

NOTE TO THE EDITOR

***In vitro* antiviral activity of *Melaleuca alternifolia* essential oil**

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Keywords

antiviral activity, essential oil, influenza virus, *Melaleuca alternifolia*, terpinen-4-ol.

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2009/0924: received 26 May 2009, revised 28 August 2009 and accepted 29 August 2009

doi:10.1111/j.1472-765X.2009.02740.x

Abstract

Aims: To investigate the *in vitro* antiviral activity of *Melaleuca alternifolia* essential oil (TTO) and its main components, terpinen-4-ol, α -terpinene, γ -terpinene, *p*-cymene, terpinolene and α -terpineol.

Methods and Results: The antiviral activity of tested compounds was evaluated against polio type 1, ECHO 9, Coxsackie B1, adeno type 2, herpes simplex (HSV) type 1 and 2 viruses by 50% plaque reduction assay. The anti-influenza virus assay was based on the inhibition of the virus-induced cytopathogenicity. Results obtained from our screening demonstrated that the TTO and some of its components (the terpinen-4-ol, the terpinolene, the α -terpineol) have an inhibitory effect on influenza A/PR/8 virus subtype H1N1 replication at doses below the cytotoxic dose. The ID₅₀ value of the TTO was found to be 0.0006% (v/v) and was much lower than its CD₅₀ (0.025% v/v). All the compounds were ineffective against polio 1, adeno 2, ECHO 9, Coxsackie B1, HSV-1 and HSV-2. None of the tested compounds showed virucidal activity. Only a slight virucidal effect was observed for TTO (0.125% v/v) against HSV-1 and HSV-2.

Conclusions: These data show that TTO has an antiviral activity against influenza A/PR/8 virus subtype H1N1 and that antiviral activity has been principally attributed to terpinen-4-ol, the main active component.

Significance and Impact of the Study: TTO should be a promising drug in the treatment of influenza virus infection.

Introduction

The essential oil of *Melaleuca alternifolia*, also known as tea tree oil (TTO), is a complex mixture of terpene hydrocarbons and tertiary alcohols mainly distilled from plantation stands of the Australian native plant *M. alternifolia*, a member of the *Myrtaceae* family. A series of standards has attempted to define and limit the variation seen in this heterogeneous mixture, because it is subject to considerable batch-to-batch variation depending on growth conditions at the plantations (Kawakami *et al.* 1990). The exact constituency of TTO varies, as well as the antibacterial, antifungal, anti-inflammatory and analgesic properties (Carson and Riley 1993; Hart *et al.* 2000; Hammer *et al.* 2003; Caldefie-Ch  zet *et al.* 2006).

Shellie *et al.* identified about 72 out of 97 possible components in *M. alternifolia* using GC and GC/MS.

Quantitative results (g% w/w) obtained by GC (FID, Flame Ionization Detector) analysis of oil samples, demonstrated that TTO is characterized by a high proportion of terpinen-4-ol (36.71%) and γ -terpinene (22.20%), and moderate levels of 1,8 cineole (2.49%), *p*-cymene (2.52%), α -terpinene (10.10%), terpinolene (3.53%) and α -terpineol (2.74%) (Shellie *et al.* 2003).

Many authors have demonstrated that TTO has a broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria, both aerobic and anaerobic, against yeasts and fungi. It is also active against clinically isolated fluconazole-resistant *Candida* strains (Hammer *et al.* 2004; Wilkinson and Cavanagh 2005; Carson *et al.* 2006).

Recently, it was also demonstrated an *in vitro* antimicrobial activity against *Mycoplasma pneumonia*, *Mycoplasma fermentans* and *Mycoplasma hominis* (Furneri *et al.* 2006).

The aims of the present study was to compare the *in vitro* antiviral activity of TTO and its constituents, terpinen-4-ol, α -terpinene, γ -terpinene, *p*-cymene, terpinolene and α -terpineol, against some DNA and RNA viruses, including influenza A/PR8 virus subtype H1N1 in Madin-Darby Canine Kidney (MDCK) cells, Herpes simplex virus type 1 (HSV-1) and 2 (HSV-2) in VERO cells, Echovirus 9 (Hill strain) in LLC-MK2 cells, Poliovirus 1 (Sabin strain), Coxsackievirus B1 and Adenovirus 2 in HEp2 cells.

TTO employed in our experiments was provided by Australian Botanical Products (Hallam, Australia). Terpinen-4-ol, α -terpinene, γ -terpinene, *p*-cymene, terpinolene and α -terpineol were obtained from Sigma Chemical Company. All the compounds were dissolved in dimethyl sulfoxide (DMSO; Sigma) to give a concentration of 10% (v/v) and diluted in maintenance medium at final concentrations ranging from 0.1% (v/v) to 0.0001% (v/v). Dilution of test compounds contained a maximum concentration of 0.01% DMSO (v/v), which was not toxic to all cell lines used.

Viruses tested working stock solutions were prepared as cellular lysates using DMEM (or RPMI 1640 for MDCK cells) supplemented with 2% heat-inactivated foetal calf serum (FCS), 0.2 g l⁻¹ of streptomycin and 200 U ml⁻¹ of penicillin G.

The cytotoxicity of the test compounds was evaluated by both measuring their effect on cell morphology (e.g. rounding up, shrinking, detachment) by light microscopy and on cell growth by the MTT method in 96-well tissue culture plates, when compared with the control cultures, after 24, 48 and 72 h. The 50% cytotoxic dose (CD₅₀) was expressed as the concentration of the compounds that inhibited cell growth by 50% when compared with the control cultures (Cutri *et al.* 1998).

The antiviral activity of tested compounds was evaluated against polio 1, ECHO 9, Coxsackie B1, adeno 2,

HSV-1 and HSV-2 viruses by 50% plaque reduction assay, as previously described (Cutri *et al.* 1998). The anti-influenza virus assay was based on the inhibition of the virus-induced cytopathogenicity on MDCK cells, as previously described (Garozzo *et al.* 2000).

The compound concentration required to inhibit virus plaque formation and virus-induced cytopathogenicity by 50% was expressed as the 50% inhibitory dose (ID₅₀) and calculated by dose-response curves and linear regression. Noninfected and infected cells in the absence of compounds served as cell and virus control, respectively.

To test possible virucidal activity, equal volumes (0.5 ml) of virus suspension (containing 10⁶ PFU ml⁻¹) and medium containing various concentrations of the compounds were mixed and incubated for 1 h at 37°C. Infectivity was determined by plaque assay after dilution of the virus below the inhibitory concentration.

Results obtained from our screening demonstrated that the TTO and some of its components have an inhibitory effect on influenza virus A/PR8 replication at doses below the CD₅₀ (Table 1).

In fact, the ID₅₀ value was found to be 0.0006% (v/v) and was much lower than its CD₅₀ (0.025% v/v). Three of the TTO components tested were effective. In particular, the ID₅₀ values were found to be 0.0025% (v/v), 0.0012% (v/v) and 0.025% (v/v) for terpinen-4-ol, terpinolene and α -terpineol, respectively. Finally, compounds α -terpinene, *p*-cymene and γ -terpinene were completely ineffective.

All the compounds were ineffective against polio 1, adeno 2, ECHO 9, Coxsackie B1, HSV-1 and HSV-2 viruses.

The study of the effect of these compounds on neutralization of virus infectivity demonstrated that none of the tested compounds showed virucidal activity against polio 1, adeno 2, ECHO 9, Coxsackie B1 and influenza A/PR8. Only a slight reduction for the TTO (0.125% v/v) against

Compounds	CD ₅₀ (%v/v)*,†	ID ₅₀ (%v/v)*,‡						
		Influenza	HSV-1	HSV-2	ECHO 9	Cox B1	Polio 1	Adeno 2
TTO	0.025	0.0006	>0.025	>0.025	>0.025	>0.025	>0.025	>0.025
Terpinen-4-ol	0.05	0.0025	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
Terpinolene	0.012	0.0012	>0.012	>0.012	>0.012	>0.012	>0.012	>0.012
α -Terpinene	0.012	>0.012	>0.012	>0.012	>0.012	>0.012	>0.012	>0.012
γ -Terpinene	0.012	>0.012	>0.012	>0.012	>0.012	>0.012	>0.012	>0.012
<i>p</i> -Cymene	0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
α -Terpineol	0.05	0.025	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05

HSV, Herpes simplex virus.

*Values are mean \pm 0.5 SD (maximal SD estimated) for three separate assays.

†CD₅₀, concentration which inhibited cells growth by 50% when compared with control culture.

‡ID₅₀, concentration which inhibited virus plaque formation and virus-induced cytopathogenicity by 50%.

Table 1 Antiviral activity of tea tree oil (TTO) and its components

HSV-1 and HSV-2 viruses was demonstrated (data not shown).

In contrast with the results reported by other authors (Carson *et al.* 2001, 2008; Schnitzler *et al.* 2001), TTO did not show any antiviral activity against the replicative cycle of HSV-1 and HSV-2.

In this case, the different mechanism of action could depend on many factors involved. In fact, the origin of TTO, the diversity of antimicrobial components and the relative concentrations or the viral serotype could influence the antiviral activity.

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