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The Activity of Cedar Leaf oil Vapor Against Respiratory Viruses: Practical Applications

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ABSTRACT

Respiratory viruses continue to cause frequent acute and chronic infections, for which few satisfactory treatments are available. Some essential oils possess antiviral properties, but these have usually been tested as liquids, which have limited applications. In this study the vapor of cedar leaf oil (CLO vapor) was evaluated for antiviral activity, in addition to its possible anti-inflammatory activity. The viruses tested, Influenza viruses, Rhinovirus, Adenovirus, and Herpes simplex viruses 1 and 2, in the form of dried films, were all inactivated by exposure to CLO vapor. In assays for influenza viral hemagglutinin (HA) the HA activity was inhibited by CLO vapor. Exposure of human lung epithelial cell monolayers to the vapor showed inhibition of rhinovirus virus-induced cytokine IL-6, but the cells themselves were not adversely affected by short exposure to the vapor. However the two major volatile components of CLO, thujone and α - pinene, did not show activity against influenza viral infectivity or hemagglutinin, indicating possible synergistic effects of the whole vapor. We conclude that CLO vapor has potential applications in the control of viral respiratory infections.

INTRODUCTION

Acute respiratory infections in humans are usually ascribed to one or more of a group of well known viruses, including influenza viruses A and B, rhinoviruses ("common cold" viruses), parainfluenza viruses, corona viruses, respiratory syncytial virus, and several adenoviruses (Gwaltney 2002). However, because of the significant differences in replication schemes among these viruses, it seems unlikely that a single antiviral drug could be effective as a generic remedy for "colds and flu", and chronic respiratory infections. In addition, the symptoms that accompany these infections are largely due to the viral induction of cytokines and chemokines, which may result in protracted inflammatory responses (Eccles, 2005; Roxas and Jurenka, 2007; Sharma *et al.*, 2009; Fedson, 2009; Oslund and Baumgarth 2011). Furthermore since the respiratory symptoms tend to be similar regardless of the virus, it is often difficult to specify the invading virus. Consequently there is a need for a non-toxic product that can inactivate various respiratory viruses, and also to control the inflammatory responses. Several essential oils have been shown to possess antiviral and antimicrobial activities (Carson *et al.*, 2006; Cermelli *et al.*, 2008; Alim *et al.*, 2009; Sadlon and Lange, 2010) and anecdotal evidence suggests that in some cases the vapor of such oils could be useful in alleviating the symptoms of respiratory infections (Sadlon and Lange, 2010). For example, the oil of Western red cedar leaves (*Thuja plicata;* Naser *et al.*, 2005) has been used traditionally among Aboriginal peoples of the Pacific North West to treat a variety of upper respiratory symptoms and wounds. The relatively mild odor is considered to be safe, pleasant and acceptable (Hudson *et al.*, 2011). Such a property could also be useful in decontaminating ventilation systems in buildings where "sick building syndrome" is a problem (Norback, 2009; Hudson *et al.*, 2011). However it is important to establish experimentally that short-term exposure to CLO vapor is harmless to cells. The objective of this study was to evaluate the ability of cedar leaf oil vapor (CLO vapor) to inactivate several viruses implicated in respiratory infections, and to inhibit the influenza virus – induced secretion of cytokine (IL-6) in cultured human lung cells, under conditions designed to reflect practical situations. In addition we examined the two major constituents of CLO for possible antiviral activity.

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MATERIALS AND METHODS

Test materials

The cedar-leaf oil was obtained from Tree of Life Essential Oil, Port Hardy, BC, Canada. The product was obtained by steam distillation of *Thuja plicata* (Western red cedar) leaves, and contained >80% thujone. Pure thujone and α - pinene were obtained from Sigma Chemical co (MO, USA)

Cells and viruses

Madin-Darby canine kidney cells (MDCK), A549 human lung epithelial cells, human epithelial cells H-1, and LLC-MK monkey cells were all acquired originally from ATCC (American Type Culture Collection, Rockville, MD), and were routinely cultivated in Dulbecco MEM (DMEM), in cell culture flasks, supplemented with 5% fetal bovine serum, at 37°C in a 5% CO_2 atmosphere (cell culture reagents were obtained from Invitrogen, Ontario CA). No antibiotics or antimycotic agents were used.

Influenza virus A/Victoria/H3N2, Influenza virus A/Denver/1/57/ H1N1, Herpes simplex virus types 1 and 2 (HSV-1 and 2), and human Adenovirus type 4 (Ad-4), were acquired from BC Centre for Disease Control, Vancouver. Rhinovirus 14 was obtained from ATCC. Influenza viruses were grown in MDCK cells with TPCK (L-1-Tosylamide-2-phenylethyl chloromethyl ketone, from Sigma Chemical co.) treated trypsin (2µg/ml). HSV-1 and -2, and Ad-4, were grown in LLC-MK cells. RV 14 was grown in H-1 cells. All viruses were measured quantitatively by plaque formation in the appropriate cells.

Cytotoxicity assay

Cell viability was measured on monolayers of human lung A549 cells grown to confluence in 6-well trays. For treatment the media were removed by aspiration, and the moist cells were exposed for various times to CLO vapor. Following a further 24 h incubation in normal medium, cell viability was measured by the lactate dehydrogenase (LDH) method, using the kit obtained from Sciencell (Carlsbad, CA, USA), and following the instructions provided. The final assay reactions were measured in a micro-plate reader at a wavelength of 490 nm.

In other experiments, the Cell Proliferation Assay Kit (XTT) (ATCC, Manassas, VA) was used according to the manufacturer's instructions. The protocol was similar to that described above. All tests were run in triplicate and mean values recorded.

Virucidal activity

The method used was a modification of our standard plaque reduction assay system (Vimalanathan et al 2005). 20 µl aliquots of virus (containing 1,000 pfu) were individually dried on the underside of the caps from sterile Eppendorf tubes, within the biosafety cabinet (10 min). 250 µl of undiluted cedar leaf oil were carefully added to each tube, the caps were replaced and exposure to oil vapor for various time periods, at 22 ºC. Caps were removed again and each exposed virus film was reconstituted in 1 ml of phosphate buffered saline (PBS). All samples (in triplicate) were then assayed for virus plaque formation in the appropriate cells as described above. Canola oil, which does not have antiviral activity, was used as a negative control. Other control tests used PBS in place of oil.

Hemagglutination (HA) Inhibition Assay

HA inhibition was measured in dried films of virus exposed to the CLO vapor, as described above for antiviral activity of CLO vapor in Eppendorf tubes. 50µl of reconstituted exposed virus were mixed with 50 µl of 0.75% suspension of human type O Rh+ erythrocytes and incubated at 22ºC for 60 min (WHO Manual, 2011). The CLO concentration that completely inhibited the hemagglutination of the virus was determined.

Anti-Cytokine (IL-6) Activity

A549 cells were grown in DMEM, in 6-well trays, to produce confluent monolayers. Cells were infected with RV14 at 1.0 infectious virus per cell (1 pfu/cell), for 1 h at 37°C. After 1h, the virus inoculum was removed and the cells were washed twice with PBS to remove any unabsorbed virus. The moist cells were exposed to CLO vapor for 60min. Controls included cells with no virus and cells (± virus) with equivalent exposure to canola oil Cell free culture supernatants were harvested after 48 h and assayed for IL-6 (according to assay kit manufacturer instructions; e-Biosciences, San Diego CA). All cultures were in triplicate and each supernatant was assayed in triplicate.

RESULTS

Antiviral activity of CLO Vapor

Results of the antiviral effects of CLO vapor are summarized in Fig. 1. The membrane-containing viruses, influenza H3N2, H1N1, B, and HSV-1 and -2, were readily inactivated by CLO vapor (Fig. 1; Influenza B and HSV-2 data were less extensive and are not shown, although the result was the same as for the other viruses) . Rhinovirus 14, which does not contain a membrane, was only partially inactivated by 30 min but was completely inactivated by 60 min exposure. Adenovirus (no membrane) was more refractory and required 2 hours of CLO vapor for complete inactivation (not shown in figure). These data suggest that viruses with membranes, i.e., most respiratory viruses, are highly vulnerable to CLO vapor.

Effects of CLO against virus HA

The oil and vapor phases of CLO were tested for effects against the influenza virus membrane protein, hemagglutinin (HA) which is also a major virulence component of the virus. The vapor was active against HA, which could at least partly explain the antiviral activity (Fig. 2). However the principal chemical components, the terpenes thujone and α-pinene, were inactive against the virus as well as HA (Table 1 and Fig.2).

Table. 1: Inhibitory Activity of CLO Vapor against HA Activity.

	HA inhibition	Antiviral activity
CLO vapor	+	+
Thujone	-	-
α- pinene	-	-

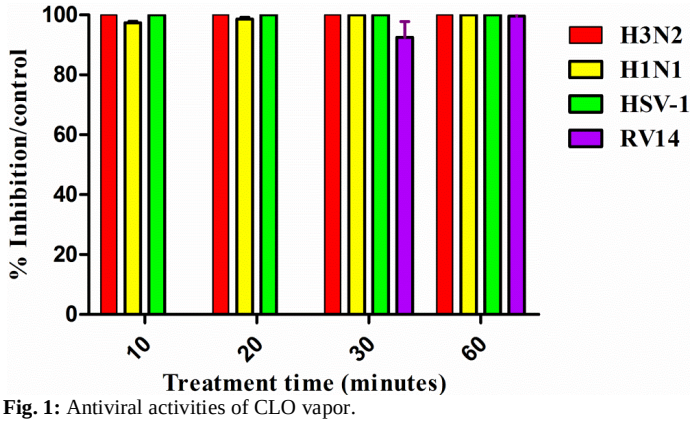

Fig. 1: Antiviral activities of CLO vapor.
Dried films of each virus were exposed to CLO vapor for the time periods indicated, as described in Methods. The films were then reconstituted in PBS and assayed by plaque formation in the appropriate cells. Controls consisted of identical dried films without exposure, or exposure to canola oil.

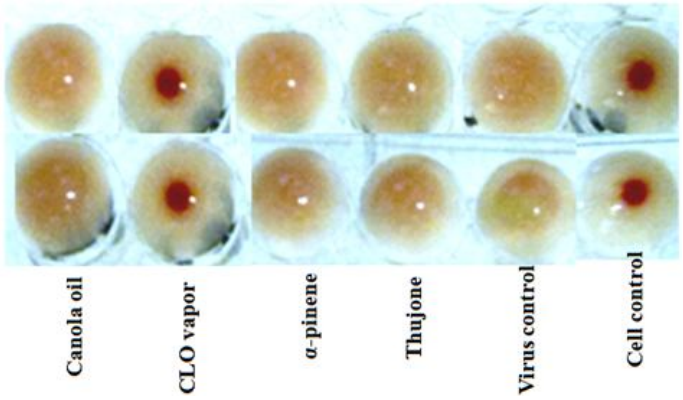

Fig. 2: Viral HA Assay plate.
Viral hemagglutination assays were conducted with human type O Rh+ erythrocytes, as described in Methods (and in WHO Manual, 2011). The presence of conspicuous red buttons in the well indicates absence of agglutination, ie. inhibition of HA activity. The other wells show normal hemagglutination.

Tests for Cytotoxicity of CLO Vapor

Cytotoxicity was evaluated by exposing monolayer cultures of human lung, A549 epithelial cells, (with culture medium removed), to CLO vapor for different times. This was followed by microscopic examination of the cells for signs of cell toxicity and assays for cell viability. No signs of cytopathology were evident, and the results of the quantitative LDH (lactate dehydrogenase) and XTT assays showed that exposures up to 60 minutes had no effect on cell viability (data not shown), in confirmation of our previous results (Hudson *et al.*, 2011).

Effect of CLO Vapor on IL-6 production

We have previously established a model system for the measurement of virus-induced inflammatory responses in human lung epithelial cell cultures (Sharma *et al.*, 2009). In this test system CLO vapor was able to inhibit the rhinovirus-induced secretion of the pro-inflammatory cytokine IL-6, as shown in Fig 3. In other words CLO vapor showed anti-inflammatory activity. Since IL-6 is frequently produced in excess in infections and inflammatory diseases, and is also responsible for some of the symptoms of colds and flu, including fever and headache, then this property of CLO vapor should be considered beneficial. Furthermore the CLO vapor did not produce IL-6 in uninfected control cells. This result complements the lack of cytotoxicity of CLO vapor.

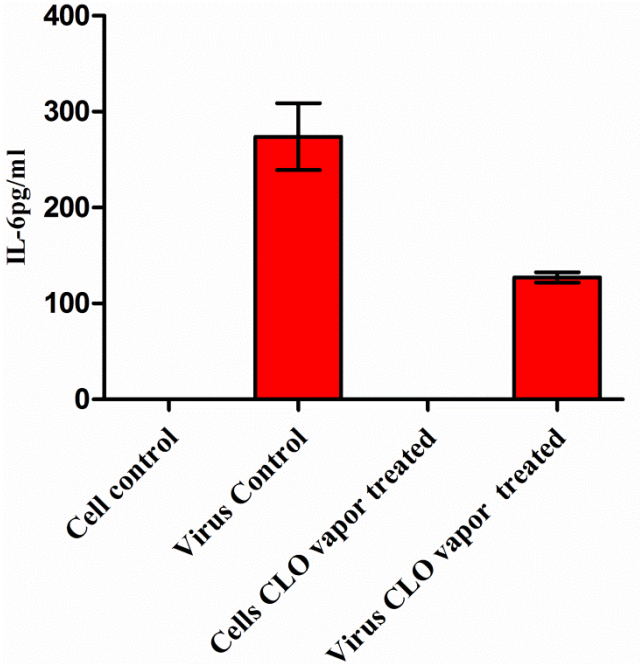

Fig. 3: Anti-inflammatory activity of CLO vapor.
Human A 549 epithelial cells were inoculated with rhinovirus 14 (1 pfu/cell), or control medium, for 60 min, followed by CLO vapor treatment for 60 min. After a further 24 h in normal medium, supernatants were removed for assay of IL-6 content, as described in Methods.

DISCUSSION

Respiratory viruses continue to cause problems within the general population, as a result of frequent acute and chronic infections, and occasional epidemics. Existing therapeutic agents are clearly inadequate, partly because of the diversity of replication schemes among these viruses, and hence the paucity of suitable molecular targets available; and partly because of the continual emergence of drug-resistant mutants. In addition the symptoms of respiratory infections generally arise from the indirect inflammatory responses to the viruses.

Some essential oils have demonstrated effective antimicrobial and antiviral properties, and in a few cases anti-inflammatory properties as well (Carson *et al.*, 2006; Alim *et al.*, 2009; Cermelli *et al.*, 2009; Sadlon and Lang, 2010).

However, these studies tested the liquid oil phases, which are generally less practical and possibly toxic for nasopharyngeal or oral applications. A few reports have indicated that the vapors of some oils might be useful for this purpose (Sadlon and Lang,

2010; Thyagi *et al.,* 2011; Hudson *et al.,* 2011), and this type of application would be in accord with anecdotal reports of the usefulness of inhaled vapors (Sadlon and Lang, 2010; Hudson *et al.,* 2011).

In order to evaluate this possibility we examined the vapor of cedar leaf oil (CLO vapor) for its ability to inactivate several viruses commonly associated with respiratory and oral mucosal infections. Also, since these viruses often induce substantial pro-inflammatory cytokine responses, we decided to evaluate the vapor for possible anti-cytokine activity in virus-infected cells. The test system was based on our previous protocol, in which dried films of the test organism or virus were exposed for various time periods to the CLO vapor, and assessed for surviving infectious virus, by means of our standard plaque assay procedures.

All of the viruses tested, Influenza virus type A and B, Rhinovirus type 14 (RV 14), Herpes simplex viruses 1 and 2, and Adenovirus type 4 (Ad 4), were completely inactivated by the treatment. In some cases, as few as 10 minutes of exposure were sufficient, whereas the non-membrane containing viruses, RV 14 and Ad 4 required significantly more exposure time.

Very little of the oil was lost to evaporation during the exposure period. This is of practical importance since the use of CLO vapor would thus be effective and economical, with potential applications to infected people suffering from respiratory symptoms (Sadlon and Lang, 2010), and on a larger scale in the ventilation systems of buildings susceptible to "sick-building syndrome" (Hudson *et al.,* 2011).

The results of the cytotoxicity tests showed no effect of this short exposure time on cell viability, in human lung epithelial cells, or on the microscopic appearance of the cells.

In our model system for the measurement of virus-induced inflammatory responses in human lung epithelial cell cultures (Sharma *et al.,* 2009), CLO vapor was able to inhibit the rhinovirus- induced secretion of the pro-inflammatory cytokine IL-6. In other words CLO vapor showed anti-inflammatory activity.

Since IL-6 is frequently produced in excess in infections and inflammatory diseases, and is also responsible for some of the symptoms of colds and 'flu, including fever and headache, then this property of CLO vapor would be considered beneficial. Furthermore the CLO vapor did not induce IL-6 in uninfected control cells. This result complements the lack of cytotoxicity of CLO vapor. In an attempt to understand the mechanism of action against influenza virus, we examined the effect of CLO vapor on influenza virus hemagglutinin (HA) a membrane protein which is also one of the major virulence components of the virus and is necessary for entry of virus into cells. HA activity was effectively inhibited, suggesting a possible explanation for the antiviral activity. However, the principal constituents of CLO, thujone and α pinene, did not display antiviral activity or inhibition against HA. This suggests that the antiviral activity may be due to synergistic effects of several oil constituents. On the basis of these results we conclude that CLO vapor possesses several basic requirements for an effective treatment against respiratory infections. Firstly it is capable of inactivating the viruses themselves, which would consequently diminish the spread of virus within a population or a building. Secondly, the treatments applied are not cytotoxic. Thirdly, it is also anti-inflammatory, as indicated by its inhibitory effect on virus-induced cytokine IL-6.

Since we have previously demonstrated that the vapor has antibacterial and antifungal activity against surface films of these organisms (Hudson *et al.,* 2011), then CLO vapor appears to have a broad spectrum of medical and health applications.

CONCLUSION

The vapor of cedar leaf oil has potentially useful bioactivities for applications to situations involving respiratory viruses. The vapor inactivates respiratory viruses on contact, and inhibits the virus-induced cytokine IL-6 production in virus-infected lung epithelial cells. No evidence of cytotoxicity has been observed.

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