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Abstract: A variety of assays were utilized to determine the effects of Leptospermum petersonii essential oil on both Candida albicans and Aspergillus fumigatus. Hyphal morphology, susceptibility of spheroplasts and uptake of propidium iodide following exposure to the oil suggest that the mode of action of L. petersonii essential oil is through direct disturbance of the fungal cell membrane. Data also confirms that the volatile component of the oil is highly antifungal, independent of direct contact between the liquid oil and the fungal membrane. The degree of inhibition was greater when fungi were directly exposed to oil volatiles compared to pre-inoculation exposure of oil volatiles into the agar. It is likely that the essential oil volatiles are acting both directly and indirectly on the fungi to produce growth inhibition.

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Title: **The effect of *Leptospermum petersonii* essential oil on *Candida albicans* and *Aspergillus fumigatus***

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Running title: Antifungal activity of *Leptospermum petersonii*

SUMMARY

A variety of assays were utili~~s~~z~~z~~ed to determine the effect of *L. petersonii* essential oil on both *Candida albicans* and *Aspergillus fumigatus.* Hyphal morphology, susceptibility of spheroplasts and uptake of propidium iodide following exposure to the oil suggest that the mode of action of *L. petersonii* essential oil is through direct disturbance of the fungal cell membrane. Data also confirms that the volatile component of the oil is highly antifungal, independent of direct contact between the liquid oil and the fungal membrane. The degree of inhibition was greater when fungi were directly exposed to oil volatiles compared to pre-inoculation exposure of oil volatiles into the agar. It is likely that the essential oil volatiles are acting both directly and indirectly on the fungi to produce growth inhibition.

Keywords: antifungal, essential oil, volatile compounds

INTRODUCTION

Several essential oils have been reported to display potent antifungal activity and it has been suggested that these oils may be viable alternative therapies for fungal infection, particularly against superficial mycoses [1,2]. The majority of studies conducted to date have concentrated on identification of bioactive essential oils and their active components, however, reports on the mechanisms of antimicrobial action of essential oils are increasing. Several essential oils, such as tea tree, oregano, lemongrass and lavender, have been demonstrated to target the cell membrane in both bacteria and fungi [3-6].

The essential oil derived from the Australian native tree *Leptospermum petersonii* (Lemon Scented Tea-Tree: family *Myrtaceae*) and the associated volatile (vapour) components have been reported to display potent antifungal activity [7, 8]. To proceed with ~~gress~~ the study of this oil as a potential treatment for fungal infection it is important to determine the mode of action. The mechanism of action of *L. petersonii* was therefore examined using a variety of assays to determine the effect of the oil on both *Candida albicans* and the hypha~~t~~e of *Aspergillus spp*. A wide range of assays were used including examination of susceptibility of spheroplasts, hyphal morphological examination using scanning electron micrographs and light microscopy, uptake of propidium iodide and leakage of cellular components following exposure to the oil.

MATERIALS AND METHODS

Leptospermum petersonii essential oil was supplied by Mr D Archer, Toona Essential Oils. *Aspergillus fumigatus* ATCC 16903 and ATCC 1022 were obtained from the American Type Culture Collection. Clinical isolates of *A. niger, A. fumigatus, A. flavus, A. terreus* and *A. nidulans* were provided by D Ellis, Adelaide. *Candida albicans* (oral isolate) was provided by S Wheeler, Charles Sturt University. *Aspergillus* fungi were maintained on Sabouraud's agar (SAB: Oxoid) or malt extract agar plates, with and without 5% glucose (Oxoid). Spores were harvested in sterile

phosphate buffered saline (PBS) containing 0.1% Tween 80 (vol/vol) from 1 week old fungal cultures. *C. albicans* was grown and maintained on yeast extract peptone glucose (YPD) broth/agar (Sigma). Glass Petri dishes were used throughout.

MIC_{100} determination: Stable emulsions of oil in aqueous medium were prepared utilising Tween 80 as described previously [9]. MIC_{100} was determined using a standard macro broth dilution method. Control tubes contained sterile water (+/-Tween). Tubes were inoculated with $1x10^5$ *C. albicans* from an 18h old culture; incubated at 30°C/24h/120 rpm; the degree of growth was determined visually. Macro broth dilution assays for *A. fumigatus* ATCC 16903 were carried out using an inoculum consisting of a suspension of ungerminated and germinated conidia as well as hyphal fragments. Briefly, media containing 0.1%, 0.05%, 0.025% or 0.0125% of oil were prepared as described above for *C. albicans*. Control tubes contained sterile water (+/- Tween). Tubes were inoculated with *A. fumigatus* spores ($2x10^4$ spores/mL in PBS) or 200µL of broth culture inoculated 24h previously with $2x10^4$ spores/mL; with the mycelium culture vortexed for 5 min prior to inoculation and incubated 35°C/48h/120rpm. For broth dilution using a germinated conidia inoculum, $2×10^4$ spores were added to each well of a 24-well cell culture plate (Sigma), incubated for 6-10h and the percentage spore germination monitored until 90% of conidia had germinated. Plates were incubated with shaking (35°C/48h/120rpm) and examined microscopically for the presence/absence of fungal growth. Test wells that displayed no visual growth were pelleted (15000rpm/25 min), the pellet resuspended in 1mL of PBS and re-pelleted/washed twice. Pellets were resuspended in 500µL of malt broth (Oxoid), plated onto SAB and malt extract agar plates, incubated (35°C/48h) and the degree of growth determined visually. The MIC was defined as the lowest concentration of oil that completely inhibited all visible growth. The mean fungicidal concentration (MFC) was determined by subculturing 500µL from the highest dilution demonstrating complete growth inhibition and from all subsequent dilutions with no visible growth on appropriate agar plates. Plates were then incubated at 37°C/24h, the degree of growth determined visually and the number of colonies recorded. The MFC was defined as the

lowest concentration at which 99.9% of the initial inoculum was killed. The results presented are the mean of three independent replicates.

~~**Time kill assay**~~

~~Time kill assays for *L. petersonii* oil against *C. albicans* and *A. fumigatus* ATCC 16903 utilised a modified macro broth dilution method. Media containing 0.2, 0.02 and 0.002% *L. petersonii* oil was prepared with the appropriate amount of Tween 80[9]. Control tubes containing no oil (+/- Tween) were prepared in the same manner. For *C. albicans* assays, tubes were inoculated with 100µL of an 18h culture of *C. albicans* adjusted to 1x10[6] cells/mL. Tubes were incubated at 30[o]C with vigorous shaking (120rpm). At 0.25, 0.5, 1, 2, 3, 4 and 24h time intervals 10µL aliquots were taken and serially diluted to 10[-4] in sterile PBS. 100µL of each dilution and the original test suspensions were lawn inoculated onto duplicate nutrient agar plates. Plates were incubated at (30[o]C/24h) and the number of colony forming units (CFU) counted. Viability of fungi was determined by adding 100µL of 0.05% Methylene blue (Sigma) to independent samples of *C. albicans* and the number of viable cells was counted via haemocytometer.~~

~~Time kill assays for *A. fumigatus* ATCC 16903 were carried out as described for *C. albicans* except that 100µL of a 1x10[5] spore/mL suspension of *A. fumigatus* was utilised as the inoculant with an incubation temperature of 25[o]C; centrifugation (15000rpm/25min) was utilised to remove essential oil and supernatant, with pellets washed 3x in sterile PBS prior to re-suspension in 500µL of malt broth and prior to plating out onto SAB and malt extract agar plates. Plates were incubated at 25[o]C/48h and the presence or absence of growth was determined visually. The results presented are the mean of three independent replicates.~~

Exposure of established fungal colonies to volatile components: Agar plates were inoculated with a 6mm fungal plug taken from the periphery of an established colony and incubated at 25^{o}C until the colony radius reached 20mm. A 30mm filter paper disc (Oxoid), impregnated with either 100µL of essential oil or 100µL of water, was attached to the lid of each

Petri dish, the dish was sealed (parafilm) and the plate exposed to oil volatiles for 0.5, 1, 3, 6, 18 or 24h; the Petri dish lid with attached disc was then replaced. Plates were re-incubated (48h), colony size measured and the percentage growth inhibition determined. The results presented are the mean of three independent replicates.

Effect of volatile components absorbed onto agar pre-inoculation: 25mL of appropriate sterile agar was exposed to oil volatiles for 0.5, 1, 3, 6, 18 or 24h after which the Petri dish lid, with attached 30mm filter paper disc (Oxoid), impregnated with either 100µL of essential oil or 100µL of water, was replaced with a fresh Petri dish lid. The agar was then inoculated with a 6 mm fungal plug taken from the periphery of an established colony of *Aspergillus,* the plates re-incubated (48h), the colony size measured, compared to control colonies and the percentage inhibition calculated. The results presented are the mean of three independent replicates.

Morphological changes following exposure to *L. petersonii* oil: Morphological changes in *C. albicans* grown in the presence of *L. petersonii* oil were determined using a modified macro broth dilution method as described above with 100µL aliquots removed after 60min and examined microscopically for any morphological changes. A minimum of 200 cells was examined from each sample. Examination of morphological changes to *A. fumigatus* ATCC 16903 was performed as described for *C. albicans* with the following modifications. 100 µL of a $1x10^6$ spore/mL suspension was inoculated into 5mL of YPD broth and incubated (25^oC/48h). Oil was added (0.5, 0.05, 0.005 and 0.0005% (vol/vol)) and the fungi incubated with shaking (25^oC/24h). Three 100µL aliquots were taken from each sample with ≥50 individual hyphae examined microscopically for morphological changes. A 2mm diameter sample of mycelia was also taken from the periphery of a fungal colony grown on a 200mg/L *L. petersonii* agar plate, stained with lactophenol cotton blue (HistoLabs), the diameter of 100 individual hyphae recorded and any morphological changes noted.

Morphological Examination of *A. fumigatus* ATCC 16903 by SEM: A Cambridge 360 cryo-scanning electron microscope at -170^{o}C/10 kV was used throughout. *A. fumigatus* ATCC 16903 was exposed to *L. petersonii* oil via the standard volatile assay described above (3 h exposure) or the agar dilution method (200 mg/L) as previously reported [9].

Cellular Leakage of 260 nm absorbing material: *C. albicans* was cultured with shaking in YPD broth at 30^{o}C/18 h, pelleted (8000g/5min), washed twice with cold **3-(N-morpholino) propanesulfonic acid (**MOPS) buffer (Sigma), resuspended in ice cold MOPS and the concentration adjusted to 5x10^{7}CFU/mL. ~~9~~Nine mL of cell suspension was added to the appropriate volume of oil/MOPS/Tween 80 to give final oil concentrations of 0.2, 0.02, 0.002, and 0.0002% [9]. ~~1~~One mL was immediately removed, centrifuged (8000g/5min) and absorbance of supernatant was read against controls (as above without *C. albicans*) at 260nm (time zero). This was repeated at 0.5, 1, 2, 3, 4, 5 and 6h. The *A. fumigatus* ATCC 16903 assay was performed as described for *C. albicans* except that *Aspergillus* was incubated with shaking in YPD broth (30^{o}C/3 days), the broth was filtered (Whatman no.1 filter paper) and the fungal mass washed twice with sterile cold distilled water and once with cold MOPS. Half a gram ~~0.5g~~ of wet weight ~~of~~ fungus was re-suspended in 9mL of cold MOPS. ~~1~~ One mL of various oil concentrations were added to final concentrations of 0.5, 0.05, 0.005, and 0.0005%. All assays were performed in triplicate on separate occasions.

Susceptibility of spheroplasts: *C. albicans* was grown with shaking (5h/35^{o}C) in YPD broth. Yeast cells were pelleted (1800g/10min), washed once with distilled water and once with 1.4M sorbitol (Sigma). Pellets were resuspended, adjusted to 1x10^{7} cells/mL in 0.04M HEPES (ph7.4), 0.5mM $MgCl_2$, 0.5% mercaptoethanol, 1.4M sorbital (Sigma), the suspension incubated (15 min/35^{o}C) with light shaking and Lyticase (10units/10^{7}cells) (Sigma) added and the mixture incubated (30^{o}C) with gentle shaking. The suspension was examined microscopically for the presence of spheroplasts every 10 min. When the majority of the *C. albicans* cells had converted into spheroplasts the suspension was pelleted (1800g/10 min) and resuspended in 10mM sodium

HEPES buffer; 2% glucose (Sigma). Nine ~~9~~ mL of spheroplast suspension was used throughout with a final volume of 10 mL to give final oil concentrations of 0.2, 0.02, 0.002, and 0.0002%: sterile PBS was utilised in control samples. The absorbance at 600 nm of the samples was recorded at 0, 10, 20, 30 and 60 min time intervals. Twenty ~~20~~µL samples of treated spheroplasts and controls were removed at each time interval and examined using phase contrast microscopy for evidence of spheroplast damage.

Uptake of propidium iodide (PI): Briefly, $1x10^6$ *C. albicans* cells were incubated with 0.2, 0.02, 0.002, and 0.0002% (v/v) *L. petersonii* oil. At 0, 15, 30, 45 and 60 min cells were pelleted (3000g/5min), washed 3x with PBS; resuspended in 1mL PBS, 1µg/mL of PI added and the cell suspension incubated on ice (20min) before the percentage of PI positive cells was determined at 620 nm (Beckman Coulter, Cell Lab Quanta flow cytometer; 15 mV argon laser).

Medium Acidification: The glucose-induced medium acidification of *C. albicans* was measured after the method of Haworth et al [10].

pH change of media after addition of *L. petersonii* essential oil: 10 mL of malt or nutrient broth emulsions of *L. petersonii* essential oil were prepared as described previously [9], with final oil concentrations of 0.1%-10%. An additional tube contained only Tween 80 at the highest concentration used to prepare the oil solutions (0.1%). The pH of each of these solutions and of broth without additives was recorded. All testing was competed in triplicate. The pH values of each concentration were compared (t-test) and results were deemed to be significant if $p<0.05$.

RESULTS

The minimum inhibitory concentration (MIC_{100}) for *A. fumigatus* ATCC 16903 was determined to be 0.05% oil (v/v), irrespective of mode of inoculation, with the oil demonstrating fungicidal activity (MFC) at the same concentration. The MIC_{100} for *C. albicans* (0.02%), however, differed from the MFC (0.04%). Ten times the MIC_{100} (0.2%) resulted in 100% growth inhibition of *C. albicans*

within 30min while 0.1xMIC_{100} (0.002%) of *L. petersonii* oil caused 91.9±3.9% growth inhibition after 24 h exposure. No viable cells were present following exposure to 10xMIC_{100} within 30 min or 1xMIC_{100} within 60 min while a 91.9±3.9% reduction in viable cell count was detected following exposure of cells to 0.1xMIC_{100} for 24 hours (Table 1). ~~The~~ A time kill assay for *A. fumigatus* ATCC 16903 was recorded as growth or no growth: 1xMIC_{100} (0.05%) caused 100% growth inhibition within 3 h but not at 2 hours or less of exposure (data not shown).

Established fungal colonies (≥20 cm diameter) exposed to *L. petersonii* oil vapours, with no direct contact between oil and fungal hyphae, demonstrated varying degrees of growth inhibition at 30 min post-exposure: namely, *A. niger* 50±10%, *A. fumigatus* 87±3.8%, *A. fumigatus* ATCC 1022 92±1.5%, *A. fumigatus* ATCC 16903 95±4.4%, *A. terrus* 58±14.4%, *A. flavus* 71±4.2% and *A. nidulans* 96.5±3%. Fungicidal activity was evident in all fungi within 1h of exposure. Established colonies exposed to *L. petersonii* volatiles appeared dehydrated with little or no spore formation. When *L. petersonii* oil vapour was allowed to absorb onto agar prior to fungal inoculation, *A. fumigatus* was the most susceptible while *A. flavus* was the least susceptible (Table ~~1~~2).

Both *A fumigatus* ATCC 16903 and *C. albicans* were examined for morphological changes following direct exposure to *L. petersonii* essential oil. After 1 h of exposure at 1x and 10xMIC_{100}, the *C. albicans* membrane became irregular with evidence of blebbing and granulation of the cytoplasm (Figure 1~~data not shown~~). *C. albicans* incubated at 10xMIC_{100} had an average length of 5.2µm while control cells averaged 9.8µm. *A. fumigatus* grown in the presence of *L. petersonii* essential oil demonstrated a heterogeneous cytoplasm (granular and/or contained vacuoles), while cell walls became irregular with significant blebbing. Some hyphal cells exhibited extensive degeneration with destruction of cell wall and complete retraction of the cytoplasm. The hyphae of *A. fumigatus* grown on agar containing 200 mg/L of *L. petersonii* essential oil were significantly thinner than the controls; with mean diameters for each being 1.5±0.4µm and 2.8±0.4 µm respectively.

Under SEM examination, the hyphae of unexposed *A. fumigatus* exhibited smooth and intact hyphae with a uniform width; however, varying degrees of cell wall degeneration and destruction were displayed following exposure to the oil volatiles. Following 3h of exposure to oil volatiles, the fungal wall displayed extensive pitting and tearing of the cell wall and/or penetration into the cytoplasm, with evidence of irregular and rough cell walls and large amounts of blebbing. Conversely, when *Aspergillus* was grown in direct contact with liquid oil (200 mg/L *L petersonii*) the hyphae appeared distorted, shrunken and, in the majority of cases, appeared to have lost structure and rigidity which caused the hyphae to collapse. Representative examples of each group are presented in Figures ~~1~~2 and ~~2~~3 respectively.

Leakage of intracellular components at 260nm was measured for both *C. albicans* and *A. fumigatus.* Only a slight increase in efflux was observed following 6 hours exposure of C*. albicans* to 10x and 1xMIC_{100} of *L petersonii* oil compared to controls: no other changes were detected, however, a high background absorbance was found in all solutions that contained *L. petersonii* oil (data not shown).

The susceptibility of spheroplasts to *L. petersonii* oil was also examined. The absorbance of unexposed spheroplasts over a 60 min time interval decreased from 0.37 ± 0.02 to 0.33 ± 0.02. Upon addition of 10xMIC_{100} of *L petersonii* oil (v/v) to the spheroplasts there was a statistically significant decrease ($p<0.001$) in absorbance readings within 10 min. Similarly, the addition of 1x and 0.1xMIC_{100} of *L petersonii* oil caused a significant decrease in the absorbance when compared to the control (both $p< 0.05$); however the decrease was smaller than that noted with 10xMIC_{100}. The decrease in the absorbance caused by the addition of 0.01xMIC_{100} of oil was not statistically significant when compared to the control ($p<0.5$).

Changes in membrane permeability were examined via the uptake of propidium iodide (PI) by *C. albicans* following exposure to *L. petersonii*. PI fluorescence for untreated *C. albicans* cell was very low (1.3±0.2%), indicating that the cells remained impermeable to PI. All *C. albicans* cells

that had greater intensity fluorescence than the control parameters were defined as PI+. Sixty eight percent of cells that were treated with 1% *L. petersonii* oil for 15 min became PI+, increasing to 92% when cells were exposed for 60 min. When cells were treated with 0.1% oil for 15 min and 60 min, 20% and 69% of the cells became PI+ respectively, however, when *C. albicans* was treated with 0.01% oil there was no significant change in the number of PI+ cells compared to the control (Figure 43).

Acidification of the fungal medium was measured for both *C. albicans* and *A. fumigatus f*ollowing exposure to *L. petersonii* oil at varying time intervals. An increase in *L. petersonii* oil concentration consistently led to a reduction in the rate of acidification of the media, with the results being more pronounced with *C. albicans.* Incubation of *C. albicans* with glucose for 60 min led to a 0.70±0.05 reduction in pH of the fungal medium. When the yeast was incubated with 10x and 1xMIC_{100} *L. petersonii* oil, reduction in the pH of the medium was 0.23±0.03 and 0.40±0.09, while, when 0.1x and 0.01xMIC_{100} of oil was added then the pH of the medium was reduced by 0.46 ± 0.12 and 0.50 ± 0.09 units respectively. Following incubation with glucose for 60min, *A. fumigatus* caused a 1.27±0.109 reduction in the pH of the medium. When the fungus was incubated with 10x and 1xMIC_{100} *L. petersonii* oil, the reduction in the pH of the medium was 0.81±0.01 and 0.84±0 units respectively. The addition of 0.1x and 0.01xMIC_{100} *L. petersonii* oil to media in the absence of microorganisms, resulted in a pH reduction of 1.01±0.02 and 1.07±0.02 units respectively. The addition of 1%, 5% and 10% *L. petersonii* oil to nutrient broth, in the absence of a fungal inoculum, resulted in a drop of pH from 7.33 to 7.286, 7.00 and 6.79 respectively, while the addition of 1%, 5% and 10% *L. petersonii* oil to malt broth resulted in a drop of pH from 5.38 to 5.29, 5.2 and 5.1 respectively.

DISCUSSION

This study investigated the antifungal mode of action (MOA) of *L. petersonii* essential oil using a variety of in vitro methods. *C. albicans* was used as the primary testing organism; however, *A. fumigatus* was also used whenever possible. A range of essential oils have been demonstrated to

have a higher *C. albicans* MIC than the citral-rich (≥60%) *L.* petersonii (0.02%), including *Backhousia citriodora* (≥90% citral) and *Cymbopogon flexuosus* (≥80% citral) [11-16] while the well known *M. alternifolia* (tea tree) essential oil has demonstrated a $Log10^3$ reduction in *C. albicans* cells at 0.5% from 30 min to one hour, indicating that *L. petersonii* oil is a more potent inhibitor of fungal growth [3, 17-19]. The MIC_{100} of *L. petersonii* oil against *A. fumigatus* (0.05%) displays more potent antifungal activity than other reported oils including *Cymbopogon citratus* (70% citral): conversely, the oil of *Artemisia asiatica* demonstrates comparable activity to *L. petersonii* against *A. fumigatus* [20-25].

Time kill studies and/or viability measurements on *C. albicans* have previously been conducted for major antifungal compounds, with compounds such as micafungin, caspofungin and voriconazole killing <99.9% of *C. albicans* at the highest concentrations tested ($16xMIC_{100}$) within 24 hours while nyastatin and amphotericin B, at $\geq 4xMIC_{100}$, killed 99.9% of the yeast cells within 4 hours [26 - 30]. The MIC_{100} of these drugs against *C. albicans* range from 0.003 µg/mL to 0.5 µg/mL; lower than the MIC_{100} of *L. petersonii* (160 µg/mL). *L. petersonii* oil at $10xMIC_{100}$ and $1xMIC_{100}$ killed 99.9% of *C. albicans* within 30min and one hour respectively. The activity of *L. petersonii* oil would therefore appear to be significantly faster than these conventional drugs.

Pharmacodynamic studies of antifungal drugs on moulds have significantly lagged behind studies based on *C. albicans*, primarily due to the lack of a suitable time kill assays and difficulty of enumerating colony forming unit data from filamentous fungi [31,32]. Some studies have, however, been conducted using conidial inoculum and concluding the experiment before the development of visual hyphae in the control [33-35]. Only amphotericin B and posaconazole have been reported to reduce the fungal load by greater than $Log10^3$ at 48 hours, while itraconazole and voriconazole had little effect on *A. fumigatus* numbers within 48 hours at concentrations 20-fold higher than the MIC. Conversely, $10xMIC_{100}$ of *L. petersonii* (0.5% v/v) resulted in complete inhibition of *A. fumigatus* within 1 hour and $1xMIC_{100}$ (0.05% v/v) caused complete inhibition within 3 hours.

Morphological examination of *C. albicans* cells following exposure to *L. petersonii* oil showed that the treated cells displayed morphological changes similar to those seen following exposure to membrane disrupting drugs or ATP synthase inhibiting drugs such as amphotericin B and polygoidal [36-38]. Significant morphological changes were also apparent when *A. fumigatus* was exposed to *L. petersonii* oil similar to effects following exposure of hyphate fungi to micafungin, aqueous garlic extract and *Cymbopogon nardus, C. citratus, Lavandula, Thymus* and *Mentha pipertia* essential oils [4]. It has been suggested that these effects may be caused by inhibition of enzymes responsible for cell wall synthesis or through alteration of fungal metabolism and that the volatile components of essential oils may play a major role in antifungal activity [4,40-43].

It is still unclear how essential oil volatiles inhibit fungal growth. It has been suggested that essential oil volatiles act directly on the mycelia [44] or that they act on fungal growth indirectly via absorption into the agar [45] or that the MOA is a combination of both [46]. In this study, growth inhibition was significantly less when the oil volatiles were first absorbed into the agar and were not in direct contact with the fungus.

The effect of the volatile components of *L. petersonii* essential oil alone on the morphology of *A. fumigatus* was examined by SEM. Following exposure to *L. petersonii* oil volatiles, varying degrees of cell wall degeneration and destruction were displayed. *C. nardus* and *C. citratus* oils and oil volatiles have been reported to cause similar damage to *A. niger* hyphae [4,5]: it has been suggested that the main target of *Cymbopogon* oils is the plasma membrane, possibly via increasing cell permeability or inhibition of cell wall synthesis enzymes. The morphological studies conducted in the present study would suggest that the mechanism of action of *L. petersonii* is directed at the plasma membrane.

Membrane damaging compounds such as amphotericin B, nystatin, natamycin, chitosan and ethanol are known to induce cellular leakage of 260nm absorbing material within one hour of

exposure of susceptible fungi [47-49]. Conversely, the azole class of antifungals, such as voriconazole, ketoconazole and miconazole, do not cause cellular leakage except at very high concentrations (≥20xMIC_{100}). *M. alternifolia* essential oil has also been reported to induce very low levels of cellular leakage in *C. albicans* at ≥6 hours post exposure [36,50]; potentially affecting membrane fluidity rather than causing direct damage and allowing leakage. In this study, while minimal changes in leakage of cellular components occurred in *C. albicans at* ≥6 hours post-exposure, no leakage was observed at any time with *A. fumigatus*: implying that the disruption of the fungal membrane is not the primary mechanism of action, contrary to the evidence supplied in the time kill studies. If *L. petersonii* does target the fungal cell membrane, rapid lysis of spheroplasts would be expected to occur post-exposure [51-53]: indeed, only 10 min of exposure to 10xMIC_{100} of the oil resulted in a rapid cell lysis. This would imply, contrary to the cellular leakage results that the oil is, indeed, directly targeting the cell membrane similar to other membrane damaging agents such as amphotericin B, ibuprofen, Polyquad, myristamidopropyl dimethylamine, benzydamine and bupivacaine [51, 53, 54]. These contradictory findings warrant further investigation, however, it is most likely that the essential oil caused significant interference in the cellular leakage analysis. Extraction of the active compounds from *L. petersonii* oil may allow accurate testing of cellular leakage of 260 nm absorbing components.

To further clarify the effect on membrane viability, propidium iodide (PI) studies were conducted. *L. petersonii* oil, at the highest concentration tested (1% v/v), demonstrated PI permeability in 68% of the cells after 15 min and 92% after 60 min; again implying that the oil is damaging the cell membrane similar to compounds like echinocandin, amphotericin and ibuprofen. Unlike *L. petersonii* oil treatment, however, *C. albicans* requires ≥3 hours exposure to these compounds to demonstrate an increase in PI permeability [53,55]. It has been reported that antifungal drugs that do not directly target the cell membrane do not induce cell permeability to PI within 6 hours [56].

Optimal cytoplasmic pH conditions in the fungal cell, regulated through the fungal plasma membrane ATP-dependent proton pump, H+-ATPase, must be maintained to ensure the

operation of essential intracellular processes [57-60]. *L. petersonii* oil was found to reduce glucose induced acidification of the media by *C. albicans* and *A. fumigatus* within 60 mins at 1xMIC_{100}. *M. alternifolia* and polygoidal have also been reported to reduce glucose induced acidification within 60 min and it has been proposed that these agents may be inhibiting H+-ATPase directly or indirectly by reducing the amount of ATP produced by mitochondrial ATPase [36,50, 61].

The pH of the media is known to influence the growth of fungi, with most organisms preferring an acidic medium [62]. Most essential oils are highly acidic and may affect the pH of the test medium, which could influence the assay and produce higher antifungal activity than is attributable to the essential oil [63]. At the highest concentration of *L. petersonii* oil tested (10%), the pH of the media was reduced by 0.29 pH units, however, the effect on the pH of the medium by the oil at effective antifungal concentration was negligible: it is therefore unlikely that direct pH modification of media plays a major role in the demonstrated antifungal activity of *L. petersonii* essential oil.

This study would support the hypothesis that *L. petersonii*'s mode of action is through direct disturbance of the fungal cell membrane. *L. petersonii* oil may also be inhibiting H+-ATPase. The volatile component of the oil is highly antifungal, independent of direct contact between the liquid oil and the fungal membrane. The results of the current study therefore provide evidence that the essential oil volatiles are acting in combination, both directly and indirectly, on the fungi to produce growth inhibition. The exact mechanism of action of the volatile component of *L. petersonii* essential oil is under investigation.

Disclosure of Conflict of Interest

There is no conflict of interest

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Table 1 – Time kill assay for *C. albicans*

Oil concentration	Time (h)						
	0.25	**0.5**	**1**	**2**	**3**	**4**	**24**
0.1 x MIC	0.53 ± 6.4	8.3 ± 4.3	34.8 ± 2.4	43.7 ± 2.8	47.9 ± 1.0	55.5 ± 4.1	91.9 ± 3.9
1 x MIC	10.3 ± 4.4	31.6 ± 6.9	100	100	100	100	100
10 x MIC	95.9 ± 0.6	100	100	100	100	100	100

C. albicans results presented as percentage growth inhibition.

Table ~~1~~2 – Percentage growth inhibition of fungi by volatile components absorbed onto agar pre-inoculation

Exposure Time (h) of Agar	*A. niger*	*A. fumigatus 16903*	*A. fumigatus 1022*	*A. fumigatus*	*A. flavus*	*A. nidulan~~a~~s*	*A. terreus*
0.5	20 ± 1.2	83 ± 1.8	79 ± 1	72 ± 3.5	15 ± 0.9	29 ± 5.7	34 ± 2.1
1	76 ± 5	97 ± 2.6	99 ± 1.6	96 ± 4.6	31 ± 3.4	62 ± 4	91 ± 4.8
3	100	100	100	100	54 ± 2	100	100
≥6	100	100	100	100	100	100	100

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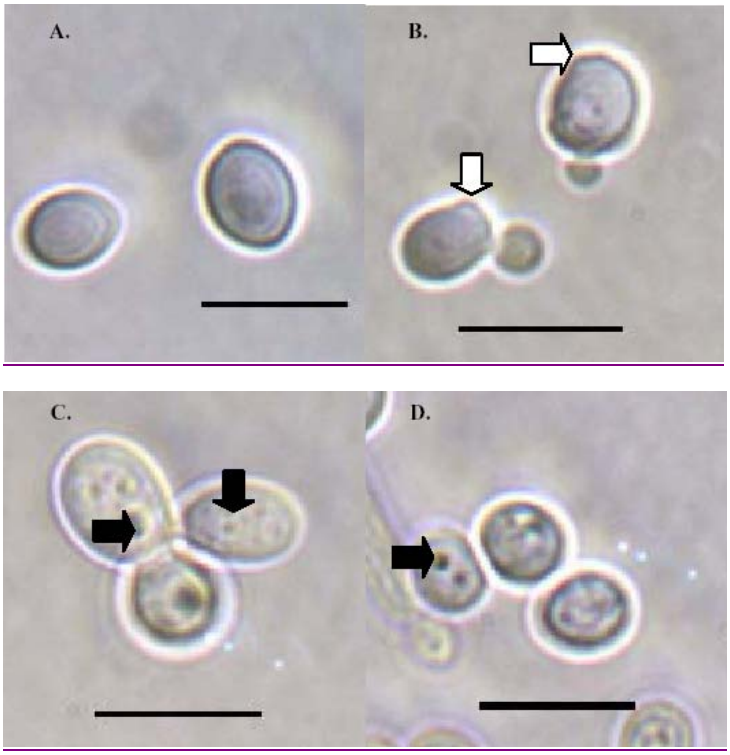
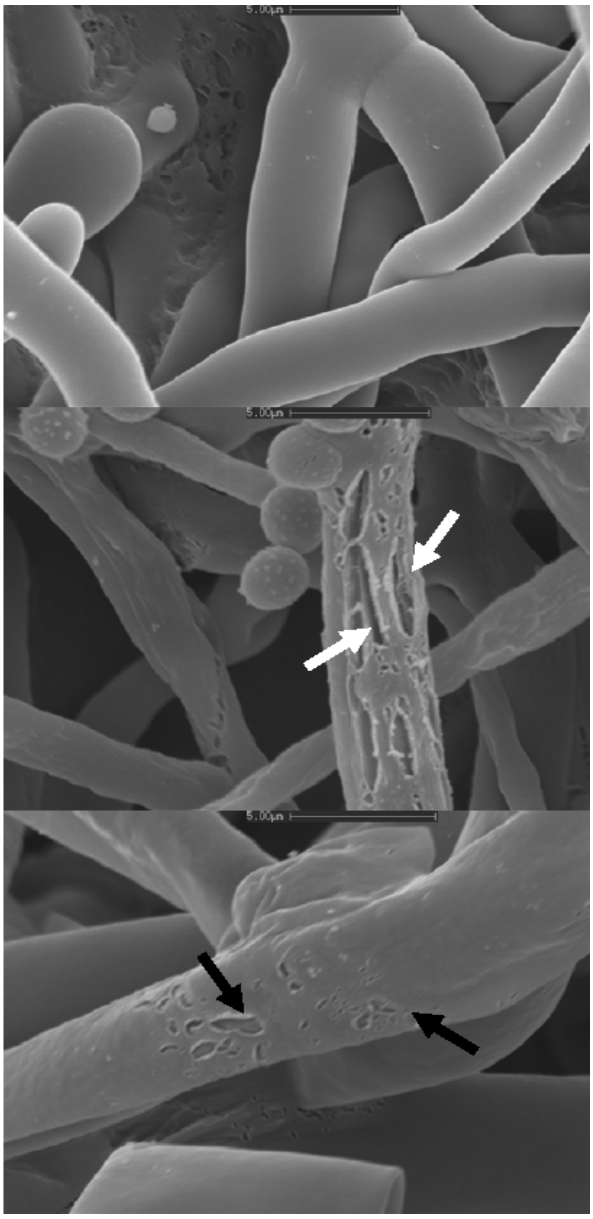
Figure 1: Morphological Changes in *C albicans* induced by *L. petersonii* oil



A. Control, **B.** 1x the MIC oil concentration. **C.** 1x the MIC oil concentration. **D.** 10x the MIC oil concentration, note the smaller size. Original magnification 1000x, Bar = 10 µm. White arrows indicate membrane damage/blebbing. Black arrows indicate granular cytoplasm.

Figure 2: Effect of *L. petersonii* volatiles on *A. fumigatus* hyphae



~~Figure 1: Effect of *L. petersonii* volatiles on *A. fumigatus* hyphae~~

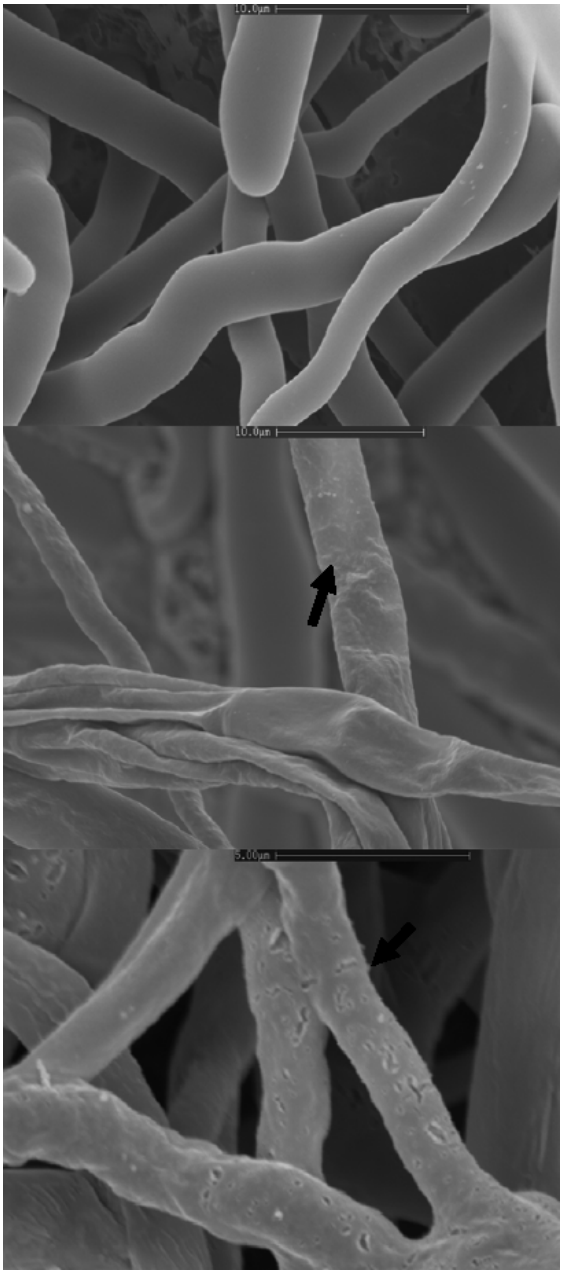
A

B

C

A - Control hyphae; B and C - hyphae following 3 hours exposure to *L. petersonii* oil volatiles. Black arrows indicate pitting and tearing damage to the cell wall. White arrows indicate penetration of cell wall into the cytoplasm. Bar = 5 µm.

Figure ~~23~~: Effect of *L. petersonii* oil on *A. fumigatus* hyphae (direct contact)



A

B

C

A: untreated hyphae; B & C: hyphae following growth on 200mg/mL *L. petersonii* oil. Black arrows indicate flattened cell wall and pitting on the cell wall. Bar = 10 µm for A. and B. and 5 µm for C.

Figure ~~34~~: Propidium iodide uptake: percentage viability of *C. albicans* following exposure to *L. petersonii* essential oil.