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Determination of *Legionella pneumophila* susceptibility to *Melaleuca alternifolia* Cheel (tea tree) oil by an improved broth micro-dilution method under vapour controlled conditions

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ABSTRACT

The aim of this study was to determine the *in vitro* activity of *Melaleuca alternifolia* Cheel (tea tree) oil (TTO) against 22 strains of *Legionella pneumophila* of different serogroup and source of isolation. Both a standard broth micro-dilution method, with slight modifications, and a micro-atmosphere diffusion method were used. Furthermore, we have established a simple sealing procedure in the micro-dilution method to determine the antibacterial activity of TTO against *Legionella* in aqueous phase. The results showed that *L. pneumophila*, quite irrespective of serogroup and source of isolation, is exquisitely sensitive to TTO, with minimal inhibitory concentration (MIC) ranging from 0.125 to 0.5% v/v, and a bactericidal activity at 0.5% v/v. In addition, we show here that TTO vapours exert critical activity, that must be controlled for reproducible MIC determinations. Overall, our data suggest that TTO could be active as anti-*Legionella* disinfectant, for control of water system contamination, especially in spas, in small waterlines or in particular respiratory medical devices.

1. Introduction

Recently, the essential oils (EOs), their constituents and products of plant secondary metabolism have been evaluated for their antimicrobial activities (Inouye et al., 2001a; Burt, 2004; Chami et al., 2004; Friedman et al., 2004; Hammer et al., 2004) in the perspective of potential therapeutic use. Among the EOs, Australian tea tree oil (TTO) is one of the most promising due to the broad spectrum of antibacterial, antifungal, antiviral and other biological activities. In particular, TTO activity has been assessed against a high number of bacterial species, using standardized or modified methodologies. However, data on *in vitro* activity of TTO against *Legionella pneumophila* are lacking, probably also due to the difficulties encountered for standardization of susceptibility methods for this fastidious-to-grow microorganism.

L. pneumophila is the causative agent of two clinical syndromes in humans: a severe pneumonia called Legionnaires' disease (Fraser et al., 1977) and a self-limited flu-like illness named Pontiac fever (Glick et al., 1978). *Legionella* is ubiquitous in freshwater environment where it survives as intracellular parasite of free-living protozoa and can be associated with biofilms (Albert-Weissenberger et al., 2007; Molofsky and Swanson, 2004). The infection occurs through inhalation or aspiration of *Legionella* contaminated aerosols, followed by replication in lung alveolar macrophages. The disease follows *Legionella*'s ability to replicate inside the phagosome, thus escaping from intra-lysosomal degradation (Horwitz and Maxfield, 1984; Tilney et al., 2001). In Europe, *Legionella* species are the causative agent of about 1.9%, 4.9% and 7.9% pneumonia cases occurring in community, health care institutions and requiring admission to intensive care units, respectively (Woodhead, 2002). Outbreaks of legionellosis have been mostly associated to artificial contaminated environments such as building water distribution systems, cooling towers, spas, and similar. Water distribution systems and aerosol-producing medical devices are main sources of health care-associated legionellosis (Stout and Yu, 2003a; Tablan et al., 2004).

Although various physical and chemical disinfection methods have been investigated to reduce *Legionella* contamination in artificial water sources (Stout and Yu, 2003b; Chang et al., 2007; Zhang et al., 2007; Sheffer et al., 2005), environmental control of *Legionella* is still a great challenge, and the optimal strategy for long-term abatement of bacterial burden remains quite problematic (Atlas, 1999; Lasheras et al., 2006).

The EOs constitute an interesting category of compounds endowed with broad spectrum antimicrobial activity (Kalemba and Kunicka, 2003; Ríos and Recio, 2005; Janssen et al., 1987). In theory, these compounds could be used alone or in combination with other disinfection methods, to prevent *Legionella* colonization in small waterlines (e.g. dental unit), and reduce colonization of medical devices present in health care facilities.

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In recent years, some studies have highlighted the efficacy of a variety of EOs in vapour state against respiratory tract pathogens (Inouye et al., 2001a). One of the first studies on the activity of EOs against *L. pneumophila*, was performed by Ishimatsu et al. (2003). These authors demonstrated, by the disk-diffusion method, the anti-*Legionella* activity of hinokitiol (beta-thujaplicin), which is a major component of the essential oil of *Chamaecyparis obtuse*. In a more recent paper, Chang et al. (2008) described the activity of other EOs extracted by *Cinnamomum osmophloeum* leaves and by different tissues of *Cryptomeria japonica* against *L. pneumophila* at 42 °C. In particular, these authors emphasized a potential role for EOs to control *Legionella* contamination of hot water systems.

Overall, the studies reported so far have been performed with different methods, and different measure outcomes have been employed, with little comparison among them. These differences have strongly influenced the determination of the anti-*Legionella* activity of EOs, thus constituting an important limitation in our understanding of their true disinfection potential for *Legionella* control. Moreover, it appears that in the two previous studies (Chang et al., 2008; Ishimatsu et al., 2003) only one strain of *Legionella* has been employed.

The aim of our study was to determine the *in vitro* activity of TTO against *L. pneumophila* serogroups (SG) 1 and 6 from different sources (stock cultures, environment, clinical) by using two different methods: the Clinical and Laboratory Standards Institute (CLSI) broth micro-dilution method (CLSI, 2006) with slight modifications, and the micro-atmosphere diffusion method (Maruzzella and Sicurella, 1960; López et al., 2005). Furthermore we have improved the conditions of the assay by careful consideration of oil activity in aqueous phase and preventing evaporation by microplate sealing.

2. Materials and methods

2.1. Essential oil Melaleuca alternifolia Cheel (tea tree)-oil and its components

Although complying with the International Standard ISO 4730 (International Organization for Standardization ISO 4730, 2004) Australian *Melaleuca alternifolia* (Maiden and Betch) Cheel oil, supplied by Variati (Milan, Italy), was analysed before use for the exact determination of single constituents by gas chromatography (GC-FID) and gas-chromatography–mass spectrometry (GC–MS), as previously reported (Mondello et al., 2006). Terpinen-4-ol and 1,8–cineole, at a concentration of 42.35% and 3.57%, respectively, were the typical constituents which characterize the standard TTO composition, in accord with the prescription of the European Pharmacopeia (The 5th Edition of European Pharmacopoeia and its subsequent supplements, 2006) and International Standard ISO 4730. Terpinen-4-ol, which was purchased from Fluka (Buchs, Switzerland) and 1,8-cineole from Sigma-Aldrich (St Louis, MO, USA), were used as positive markers. Both components were >97% pure.

2.2. Bacterial strains and culture media

Twenty-two *L. pneumophila* strains from stock collection, clinical and environmental isolates were tested. They included: *L. pneumophila* SG 1 (Lp 1) ATCC 33152, *L. pneumophila* SG 3 (Lp 3) ATCC 33155, *L. pneumophila* SG 6 (Lp 6) ATCC 33215, *L. pneumophila* SG 8 (Lp 8) ATCC 35096, 6 Lp1 clinical isolates, 5 Lp1 environmental isolates, 5 Lp6 clinical isolates and 4 Lp6 environmental isolates. All strains were stored at −70 °C in sterile skimmed milk until use. Subculture of frozen bacteria were grown on buffered charcoal yeast extract agar supplemented with 0.1% alpha-ketoglutarate (BCYE-α, Oxoid, LTD, Basingstoke, England). Plates were incubated at 36 ± 1 °C in humidified air with 2.5% CO_2 for 72 h. *E.coli* ATCC 25922 was used as quality control.

The micro-dilution test was performed using buffered yeast extract broth (BYEB) prepared with Bacto yeast extract (Becton Dickinson and Company Sparks, MD, USA) added with *Legionella* BCYE growth supplement (Oxoid LTD, Basingstoke, England). Tween-80 (Sigma-Aldrich, St Louis, MO, USA) was added to BYEB to facilitate oil solubility (Hammer et al., 1998). After preparing micro-dilution trays with EO solutions the filled trays were sealed in plastic bags and immediately placed in a freezer at ≤−20 °C until needed.

2.3. Micro-dilution method for TTO antibacterial testing against Legionella

2.3.1. Legionella inoculum determination

Preliminary experiments carried out with the standardized inoculum described for antibiotic susceptibility testing according to the CLSI protocol M7-A7 (CLSI, 2006) did not allow a realistic, reproducible evaluation of TTO against *Legionella*. To evaluate the inoculum size, 72 h agar cultures of *Legionella* were suspended in sterile distilled water to 0.6 optical density measured at 600 nm (≅10^9 CFU/ml), vortex-mixed and diluted to give the following concentrations: ≅10^5, 10^6, 10^7, 10^8 CFU/ml. 50 µl of each diluted suspension were added in duplicate in two-fold dilutions of TTO-solution to give approximate concentrations of 10^4, 10^5, 10^6, 10^7 CFU/well (final volume 100 µl/well) in 96-well microtitre plates. The final TTO concentrations tested ranged from 0.0078% to 4% v/v. The quality control strain used in this study was as described in CLSI standard protocol. The viable inoculum was determined by CFU enumeration (in BCYE-α agar medium). Experiments were performed with the final inoculum size approximating 5×10^4 CFU/ml for *E.coli* ATCC 25922.

2.3.2. TTO solubilization

Preliminary experiments were performed to evaluate the minimal concentration of Tween 80 needed to solubilise TTO and able to maintain *Legionella* viability. Serial dilutions of Tween 80 in BYE-α broth from 1% to 0.0015% v/v concentration were performed in duplicate in 96-well microtitre plates. 10^8 bacteria/ml were inoculated at a final concentration of ~2.5×10^6 CFU/well. After 72 h at 36 ± 1 °C in 2.5% CO_2 atmosphere, optical density was measured spectrophotometrically at 600 nm, and the number of viable *Legionella* cells in each well was determined by CFU counting in BCYE-α agar medium.

2.3.3. Determination of minimum inhibitory and bactericidal concentration

Susceptibility testing to TTO was performed according to the CLSI broth micro-dilution method, with the following modifications: 1) BYEB instead of Muller–Hinton broth; 2) emulsifier Tween 80; 3) inoculum size 10^8 instead of 10^5 CFU/ml; 4) use of a sterilised Transparent Microplate Sealer (TMS) during incubation for 72 h. TTO was diluted using BYEB in the presence of Tween 80 at 0.001% v/v. Aliquots of 50 µl of two-fold dilutions of TTO solution were dispensed in 96-well microtitre plates. The final concentration of the essential oil ranged from 0.0078% to 4% v/v for TTO. The inoculum was prepared from 72 h agar cultures of *Legionella*, suspended in sterile distilled water to 0.6 optical density (measured at 600 nm), which corresponded to about 10^9 CFU/ml, as determined by a pre-established standard curve relating optical densities to the CFU. The suspension was vortex-mixed and diluted to give 10^8 CFU/ml. 50 µl of each diluted suspension (10^8 CFU/ml) were added in duplicate in two-fold dilutions of TTO-solution to give final concentration of ~ 2.5×10^6 CFU/well. Microtitre plates were then incubated at 36 ± 1 °C with 2.5% CO_2 for 72 h covered with or without a sterilised Transparent Microplate Sealer (TMS) (AMP Llseal, Greiner Bio-one, Germany). The control of bacterial growth was performed in TTO-free medium added with 0.001% v/v Tween 80. Each tray included a sterility column (eight wells) non inoculated.