

Title: Antifungal activity of *Leptospermum petersonii* oil volatiles against *Aspergillus* spp in vitro and in vivo

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ABSTRACT

Objectives This study investigates the volatile (vapour) component of an essential oil derived from the Australian native *Leptospermum petersonii* as a potential treatment for aspergillosis.

Methods The *in vitro* antifungal effects of the volatiles were assayed by a variety of methods. *In vitro* mammalian cell toxicity of the oil and the oil volatiles was also determined prior to animal testing. Efficacy of the volatiles *in vivo* was assessed using a murine model.

Results *L. petersonii* oil volatiles were found to be potent inhibitors of fungal growth *in vitro* with fungicidal activity displayed following short exposure times (≤ 1 hour). No significant mammalian cell toxicity was found to be associated with the volatiles. In the absence of treatment, *A. fumigatus* infection of animals resulted in an increase in inflammatory cell counts and high fungal burden within the lung tissue. Chitin levels in treated animals were significantly reduced compared to control animals. No viable fungi could be recovered from animals that had completed the treatment regimen.

Conclusions The significant reduction in fungal burden in the lungs of infected animals by the volatiles of *L. petersonii* oil was larger than that reported for conventional antifungal drugs of choice.

INTRODUCTION

The profile of patients considered at risk for potentially fatal fungal infections, such as invasive pulmonary aspergillosis (IPA), continues to expand due to both the increase in incidence of immunosuppressive diseases and an increasing use of immunosuppressive regimes.¹ Invasive aspergillosis is a devastating opportunistic infection, the causative organism is difficult to avoid, diagnosis of the disease can be problematic and prognosis is poor: even with treatment the mortality rate of IPA can approach 80%.^{2,3} Consequently, novel, broad spectrum, non toxic antifungal compounds, appropriate for empirical use and not prone to selection of resistant organisms are required. One potential source for novel antifungal compounds may be plant essential oils.

This study investigated the volatile components of *Leptospermum petersonii* as a potential treatment for aspergillosis by measuring the *in vitro* antifungal activity, by a variety of methods, on *Aspergillus fumigatus*, and then testing efficacy in a small scale *in vivo* trial. The *in vitro* mammalian cell toxicity of the oil and the oil volatiles were also examined to ensure low toxicity prior to animal testing.

MATERIAL AND METHODS

***In vitro* analysis of antifungal activity**

The activity of *L. petersonii* oil volatiles was determined using a modified micro-atmosphere method as described by Utama et al. 2002⁴, using 100 mm x 1.5 mm glass Petri dishes and 100 μ L of essential oil. The oil was removed at 0.5, 1, 3, 6 or 18 h intervals and the plates re-incubated at 35°C for 48 hours. *Aspergillus fumigatus* was maintained on malt extract agar.

The activity of *L. petersonii* oil volatiles using the method of delivery employed in the *in vivo* studies was also examined. Briefly, 500 μ L of *Aspergillus* spore suspension (1×10^6 spore/mL) was either lawn inoculated onto an agar plate or placed onto a 30 mm filter paper disc on a Petri dish lid. Both the inoculated agar plate and the plate with attached filter disc were independently placed into a 300 mm diameter glass vacuum desiccator modified for delivery of essential oil volatiles, with no desiccant inserted. One of the two desiccator outlets was connected via silicon tube to a 500 mL Erlenmeyer flask containing 10 mL *L. petersonii* oil, while the other remained open to the ambient air. A pump (Aquatic Animal, Flow 2.0 L/min, Capacity 120 L, Pressure 0.012 MPa) was connected to the flask side-arm to provide a pressure gradient from flask to desiccator. Test samples were positioned 2 cm below the outlet for the essential oil volatiles and exposed for 1 h to heated oil (80°C), heated empty flask (80°C), room temperature oil or ambient air. Exposed spore inoculated and control filter discs were placed onto the surface of a sterile agar plate prior to incubation. All samples were then sealed and incubated (35°C/48 h). Colony counts and colony size were determined and compared to control plates. All results presented are the mean of three independent replicates.

Mammalian cell toxicity testing

HEp-2 cell toxicity was tested using the XTT Cell Proliferation Kit II (Roche) in a 96 well flat bottom microtitre plate (Sigma). The concentration of oil tested ranged from 1% to 0.001% (v/v). The IC₅₀ (50% inhibitory concentration) was calculated using non-linear regression.

For evaluation of mammalian cell toxicity of the volatile component of *L. petersonii* essential oil, 300 μ L of a 1×10^5 cell/mL HEp-2 cell suspension was added to each of 6 wells in two 24 well flat bottom plates. The plates were incubated (37°C/5% CO₂) until the control monolayers reached 75% confluent, at which time the media was removed and replaced with 100 μ L of fresh media. One HEp-2 seeded plate was placed inside a 30 cm glass desiccator and *L. petersonii* oil volatiles from heated oil (80°C) were administered for 60 min as described above for *in vitro* antifungal testing. Control plates were placed in a separate desiccator containing only ambient air. Six cell-free control wells in each plate contained only 100 μ L of media. Media was then

discarded from all wells containing cells and replaced with 500 μ L of fresh media. The effect on cell proliferation was determined using colorimetric assay as described above.

***In vivo* testing of anti-*Aspergillus* activity of *L. petersonii* volatiles**

Adult male BALB/c mice (20 – 30g) were housed in groups and provided with feed and water ad libitum. All procedures involving animals were approved by the Charles Sturt University Animal Care and Ethics Committee (approval number 04/041). Mice were immunosuppressed using an intra-peritoneal injection of 250 mg/kg of cyclophosphamide (Sigma-Aldrich) administered as a single injection 3 days prior to infection and 1 day post-infection, with uninfected control animals following the same schedule. 500 mg/L of tetracycline (Sigma) was added to the drinking water of all animals following cyclophosphamide treatment.

Animals were randomly allocated to one of 5 groups: Group 1 - uninfected, untreated controls (n = 5); Group 2 - uninfected, treated controls (n = 5); Group 3 - infected, untreated controls (n = 11); Group 4 - infected, early treatment (n = 11); and Group 5 - infected late treatment (n = 12). Animals in groups 3, 4 and 5 were inoculated intranasally with 25 μ L of a 1×10^7 spores/mL *A. fumigatus* conidial spore suspension following the method of Cenci *et al.* (2000).¹ Commencing one day post-inoculation, animals in groups 2 and 4 received treatment with *L. petersonii* volatiles, while those in group 5 commenced treatment 5 days post-inoculation. Treatment with *L. petersonii* oil volatiles occurred daily for three days with all treatments following the same procedure. Individual mice were placed in a 30 cm diameter glass desiccator and the essential oil volatiles from heated oil (80°C) administered for 1 h as described above for *in vitro* studies. Control animals (groups 1 and 3) underwent the same procedure but were exposed to ambient air for 1 hour daily, rather than essential oil volatiles. Following each treatment animals were returned to their cages and monitored closely for signs of distress or irritation. All animals were sacrificed 9 days after inoculation using an overdose of sodium pentobarbitone (i.p: 200 mg/kg). The lungs were removed, with one immersion fixed in 10% neutral buffered formalin at room temperature for 6 h while the other was placed on ice for determination of fungal load. Fixed tissues were stored in 70% alcohol prior to routine histological processing.

Lung specimens were embedded in Tissue-Tek III embedding wax and sectioned at 5 μ m using a rotary microtome. Serial sections were mounted on glass microscope slides with every 5th and 10th slide stained using Mayers haematoxylin and eosin or Grocotts Methenamine Silver stain respectively. The degree of accumulation of inflammatory cells and the presence or absence of oedema, fungal hyphae and granulomas was recorded. Inflammatory cell counts (alveolar macrophages and neutrophils) were determined by examination of 50 fields of view (FOV) from three different lung sections, each separated by >200 μ m. Results were expressed as the

number of macrophage and/or neutrophils per FOV (x 1000 magnification). Animals that died prior to sacrifice were excluded from this analysis due to the presence of post mortem blood in the lung. Differences between groups was determined statistically using ANOVA, with $p < 0.05$ considered statistically significant.

Fungal load in lung tissue was determined using two methods: colony forming unit (cfu) count and chitin content. To determine cfu count, pre-weighed lung tissue was homogenised in a 7 mL glass homogeniser (Pyrex). The resulting suspension was diluted 1:10 in PBS and 100 μ L plated out on malt extract agar and incubated at 25°C for 48 h. Resulting colonies were counted and the colonies per gram of lung tissue were calculated.

Chitin content was determined by the method of Lehmann & White (1975).⁵ Chitin content was expressed as a unit of glucosamine equivalent. Chitin content was adjusted to give the total μ g of glucosamine per lung by multiplying the pre-weighed analysed tissue by the weight of the entire lung as measured before sectioning.

RESULTS AND DISCUSSION

***In vitro* analysis of antifungal activity**

L. petersonii essential oil volatiles displayed antifungal activity against *A. fumigatus* in a short time frame. These results demonstrate that *L. petersonii* volatiles appear to be one of the most active essential oils reported for inhibition of *Aspergillus* fungi.^{4, 6}

Oil volatiles have a potential advantage as a treatment option for lung infections such as aspergillosis as they can be inhaled directly into the lung; consequently a delivery system was developed to allow administration of essential oil volatiles to infected animals. Using this method *L. petersonii* volatiles at room temperature did not inhibit *A. fumigatus*, however, when the oil was heated to 80°C, there was 100% growth inhibition.

Mammalian cell toxicity

The IC₅₀ for *L. petersonii* essential oil against HEp-2 cells was 0.04% (v/v). The majority of essential oils have been shown to have an associated toxicity when applied directly to skin or ingested and this result implies this is the same for *L. petersonii*.⁷

In contrast, exposure of HEp-2 cells to *L. petersonii* essential oil volatiles for one hour had no effect on cell proliferation (99.68±0.93% cell survival). There is little published data that

discusses the cytotoxicity of essential oil volatiles, however it was demonstrated that there was no foetal toxicity when rats were exposed to citral via inhalation for 6 hours per day.⁸ Volatiles can be administered directly to the infection site of *Aspergillus* via inhalation, with deep tissue penetration achieved easily, making *L. petersonii* oil volatiles a potential chemotherapeutic agent for treatment of aspergillosis.

***In vivo* analysis**

A summary of the data from the preliminary *in vivo* analysis is shown in Table 1. Animal models of aspergillosis are well described in the literature. The intranasal inoculation route was utilised in this study as it is the least invasive and most closely reflects the inoculation route in humans.³ Consistent with previous studies, examination of the histological sections of animals infected with *Aspergillus* revealed a significant rise in the number of both macrophages and neutrophils in infected lung tissue with the common pattern of inflammation in *Aspergillus* infected lungs of neutrophils and macrophages surrounding fungal components which have grown in or near the peribronchial region.⁹ The high number of neutrophils and macrophages found in the lungs, despite immunosuppression of the animals, is consistent with previous studies that have suggested that the fungal infection stimulates maximum recruitment of the limited number of remaining and newly formed inflammatory cells, leaving very small numbers in the peripheral blood.^{9, 10}

Staining of infected but untreated lung tissue sections revealed extensive signs of hyphal invasion throughout the lung tissue, with branching, septate hyphae, indicative of invasive *Aspergillus*. When treatment was completed in full for the early treatment group (group 4) and the late treatment group (group 5), no fungi were detectable within the lung tissue via histological analysis, chitin content analysis or cfu count. Conventional antifungal treatments such as liposomal amphotericin B (AmBisome®) and fluconazole have been reported to produce no statistically difference in cfu counts when compared to control groups with voriconazole and amphotericin B leading to an approximate log 1 reduction in lung cfu counts and itraconazole reported to reduce the cfu count by 50%.^{11, 12} It is evident that the volatiles of *L. petersonii* were effective at reducing the fungal load when compared to conventional treatments; with infected control animals having an average of log₁₀ 2.42 of *Aspergillus* colonies per gram of lung tissue while no viable *Aspergillus* could be recovered from the lung tissue of any animal that completed the treatment regimen.

Quantification of lung burden with cfu can, however, be problematic and unreliable as it may underestimate fungal burden through mycelial or spore clumps giving rise to single colonies or overestimate as hyphal fragments of varying lengths can result in unique colonies. Therefore, other

parameters of fungal cells, such as chitin analysis, should also be evaluated in order to accurately quantify the fungal biomass. In this study, no chitin was detected in any uninfected animal or the one animal in the infected control group (group 3) which did not develop aspergillosis, while the level of chitin within the lungs of infected control animals fell within the published data range of 5.5 to 1148 µg glucosamine per lung^{13, 14} When mice received the full *L. petersonii* oil treatment from 1 day post-infection (group 4), the average level of chitin fell 100 fold. Similarly, animals that received the treatment from 5 days post infection (group 5) demonstrated an average chitin content decrease of 50 fold.

The results of this study clearly demonstrate that no viable fungi and little or no chitin could be detected in the lung tissue of any animal that completed the *L. petersonii* volatile treatment regimen, whether treatment was initiated within 24 hours of infection or delayed until establishment of *Aspergillus* infection. The mechanism of antifungal action of *L. petersonii* essential oil and potential interaction with conventional drugs remains unknown; however, the significant reduction in fungal load via simple inhalation of *L. petersonii* essential oil volatiles requires further investigation.

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TRANSPARENCY DECLARATION

None to declare

Table 1: Mean macrophage and neutrophil counts per field of view and cfu counts and chitin level per lung.

	Group 1 Control	Group 2 Treatment Control	Group 3 Infection control	Group 4 Early Treatment (infected)	Group 5^e Late Treatment (infected)
No. of animals with visible hyphae in stained lung sections	0/5	0/5	10/11	3 ^b /11	2/10 ^e
No. of animals with an increase in inflammatory cells in lung tissue	1 ^a /5	2 ^a /5	11/11	10/11 ^c	10/10 ^e
No. of animals with oedema in lung tissue	0/5	0/5	8/11	4/11 ^c	5/10 ^e
No. of animals with granulomas present in lung tissue	0/5	0/5	6/11	3/11 ^d	0/10 ^e
Mean macrophage count per field of view	1.34 ± 0.23	1.61 ± 0.15	6.29 ± 1.17	2.69 ± 0.55	3.06 ± 0.49
Mean neutrophil count per field of view	1.02 ± 0.73	0.93 ± 0.27	4.72 ± 1.50	1.54 ± 0.38	2.28 ± 0.73
Mean cfu count per gram of lung tissue (log)	0	0	2.51 ± 0.84	0.59 ± 1.10 ^c	0
Mean chitin level per lung (µg of glucosamine)	0	0	11.57	0.12 ^c	0.27 ^e

Key:

^a slight increase:

^b cfu only detected in animals that died prior to completion of treatment:

^c three animals in this group died prior to completion of treatment. No chitin or viable fungi were detected in the lung tissue of any animal that completed the treatment regimen;

^d granulomas visualised in one animal that died prior to completion of treatment and two animals that completed treatment;

^e animals in group 5 that died prior to commencement of treatment were not included

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