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

J. R. Hood1,2, D. Burton3, J. M. Wilkinson1 and H. M. A. Cavanagh1*

1School of Biomedical Sciences, Charles Sturt University, Booroomba Street, Wagga Wagga NSW 2650, Australia; 2Surveillance Branch, Office of Health Protection, Department of Health and Ageing, Canberra ACT 2601, Australia

*Corresponding author. Tel: +61-2-6933-2501; Fax: +61-2-6933-2587; E-mail: hcavanagh@csu.edu.au

Received 22 September 2009; accepted 12 October 2009

**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 h). No significant mammalian cell toxicity was found to be associated with the volatiles. In the absence of treatment, *Aspergillus 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 with 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.

**Keywords:** *L. petersonii*, essential oils, volatile compounds

**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, nontoxic 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 their efficacy in a small-scale *in vivo* trial. The *in vitro* mammalian cell toxicity of the oil and the oil volatiles was also examined to ensure low toxicity prior to animal testing.

**Materials and methods**

**In vitro analysis of antifungal activity**

The activity of *L. petersonii* oil volatiles was determined using a modified microatmosphere method, as described by Utama et al.,4 using 100×1.5 mm glass Petri dishes and 100 µL of essential oil. The oil was removed at 0.5, 1, 3, 6 or 18 h intervals and the plates reincubated at 35°C for 48 h. *A. 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 µL of *Aspergillus* spore suspension (1×10⁶ spores/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 the delivery of essential oil volatiles, with no desiccant inserted. One of the two desiccator outlets was connected via a silicon tube to a 500 mL Erlenmeyer flask containing 10 mL of *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 the flask to the 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...
Animals in Groups 3–5 were inoculated intranasally with 250 μL of a 1×10⁸ L. petersonii cell suspension adjusted 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% confluence, at which time the medium was removed and replaced with 100 μL of fresh medium. 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 μL of medium. Medium was then discarded from all wells containing cells and replaced with 500 μL of fresh medium. The effect on cell proliferation was determined using a colorimetric assay, as described above.

**Mammalian cell toxicity testing**

HEP-2 cell toxicity was tested using the XTT Cell Proliferation Kit II (Roche) in a 96-well flat bottom microtiter 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 μL of a 1×10⁹ cells/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% confluence, at which time the medium was removed and replaced with 100 μL of fresh medium. 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 μL of medium. Medium was then discarded from all wells containing cells and replaced with 500 μL of fresh medium. The effect on cell proliferation was determined using a colorimetric assay, as described above.

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

Adult male BALB/c mice (20–30 g) 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 04/04/1). Mice were immunosuppressed using an intraperitoneal injection of 250 mg/kg 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. Following cyclophosphamide treatment, 500 mg/L tetracycline (Sigma) was added to the drinking water of all animals.

Animals were randomly allocated to one of five 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 3–5 were inoculated intranasally with 25 μL of a 1×10⁷ spores/mL A. fumigatus conidial spore suspension following the method of Cenci et al.³ Commencing 1 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 3 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 h 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 (intraperitoneally, 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 the fungal load. Fixed tissues were stored in 70% alcohol prior to routine histological processing.

**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 the inhibition of Aspergillus fungi.⁴ ⁷ 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 it is the same for L. petersonii.⁸

In contrast, exposure of HEP-2 cells to L. petersonii essential oil volatiles for 1 h had no effect on cell proliferation (99.68 ± 0.93% cell survival). There are few published data that discuss the cytotoxicity of essential oil volatiles; however, it was demonstrated that there was no fetal toxicity when rats were exposed to citral via inhalation for 6 h/day.³ 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.
Antifungal activity of *Leptospermum petersonii* oil volatiles

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

<table>
<thead>
<tr>
<th></th>
<th>Group 1, control</th>
<th>Group 2, treatment control</th>
<th>Group 3, infection control</th>
<th>Group 4, early treatment (infected)</th>
<th>Group 5, late treatment (infected)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of animals with visible hyphae in stained lung sections</td>
<td>0/5</td>
<td>0/5</td>
<td>10/11</td>
<td>30/11</td>
<td>2/10c</td>
</tr>
<tr>
<td>No. of animals with an increase in inflammatory cells in lung tissue</td>
<td>10/5</td>
<td>20/5</td>
<td>11/11</td>
<td>10/11</td>
<td>10/10c</td>
</tr>
<tr>
<td>No. of animals with oedema in lung tissue</td>
<td>0/5</td>
<td>0/5</td>
<td>8/11</td>
<td>4/11c</td>
<td>5/10c</td>
</tr>
<tr>
<td>No. of animals with granulomas present in lung tissue</td>
<td>0/5</td>
<td>0/5</td>
<td>6/11</td>
<td>3/11c</td>
<td>0/10c</td>
</tr>
<tr>
<td>Mean macrophage count per field of view</td>
<td>1.34 ± 0.23</td>
<td>1.61 ± 0.15</td>
<td>6.29 ± 1.17</td>
<td>2.69 ± 0.55</td>
<td>3.06 ± 0.49</td>
</tr>
<tr>
<td>Mean neutrophil count per field of view</td>
<td>1.02 ± 0.73</td>
<td>0.93 ± 0.27</td>
<td>4.72 ± 1.50</td>
<td>1.54 ± 0.38</td>
<td>2.28 ± 0.73</td>
</tr>
<tr>
<td>Mean cfu count per gram of lung tissue (log)</td>
<td>0</td>
<td>0</td>
<td>2.51 ± 0.84</td>
<td>0.59 ± 1.10c</td>
<td>0</td>
</tr>
<tr>
<td>Mean chitin level per lung (µg of glucosamine)</td>
<td>0</td>
<td>0</td>
<td>11.57</td>
<td>0.12c</td>
<td>0.27c</td>
</tr>
</tbody>
</table>

aSlight increase.
bData on cfu only detected in animals that died prior to completion of treatment.
cThree 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.
dGranulomas visualized in one animal that died prior to completion of treatment and two animals that completed treatment.
eAnimals in Group 5 that died prior to commencement of treatment were not included.

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 utilized 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 that have grown in or near the peribronchial region.5 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.5,10

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 significant difference in cfu counts when compared with control groups. In contrast, voriconazole and amphotericin B result in an ~log 1 reduction in lung cfu counts and itraconazole is 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 with conventional treatments, with infected control animals having an average of log_{10} 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 the lung burden with cfu can, however, be problematic and unreliable, as it may: (i) underestimate the fungal burden through mycelial or spore clumps giving rise to single colonies; or (ii) overestimate the fungal burden, 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) that did not develop aspergillosis, while the level of chitin within the lungs of infected control animals fell within the published data range of 5.5–1148 µg of 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 h 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.

Acknowledgements

We wish to thank Mr D. Archer, Toona Essential Oils Buddina QLD, Professor D. Ellis, Women’s and Children’s Hospital, Adelaide and Ms
S. Wheeler, Charles Sturt University, for supplying the L. petersonii oil, clinical isolates of Aspergillus and HEp-2 cells utilized in this study, respectively.

Funding
This project was supported by an Australian Postgraduate Award and a Charles Sturt University (internal) research grant.

Transparency declarations
None to declare.

References