or lengthy delay of the exponential growth phase in these two yeasts.

For practical application, these results and proposed explanations may serve to illustrate that EOO is most effective at inhibiting yeast growth when applied in high enough concentrations to kill yeast cells initially, with enough time to act on the yeast before exponential growth begins.

CONCLUSION

From the data gathered during this experiment, *Origanum vulgare* shows great potential as a natural anti-yeast agent, in addition to its already tested bactericidal and fungicidal abilities. It is especially effective against *C. neoformans*, *R. rubrum*, and *C. albidus*, which are important threats to patients with compromised immune systems. Different yeasts respond in different ways to this natural oil over time, and the oil's efficacy may be related to the speed with which the yeast enters the exponential growth phase as well as to the concentration of the oil itself. Further testing is needed to verify how inoculum size and length of lag phase affect the action of EOO on yeast growth. It is also imperative that the effects of different growing/harvesting methods upon oil content and antifungal action be determined. Unless an essential oil with uniform chemical content can be dependably produced, it will never be possible to observe consistent values for the MIC of essential oil of oregano against any pathogen. But as current studies indicate, EOO holds great promise for combating many fungal pathogens[6, 22, 24, 31]. With further experimentation and continued *in vivo* testing, *Origanum vulgare* could serve as a highly effective anti-fungal agent in the world of medicine.

FIGURES AND TABLES

Figure 1: Time Dependent Growth of *Candida albicans* in the Presence of Essential Oil of Oregano at 37 °C

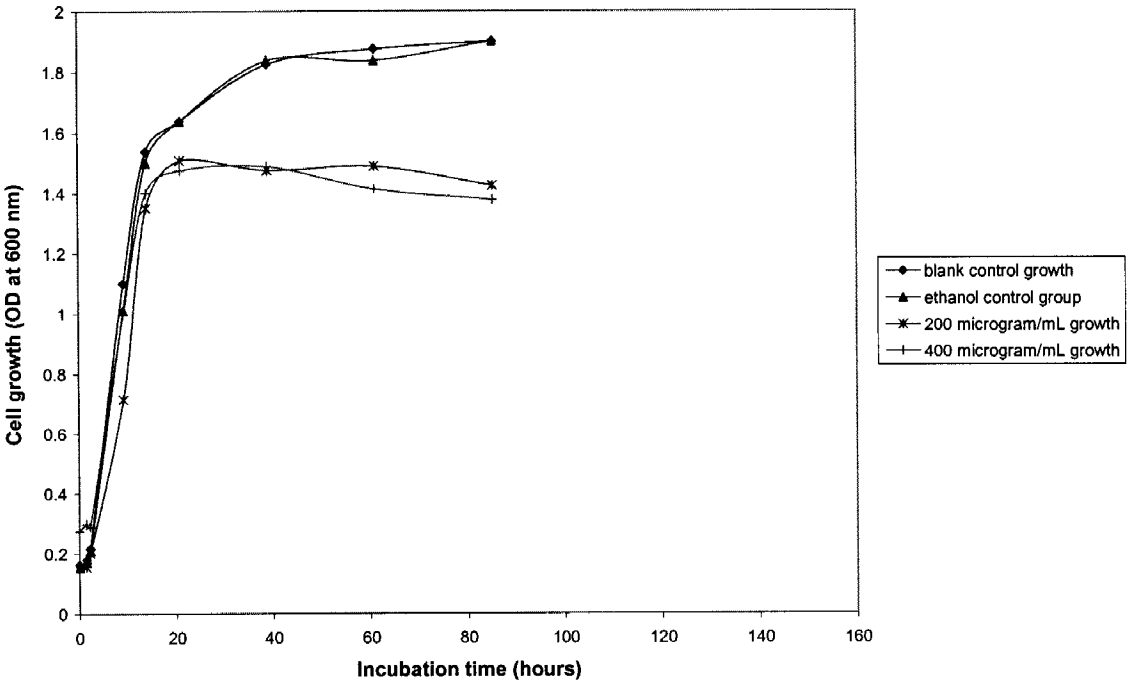

Figure 2: Time Dependent Growth of *Cryptococcus albidus* in the Presence of Essential Oil of Oregano at 37 °C

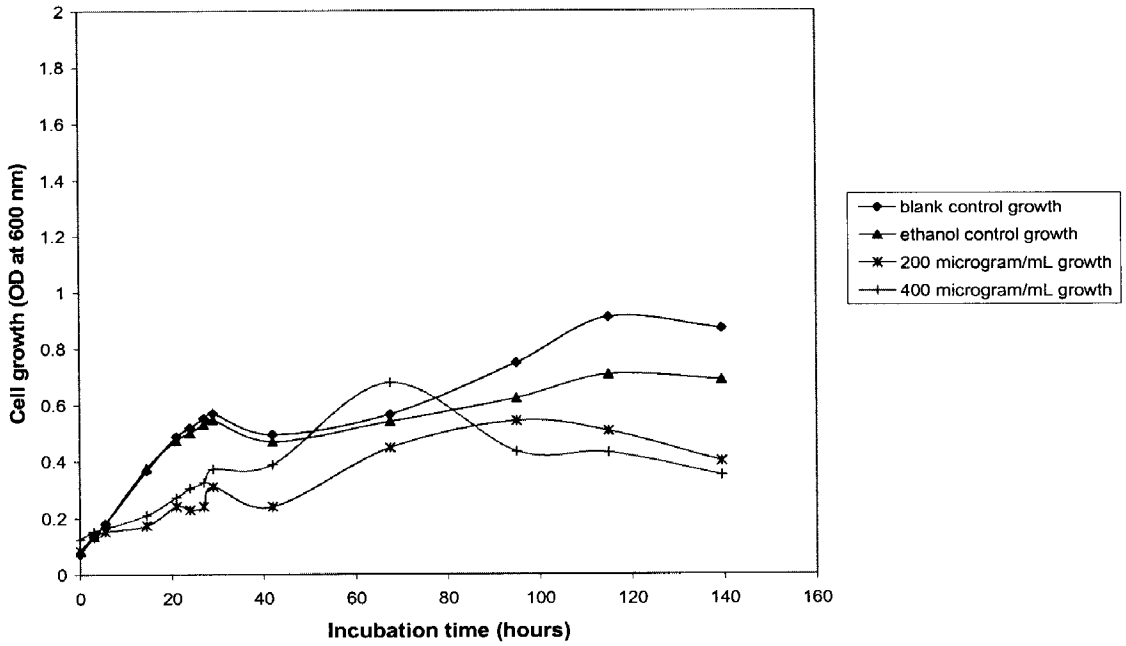

Figure 3: Time Dependent Growth of *Cryptococcus neoformans* in the Presence of Essential Oil of Oregano at 37 °C

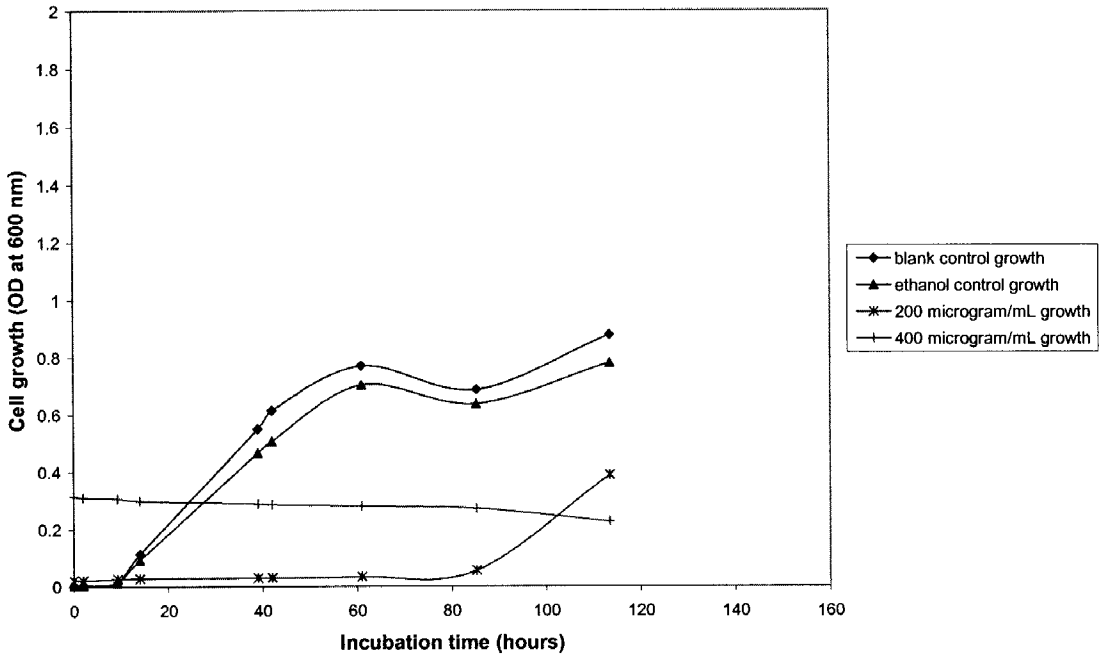

Figure 4: Time Dependent Growth of *Rhodotorula rubrum* in the Presence of Essential Oil of Oregano at 37 °C

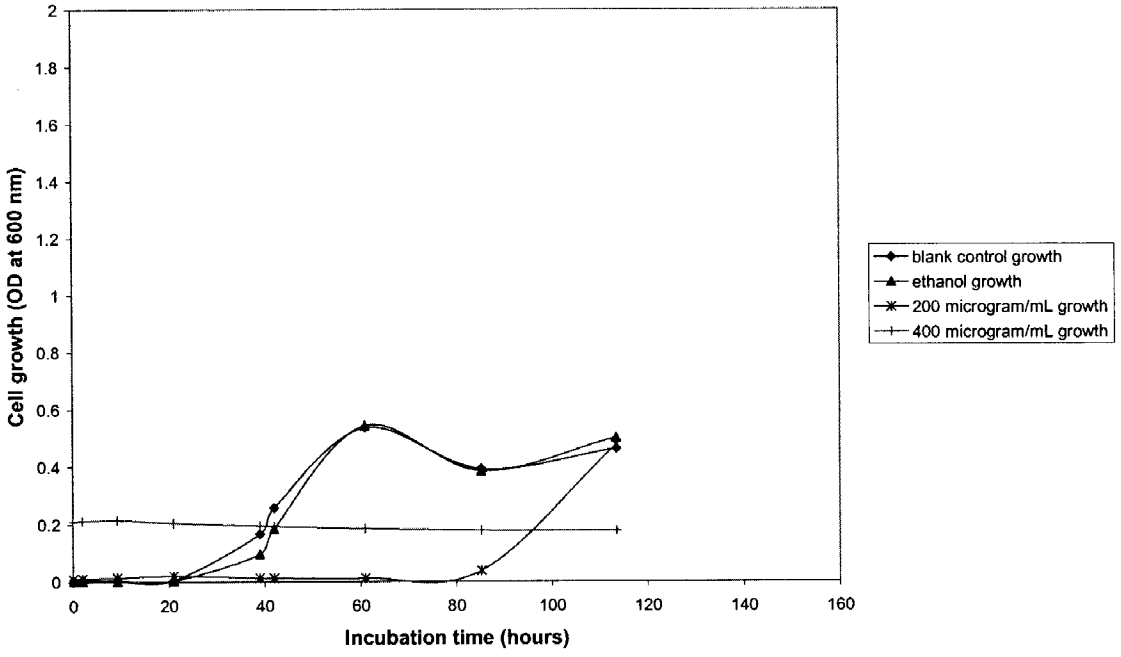

Figure 5: Absorbance of EOO + Media During Incubation Period at 37 °C

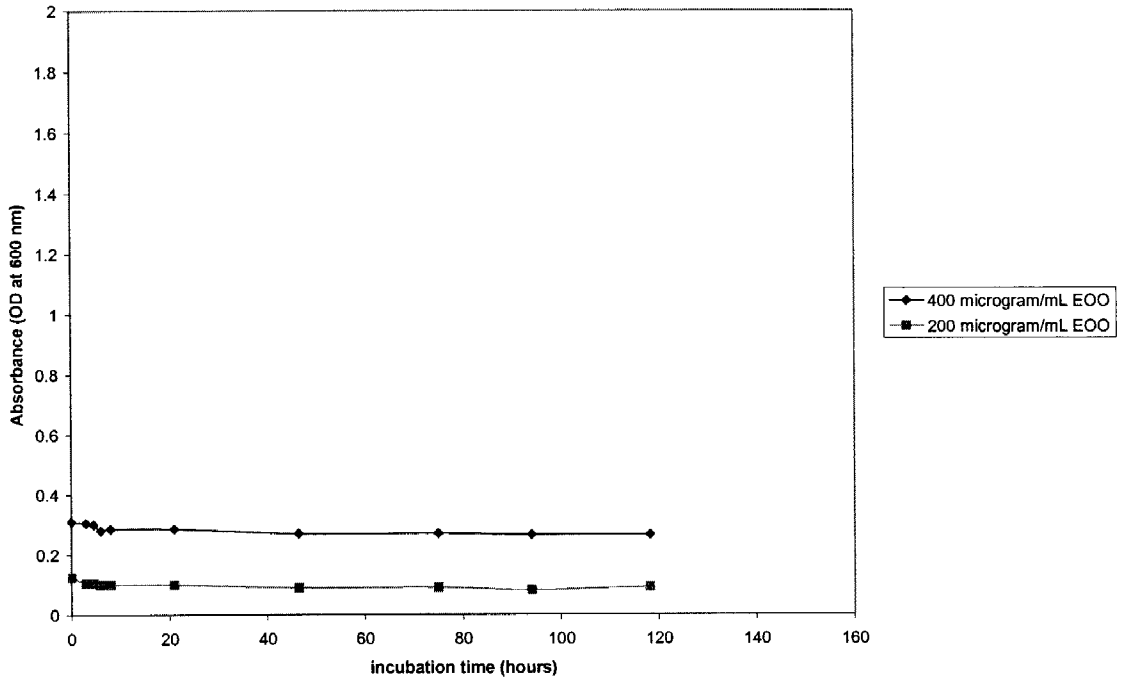

Table 1: **Liquid Media Test Groups: Volume of Ingredients Added**

	YEPD broth	yeast preculture	essential oil of oregano (EOO)	ethanol
blank control group	4.5 mL	0.5 mL	0 μL	0 μL
ethanol control group	4.5 mL	0.5 mL	0 μL	20 μL
200 μg/mL group	4.5 mL	0.5 mL	5 μL	15 μL
400 μg/mL group	4.5 mL	0.5 mL	10 μL	10 μL

Table 2: **Liquid Media Blanks: Volume of Ingredients Added**

	YEPD broth	yeast preculture	essential oil of oregano (EOO)	ethanol
blank control group	5 mL	0 mL	0 μL	0 μL
ethanol control group	5 mL	0 mL	0 μL	20 μL
200 μg/mL group	5 mL	0 mL	5 μL	15 μL
400 μg/mL group	5 mL	0 mL	10 μL	10 μL

Table 3: **Disk diffusion inhibition test results for *Candida albicans* (measurements include size of disk as approximately 7 mm)**

	Average diameter of inhibition zone (mm)		
time elapsed (hours)	Ethanol control	500 μg EOO	1000 μg EOO
15.5	7	31	32.7
41.75	6.7	25.3	31.7
47.25	7	23.7	29.7
67.5	7	22	29.7
112.5	6.7	20.7	27.3
117	7	20.7	25.7
139	7	21	26.7
161.5	7	21	26.3
185	7	22	27

Table 4: **Disk diffusion inhibition test results for *Cryptococcus neoformans* (measurements include size of disk as approximately 7 mm)**

	Average diameter of inhibition zone (mm)		
time elapsed (hours)	Ethanol control	500 μg EOO	1000 μg EOO
192	7	12.7	14.8
216.5	7	6.2	14.5

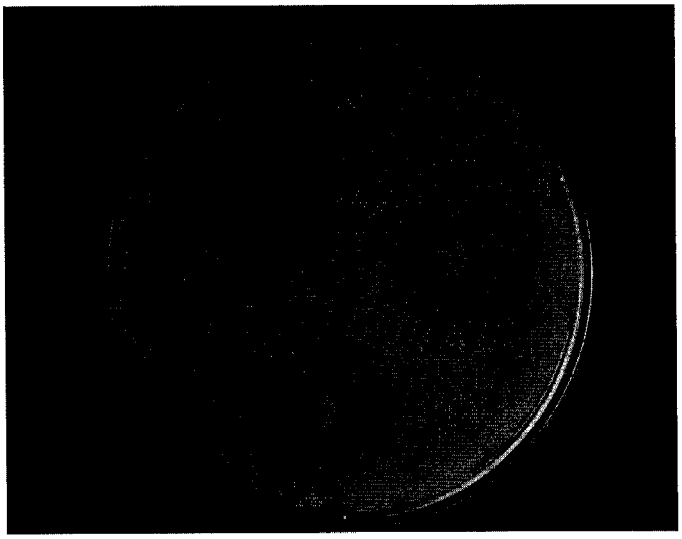

Fig. 6a: *C. albicans* growth after 139 hours with 500 μg EOO on each disk

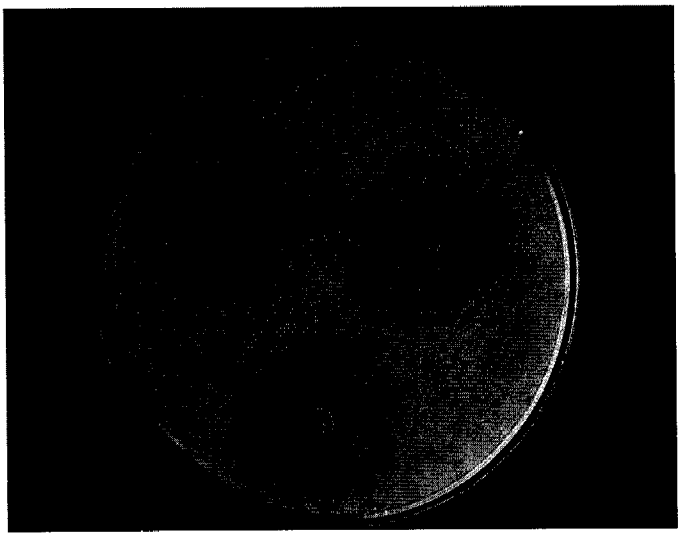

Fig. 6b: *C.albicans* growth after 139 hours with 1000 μg EOO on each disk

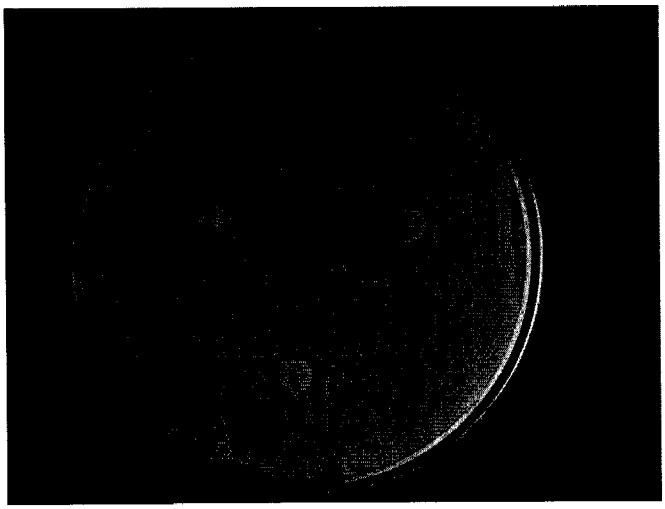

Fig. 6c: *C. albicans* growth after 139 hours with ethanol control disks

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