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Table of contents

THE ACTIVITY OF CEDAR LEAF OIL VAPOR AGAINST HUMAN CORONA VIRUS OC43 (HCoV-OC43)

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Objective:

To evaluate the antiviral effect of CLO vapors against Human coronaviruses OC43 (Beta coronavirus) and 229E (alpha coronavirus)

Materials and Methods:

The cedar-leaf oil was obtained from Inmicro Indoor Air Inc. Thuja plicata (Western red cedar) leaves, Lot No 74916.

Cells, viruses and antibody

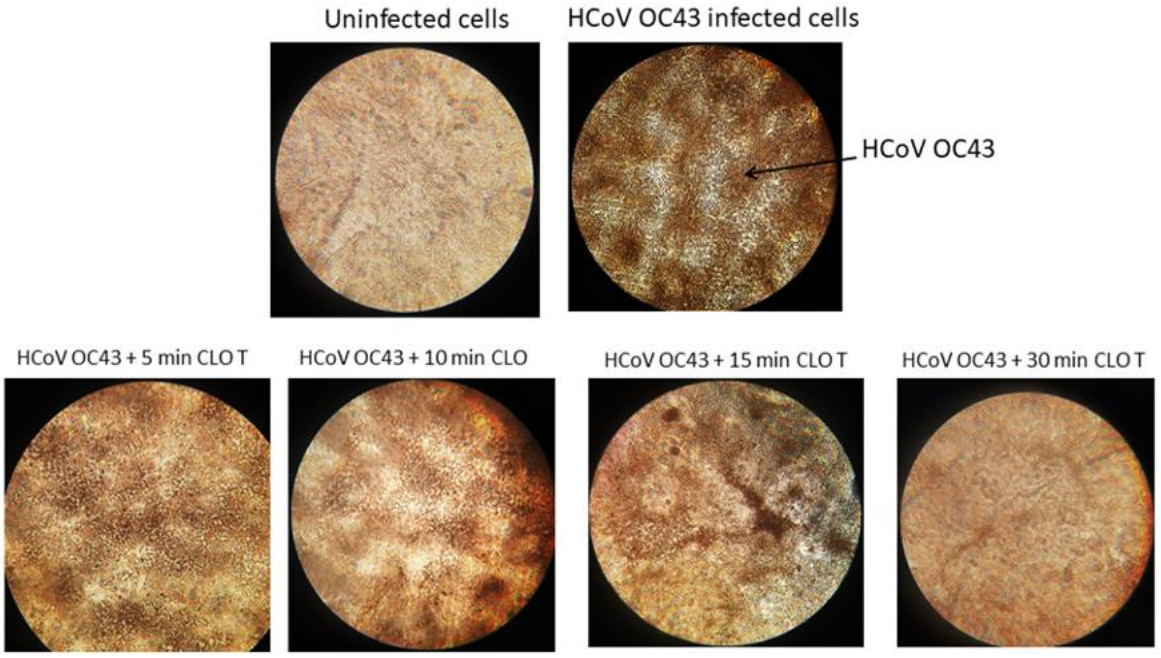
HCoV-OC43 (ATCC: VR-759) was grown on HCT-8 cells in modified RPMI 1640 medium (cell culture reagents were obtained from Thermo fisher, Ontario, CA) and infectious titre for OC43 was 1×105 TCID50/ml.

Indirect immunoperoxidase assay (IPA) was performed as described in (Lambert et al 2008).

Mouse monoclonal antibody to HCoV-OC43, secondary antibody and HRP were purchased from Sigma Aldrich.

Virucidal activity:

The method used was a modification of our assay system (Vimalanathan and Hudson 2013). 20 µl aliquots of virus (containing 1000 TCID50) were individually dried on the sterile glass slides, in the biosafety cabinet (10 min). 2mL of undiluted cedar leaf oil were carefully added to 20mL glass vial, then glass slides containing virus were placed on top of the glass vial containing cedar oil and expose to vapor and allowed for various time periods (5, 10, 15 and 30 minutes) at 22 C. Slides were removed each dried exposed film was reconstituted in RPMI media. All samples (in triplicate) were then assayed for virus foci plaque formation in the HCT cells. Canola oil, which does not have antiviral activity, was used as a negative control.



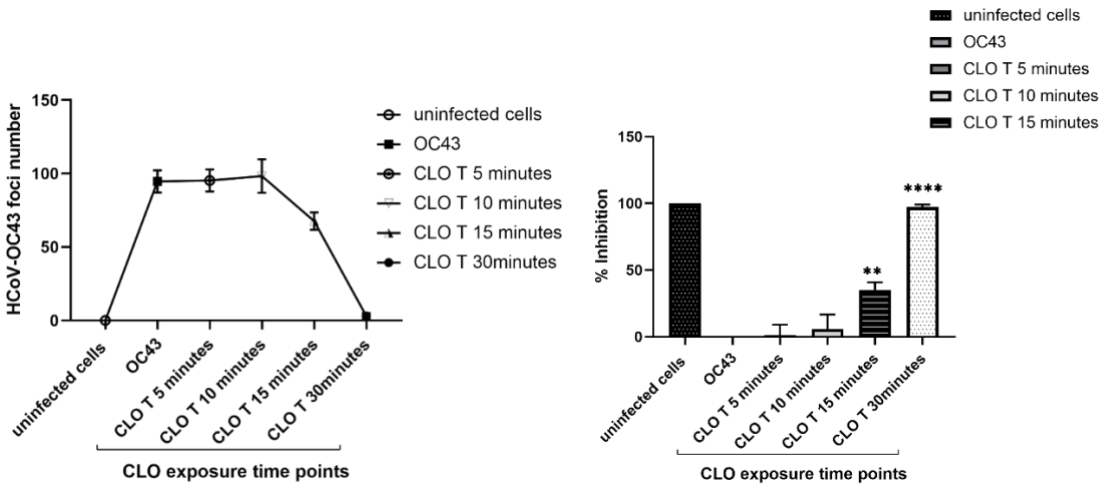


Fig. 1&2 Ant coronavirus (HCoV-OC43) activity of CLO vapor.
Dried films of HCoV-OC43 were exposed to CLO vapor for the time periods Indicated (5, 10, 15 and 30 minutes) as described in Methods. The films were then reconstituted in PBS and inoculated to HCT-8 cells after 4 days, indirect immunoperoxidase assay was performed and counted stained foci, untreated virus control was identical dried films without exposure, or exposure to canola oil.

Results and conclusion:
Antiviral activity of CLO Vapor
Results of the antiviral effects of CLO vapor are summarized in Fig. 1 and 2. Human corona virus OC43 was partially inactivated by CLO vapor after 15 minutes of exposure and completely inactivated by 30 min exposure.

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Review article

Cedar leaf oil vapor in buildings and forests: Health benefits & mechanisms

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Key words: Cedar leaf oil, terpenes, sick building syndrome, forest bathing, antimicrobial, anti-inflammatory.

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Abstract

Many people today experience symptoms indicative of chronic stress, which is manifest as persistent or frequent inflammation in one or more parts of the body. These conditions may be the result of current lifestyles, including the buildings that they work or live in, a situation referred to as sick building syndrome (SBS). Relief may be obtained by incorporating cedar leaf oil vapor into ventilation (HVAC) systems, or by spending time in a forest containing cedar-like trees or similar conifers that exude volatile compounds (terpenes) from their leaves, an experience which often leads to an improvement in health parameters. This procedure is referred to as forest bathing, or nature therapy. Many studies recently have confirmed the beneficial effects of these leaf oils and their vapors, according to a variety of laboratory experiments and field trials. Thus the cedar leaf oil terpenes have been shown in various models to inactivate many viruses and microbes directly or indirectly through activation of natural killer cells. Furthermore the terpene mixtures, and some individual tepenes, can interfere in the production of pro-inflammatory cytokines and other inflammatory mediators. This review discusses the evidence and suggests a common mechanism to explain how the leaf oil compounds, the terpenes, could relieve the chronic inflammation and stress, and consequently lead to a restoration of homeostasis.

Introduction

Plant-derived oils have been used for centuries, in different parts of the world, for many pharmaceutical, food and cosmetic applications, pest control, and for the treatment and prevention of various diseases and their symptoms, as for example in aromatherapy. These have usually been applied on an individual basis, and often as vapors for controlling respiratory problems [1-5].

Recently there have been advances in the application of oil vapors derived from the leaves of cedar and related trees (see Table 1), targeting groups of individuals for the purpose of improving or optimizing health. Two such advances are discussed in this review, namely 1) alleviation of "sick building syndrome (SBS)" otherwise known as "built environment intolerance (BEI)" [6-11], and 2) "forest bathing" or "ShinrinYoku", a popular form of "nature therapy" [12-19].

Many people in current societies suffer chronic stress, involving persistent low- level inflammation, which leads to preventable diseases [20-23]. Consequently, scientists are searching for safe remedies that can alleviate the inflammatory responses. Several groups of investigators have carried out basic studies for the purpose of confirming previous anecdotal reports on the oil vapors obtained from different species of cedar-related trees, and understanding possible mechanisms of action. These studies have revealed potent antimicrobial and antiviral activities, as well as significant immune-related activities, including anti-inflammatory activities, all of which together indicate potential beneficial effects of these oil vapors on physiological and mental health parameters. I will first discuss the results of these studies, and in the following general discussion I will attempt to present an overall mechanism to explain the benefits that may result from these treatments.

Table 1 summarizes taxonomy and common names of the Thuja and other species of "cedar – like trees" (members of the Cupressaceae, cypress family) used as sources for the oils referred to in this review. Most of the experimental work has been conducted with steam distilled leaf extracts that comprise typically 20-30 chemically discrete terpenes, although unfortunately some reports contain ambiguous information about their source materials, whether leaf, branches, stems or wood, fresh or old, and discarded foliage. These factors are likely to result in differences in composition of the major bioactive ingredients, the terpenes (sometimes referred to as the more generic term terpenoids).

Sick Building Syndrome (SBS)

Also known as "built environment intolerance (BEI)", this term was coined to describe the recently recognized health problem associated with living or working in buildings with limited air circulation, particularly large office buildings and apartment blocks. The air within

these buildings, and the indoor surfaces exposed to this air, often contains potentially pathogenic micro-organisms (bacteria and fungi/molds and their spores and toxins) and viruses, some of which could cause or enhance chronic respiratory problems, especially in individuals with asthma, allergies, and similar predisposing factors [6, 8]. Some of these organisms may also produce biofilms that are difficult to remove by conventional means [24]. Other exogenous factors, such as humidity, can also play a role [11].

In addition, individuals vary considerably in their capacity to counteract the offending micro-organisms or other triggers of symptoms such as environmental toxins/stressors [22, 25]. Thus our immune network, which is in continuous communication with our metabolic and other body networks, as well as our individual microbiomes [26-28], may not always remain optimal, with the result that a particular individual could be chronically distressed by the classical symptoms of SBS, including headaches, nausea, fevers, and exacerbation of asthma and allergic reactions. Persistence of these states has been incriminated in long-term inflammatory diseases [20].

One approach to this problem would be the periodic removal or substantial reduction in the micro-organisms inhabiting ventilation ducts and surfaces by a broad spectrum antimicrobial agent. However most of the existing products available are relatively specific and are toxic, and can only be used safely in empty buildings.

Antimicrobial activities of CLO Vapor

Earlier studies, involving the use of small animals and cell cultures, were discussed in a 2005 review [29]. In spite of the uncertainty of some of the source materials, there were strong indications that CLO, in liquid form, could provide beneficial effects in the form of antimicrobial and positive immune effects.

Tsiri *et al* [30] reported a detailed study of the chemical composition and antimicrobial activities of two varieties of *Thuja plicata* and two varieties of *Thuja occidentalis* oils derived by steam distillation of the leaves obtained from trees grown in different parts of Poland. The principal components of the oils were generally similar to each other, comprising the terpenes, alpha and beta thujones, fenchone, sabinene and terpinen-4-ol, together with smaller amounts of many other terpenoids. This analysis corroborated other studies of compositions of oils from Thuja species [30 31].

These oils were evaluated quantitatively (MIC, minimum inhibitory concentration) for antimicrobial activities against 6 bacteria, two gram-positives and four gram-negatives (see Table 2). All of them were susceptible to all four oils. In addition three species of Candida yeasts were tested by the same methodology and were found to be susceptible [30], (Table 2).

Based on anecdotal evidence and limited field trials conducted in British Columbia, we decided to investigate the oil and oil vapor, CLO and CLO-vapor, derived from Western red-cedar leaves [8, *Thuja plicata*]. The terpene content of the oil was qualitatively typical for Thuja species, with approximately 75% thujones alpha and beta. Several gram–positive and gram-negative bacteria, including potential environmental pathogens, were readily killed by exposure to the oil (CLO). The results are summarized in Table 2. Of particular note is the finding that CLO vapor was also effective against these same organisms, although longer times of exposure were required. Different quantities of bacteria were susceptible, and time course studies showed significant but slight differences in susceptibility. But in general these activities indicated potencies comparable to many other essential oils (e.g. tea-tree oil, [4, 8]). Tests indicated that the activities of CLO were bactericidal rather than bacteriostatic.

The possibility of photosensitivity was also evaluated, as many other herbal extracts, including oils, have been reported to be photoactive [3, 32]. However, the potency of CLO was not affected by the presence or absence of light [8].

Purified spores of *Bacillus subtilis* were also susceptible to CLO, but longer exposure to the oil was required in this case, a result comparable to studies with various other antimicrobial compounds [33, 34]. This may be a reflection of the more complex nature of spore walls in comparison with the vegetative bacteria [34].

Two fungi, *Candida albicans* and *Aspergillus niger*, were evaluated by techniques analogous to antibacterial MIC. Both organisms were susceptible, although *Aspergillus* was less sensitive and required longer exposure times [8]. CLO vapor was also effective against these fungal organisms, although longer exposure was required in comparison to the liquid oil.

Manoharan *et al* [24] found that CLO derived from *Cedrus libani* (Cedar of Lebanon) was a very effective agent against biofilm formation by the medically important yeast *Candida albicans*, and this was attributed to its inhibition of the hyphal growth, although the oil was not so effective at controlling growth of the planktonic (yeast) cells of the organism. Gene expression studies revealed that several proteins associated with hyphal growth and adhesion were affected by the treatment, thus explaining the inhibition of biofilm formation.

I should point out that other studies have described antimicrobial activities of oils obtained from cedar wood [35]. These not surprisingly contain some of the terpenoids found in the leaf oil, but they also contain additional compounds, including potentially toxic ones not usually found in the leaf oil.

Table 1. Cedar-like Trees (Cupressaceae-cypress family) discussed in this review. (Ref. 19 and Wikipedia, cypress family; Thuja species 2019).

Official Name	Common Names
Thuja plicata Don ex D. Don	Western red cedar; arborvitae; giant cedar
Thuja occidentalis L.	Northern/eastern white cedar; American/eastern arborvitae
Thuja koraiensis Nakai	-
Thuja standishii (Gordon) Carriere	-
Thuja sutchuensis Franch	-
Cryptomeria japonica D. Don	Sugi; Japanese cedar/redwood
Chamaecyparis obtusa	Korean/Japanese Hinoki
Cedrus libani (Cedar family)	Cedar of Lebanon

Table 2. Summary of Bioactivities of CLOs. (See text for details).

Source of CLO	Bioactivities	Relative potency (++, +)
Thuja species	Antibacterial:	
	Gram negative	++
	Gram positive	++
	Bacterial spores	+
Thuja sp	Antifungal	+ to ++
Thuja sp	Microbial biofilms	++
Thuja sp	Antiviral	++
Thuja sp	Anti-cytokine IL-6	+
Thuja plicata wood oil	inflammatory markers	Inhibited
Chamaecyparis obtusa *Cryptomeria japonica*	Inflammatory markers: NFkB, NO synthase, Cox-2	Inhibited
Different species	cytotoxicity	Not detected
Different sp	NK cell bioactivities	Increased
Different sp	insecticidal	++
Individual terpenes	Various activities re: cytokines, inflammatory mediators, NFkB, neurotransmitters, ion channels	- to ++

Antiviral activities

Viruses in general have often been incriminated as causes or triggers of the respiratory problems that initiate SBS and similar syndromes. Respiratory viruses such as various strains of influenza virus, respiratory syncytial virus, corona viruses, and rhinoviruses (common cold viruses), are frequent inhabitants of indoor air along with the microbes discussed above [36, 37]. But dozens of additional viruses are known to infect respiratory tracts of humans; consequently it has been a challenge to come up with a satisfactory "antiviral" approach for such an environment [38].

Nevertheless, the realization that essential oils could be a solution to this problem prompted us to study the potential of CLO vapor as a safe generic antiviral that could be administered through the HVAC (heating ventilation and air conditioning) system of a building. This rationale is supported by the knowledge that most known respiratory viruses contain a relatively simple but essential membrane that should be sensitive to an agent that can also kill microbes with more complex cell walls.

In quantitative tests (plaque assays, analogous to MICs for microbes), several strains of influenza virus and herpes viruses were readily killed by as little as a few minutes exposure to CLO vapor (*Thuja plicata*) [38]. Even rhinovirus and adenovirus, which are respiratory viruses without membranes, were also susceptible to CLO vapor, although in these cases somewhat longer exposures were required. In the case of influenza virus, the vital membrane protein hemagglutinin (HA) was a target for CLO, which could explain how the virus lost its infectivity.

Interestingly, two of the individual Thuja terpenes by themselves, alpha thujone and alpha pinene, failed to kill the influenza virus, suggesting that the antiviral activity of CLO vapor was due to the combined effect of many of the volatile constituents [38].

The fact that the vapor of CLO was capable of acting as a potent antiviral with a broad range of targets, is a major advantage over oils that need to be used in liquid form. I shall return to this point in the general discussion.

Insecticidal activities

Keita *et al* [31] evaluated the ability of *Thujaoccidentalis* leaf vapors to kill cowpea weevil adults (bruchids, responsible for ruining stored food products). 100% mortality was achieved at oil concentrations significantly lower than those used with traditional oil insecticides. A recent review discussed the prospects for control of insect pests by means of individual terpenes, based on QSAR (quantitative structure-activity relationship) analysis, against several insects relevant to human health [5]. Activities varied considerably according to chemical structures. However, a previous study [39] of *Salvia lavandulaefolia* essential oil and some of its individual terpenes indicated that the whole oil was more effective, in an enzyme inhibitory assay against acetyl cholinesterase, than isolated single terpenes. This should not be surprising since It is well known that the bioactivities of medicinal plant extracts are often more potent than their individual components [40, 41].

Anti-inflammatory cytokines

In our laboratory we established a model system for the detection and measurement of rhinovirus-induced inflammatory responses in human lung epithelial cells. In this system the pro-inflammatory cytokine IL-6 was secreted in substantial quantities, but in infected cells exposed for 60 minutes to CLO vapor the amount of IL-6 was decreased by more than 60% [38]. Since IL-6 is often produced in respiratory infections, and is usually considered as a pro-inflammatory cytokine, then this anti-inflammatory property of CLO vapor could be significant. In addition, exposure of control cells to the vapor did not cause any cytotoxic effects.

Forest bathing-experimental analyses

The concept of forest bathing (a translation from the Japanese *Shinrin Yoku*, [16]) as a natural and healthy approach to relieving the stress associated with urban lifestyles has been discussed in many popular sources, and in recent scientific reviews. It may be considered as a type of Nature Therapy, which has become very popular in many parts of the world that are still endowed with forested areas, eg. parts of Japan, Korea, coastal British Columbia [16, 19]. Accordingly, many reports have been published claiming improvement in certain physiological and psychological parameters following exposure, short or prolonged, to forest environments. The latter have been explained by the presence of volatile components, so-called "phytoncides", especially terpenoids, in the ambient air.

However, a number of recent reviews have criticized some of the earlier studies of this phenomenon because of inadequate scientific analysis, including too much reliance on subjective questionnaires and inadequate statistical significance. Consequently I will focus on studies that have been accompanied by acceptable laboratory evaluations. These typically compare blood samples from exposed and non-exposed subjects for specific parameters of immune function, such as natural killer cells (NK cells) and cytokines, accompanied by measurements of standard blood and urine parameters for stress indicators.

Immune parameters: NK (Natural Killer) cells

Li and co-workers carried out many field tests on subjects exposed to local forest environments, in which the most abundant trees were cedar – related, such as Japanese cedar (Sugi, or *Cryptomeria*), and Hinoki (*Chamaecyparis obtusa*), and known to contain many volatile terpenoids. Subjects were tested for several blood and urinary parameters, NK cell activity, and stress-related hormones, cortisol, adrenaline and noradrenaline [16, 17].

NK cells (natural killer cells) were chosen because they were known to be vital components of the innate immune defenses, and have properties associated with killing tumor cells and virus-infected cells, in addition to other possible roles. The mechanisms involve a variety of different molecular interactions between the NK cells and membrane proteins of the target cells, depending on the exact virus or type of tumor cell [42]. Following the initial interactions, the NK cells secrete cytolytic proteins including perforin, which damages the target cell membrane, and accompanying proteins such as granzymes, which program the target cells to proceed through apoptosis and death. Standard assays for NK cell activity in blood include measurement of the amount of cell killing by the test sample against a certain human lymphocyte cell line, as well as specific assays for perforin and granzymes [42-44].

Exposure of test subjects to the forest environment resulted in increases in the various NK cell parameters, and small decreases in the stress hormone levels [43, 44]. These results were confirmed in experiments in which subjects spent several nights in a hotel room, during which they were exposed to vaporizers containing "stem-oil" derived from *Chamaecyparis obtusa* [45].

However most of the changes in parameters tested were relatively small and in some cases of questionable significance. In addition in cases where individual subject data were plotted, there appeared to be quite different responses among the individuals. This could be a reflection of well-known findings in studies on physiological and psychological effects of natural phenomena, in which grouped data tend to be heavily influenced by individual high or low responders. Thus certain individuals could be strongly affected by the

forest exposure, or vapor exposure, whereas others may be unaffected.

Immune Parameters: Anti-inflammatory effects

A comprehensive study was recently reported by Raha *et al* [45], which included a detailed analysis of the mechanism of a significant anti-inflammatory effect in experimental rats, and in human lung cells following treatment with CLO. The rats were continuously exposed to vapors from Korean hinoki leaf oil (*Chamaecyparis obtusa*). Lung tissues from the treated and control rats were subsequently examined histologically. No toxic effects were seen in the treated animals, but their alveolar capacity had been improved by the exposure.

An in vitro model system of inflammation was established by treating cultures of WI38 cells (a standard cell line originating from human lung fibroblasts) with a microbial lipopolysaccharide preparation (LPS). In this system molecular markers of inflammation were seen, such as induction of nitric oxide synthase, activation of cyclooxygenase-2 (Cox-2), and inhibition of NF-kB, which is an important protein transcription factor that can control many other intracellular pathways involved in inflammatory responses (see below). These changes were reversed however by pre-treatment of the WI38 cells with exposure to the leaf oil. In other words, the oil, which was verified to contain the typical terpene composition expected, acted as an anti-inflammatory [45].

In a different approach to the subject of inflammation, Han and Parker [46] used a preparation of oil distilled from the heartwood of *Thuja plicata*, which has a chemical profile distinct from CLO, being rich in methyl thujate, methyl myrtenate, and terpinen-4-ol. This product, which has been used in the treatment of various skin conditions, was diluted and incubated with a commercial human skin cell model that had been cultured with a collection of known inflammatory mediators. They found that a non-toxic concentration of the oil decreased the level of several proteins associated with inflammation. They also investigated the effects of the oil on gene expression and protein production of a panel of more than 20,000 genes. There were numerous significant changes in 200 or so genes, many of which coded for proteins involved in inflammatory and wound repair pathways.

Thus the oil acted as an anti-inflammatory, or inflammation regulator. The significance of these results will be discussed below, in relation to other bioactivities of CLOs.

Bioactivities of individual terpenes

In addition to the individual studies mentioned above, there have been many reports of specific activities of individual terpenes in laboratory cell culture models and animals, many of them having the objective of finding suitable replacements of existing drugs by safe "natural" products. The targets for these tests were usually specific cytokines, inflammatory mediators, transcription factors such as NFkB, neurotransmitters, or ion channels. Some terpenes showed multiple stimulatory or inhibitory activities in certain tests, while others showed no evident activity [47-54]. It is not clear to what extent these results are relevant to the applications of total CLOs and their vapors.

Discussion & mechanisms

It might appear that I have been reviewing experimental results pertaining to two separate situations, namely: sick building syndrome on the one hand, and forest bathing, otherwise known as forest therapy or nature therapy, on the other. But in fact these situations are similar in that they consist of individuals exposing themselves to the volatile components from the leaves of cedar-like trees, which I have conveniently designated as CLO vapor, for the purpose of improving their health and reducing stress. In practice only certain parts of the body are exposed directly to the components of the CLO vapor, namely skin and respiratory surfaces, so we need to invoke a holistic role for the various body networks in their attempts to restore and maintain homeostasis as the need arises. Thus improvements in physiological functions associated with heart, lungs, blood, mental health, and stress indicators, as well as antiviral and antimicrobial responses, must be considered as parts of the overall effects of the terpenes in the oil vapors. Therefore we need a common mechanism of action.

How do people become "stressed out" and depressed? Many scientists and health advisors would agree that our current lifestyles, in urban and rural societies, in contrast to pre-industrial age lifestyles, are full of stressful stimuli, including environmental stressors that did not exist centuries ago, such as multiple toxins, and novel types of radiation from our many electronic devices [20]. Increasingly these situations are likely to lead to inflammatory responses that could become chronic and give rise to depression and possibly other abnormal mental states. In effect, stress at the cellular level is amplified by our body networks to produce physiological and mental stress [21-23]. Thus the inhabitants of inadequately ventilated buildings (poor HVAC systems), and those who deliberately expose themselves to forest essential oils, are all seeking restoration of health (homeostasis).

So how can a collection of approximately 20-30 terpenes from a specific species of conifer manage to accomplish this? This is even more of a challenge to explain when we consider that a forest is not a plantation, but a mixture of tree species, each with its own characteristic collection of terpenes, although usually these comprise just a few dominant terpenes with a larger number of minor compounds. Consequently at any time the ambient air

could be full of hundreds of different terpenes. But is it possible that certain individual terpenes work just as well? Studies referred to above indicated that a few individual terpenes are bioactive according to laboratory tests with model systems. How does this relate to exposure to CLO vapor?

Plants in general synthesize tens of thousands of different terpenes, by means of a common pathway from isoprenoid units, with various additional side chains that give each terpene a different chemical structure yet retain some common basic properties, such as a hydrocarbon chain that is responsible for their strong lipophilic nature. This property allows them easy access to and penetration through skin epithelia, and some of them have been advocated as adjuvants to help certain drugs to penetrate the skin [55]. Other properties of the terpenes are due to their various side chains, which explains why they do not all have the same physiological effects on humans. Although some individual terpenes possess multiple bioactivities, it is likely that the more widespread activities are a property of a particular mixture of terpenes, possibly in synergistic relationship between different compounds. Synergism is a property found frequently in extracts of medicinal plants, and can sometimes explain why they often possess properties different from individual constituents [37, 40, 41].

Viruses, especially the respiratory viruses, are a continuous threat to the health of humans everywhere, including many of the buildings we inhabit [36, 37]. Because of their variety and their frequency of mutations, the concept of specific antiviral drugs is impractical. However, in terms of chemical structures viruses are relatively simple, comprising RNA or DNA genes in association with one or more viral specific proteins and often surrounded by a lipoprotein membrane. Consequently they should afford easy targets for multiple terpenes. The results summarized above confirm this in the case of CLO liquid and vapor (Table 2). When viruses infect cells they often induce signaling pathways that result in the production of interferons and other cytokines that are excreted and may affect neighboring cells. Influenza virus for example has often been implicated in "cytokine storms", which can lead to severe inflammatory responses in cases of bronchitis and pneumonia [37, 56]. Several respiratory viruses have also been shown to enhance the presence of bacterial receptors, which in turn lead to secondary bacterial infections and more serious lung disease [56].

Many viruses, as exemplified by herpes viruses, establish chronic or latent infections following resolution of the initial acute infection, and may give rise to recurrent disease months or years later. Herpes simplex (cold sores, genital sores) and Herpes zoster (shingles) are common examples. Such chronic infections may act as persistent stressors with consequent low grade but significant inflammation. Thus viruses continue to pose threats to health throughout life.

Bacteria and fungi (molds) are larger and more complex organisms, usually with cell walls, but are also vulnerable to CLO liquid and vapor. Certain individual terpenes can show antibiotic effects, and this property seems to depend on the chemical nature of their side chains. Even biofilms composed of bacteria or fungi can be inactivated (Table 2). In all these cases one or more terpenes could be responsible for binding to or modifying various chemical target molecules or rendering them dysfunctional, and this could result in growth inhibition or death of the virus or microbe.

In several of the studies described above, cultured human cells were found not to be adversely affected by exposure to CLO vapor, or by incubation of the oil itself in low concentrations, according to microscopic appearance and cell viability tests. In other words CLO vapor was not cytotoxic.

In terms of indirect effects of CLO vapor on human cells, the relative ease of access and permeation of a mix of terpenes to skin tissues could enable binding of terpenes to a multitude of important receptor molecules that are prevalent on and inside cell membranes, and which are components of numerous signaling networks. For example epithelial cells, and several types of lymphoid cells, such as NK cells (natural killer cells), as well as monocytes or macrophages, inhabit the epidermis and respiratory mucosa, and many of these cells carry different types of ion channels, hormone receptors, neurotransmitters, and other protein or lipoprotein receptors in their membranes. These are all potential target molecules for terpenes. In addition some of these surface receptors also link up with the intracellular components of additional signaling pathways and their transcription factors such as NFkB that ultimately control important elements of the inflammatory and cytokine responses. I will use an example to illustrate how this scheme could work.

A common factor in numerous signaling pathways is the NFkB complex of proteins and the associated inhibitory IkB complex, both of which comprise several individual proteins. A variety of natural products, including some terpenes tested, can affect the function of different proteins in these complexes and consequently could modulate signaling pathways, including inflammatory responses.

Thus, the terpenes could likely find a variety of proteins or lipoproteins (the receptors), for which they have chemical affinity, in the cell membranes. This would trigger a set of programmed responses within the cell, involving adaptor proteins and binding to sites in the NFkB protein complex, which itself is normally inhibited, or held in check, by another cluster of proteins referred to as the IkB. If the terpene or other molecule can catalyze the phosphorylation of one of the proteins in the IkB

complex, the IkB is removed and degraded, thus allowing the NFkB to become "active". The latter is then translocated to the nucleus of the cell, where it finds and stimulates appropriate DNA binding sequences on various genes involve in inflammatory responses, such as cytokines and other inflammatory mediators [49].

Studies with individual pure terpenes have revealed a variety of reactions with different parts of this pathway, including binding with IkB proteins and interference in the DNA binding steps. But there are also many terpenes that appear not to influence this pathway [as discussed above]. The consequences of these terpene - protein interactions would likely impact most of the internal organs and tissues of the body indirectly since they are all interconnected by the various networks of communication molecules, such as cytokines, chemokines and neuro-endocrines [58] (see Figure 1).

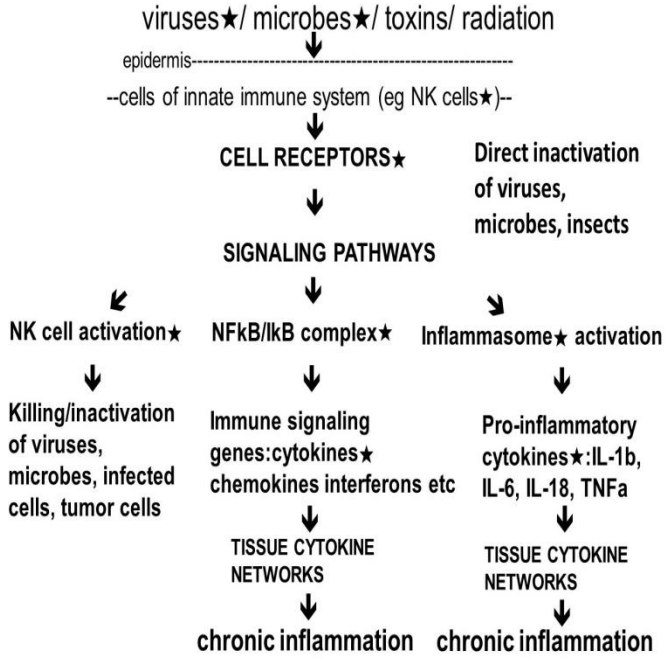

Figure 1. Scheme showing pathways leading to chronic inflammation.
—star (★) indicates known targets for CLO terpenes.

Among the cells that the terpenes would encounter on first contact with the epithelial surfaces are the Natural Killer cells (NK cells) that I discussed above.

These cells are activated by various cytokines and are ready to respond to foreign or modified proteins, ie-non-self proteins, in tumor cells and virus-infected cells, as well as some microbial cells [25].

We can think of stress at the cellular level, where any cell that is exposed to a potential pathogen (virus or microbe), or radiation, or a noxious chemical or particle, triggers a response (acts as a "stressor") to counteract this threat to its integrity. There are also intracellular stressors that have to be dealt with during the course of individual cell maintenance. Numerous recent studies have elucidated some of the signaling pathways involved in stress at the cellular level (various pattern recognition receptors, PRRs; damage associated molecular patterns, DAMPs; pathogen or microbe associated molecular patterns, PAMPs or MAMPs, [25]). Internal and external triggers induce the activation of different pathways that attempt to restore cell integrity, or sacrifice the cell by means of programmed cell death cascades.

Many of these responses go through NFkB or similar pathways and can result in the activation of a complex of cellular proteins referred to as an inflammasome, which has the primary objective of producing substantial amounts of the important pro-inflammatory cytokines such as IL-1 beta, IL-6, IL-18, and TNF alpha. This mature inflammasome is then transported to the cell membrane and the cytokines excreted into the extracellular milieu, possibly within exosomes (microvesicles), where they can circulate and influence other cells [25, 59]. Many other signaling pathways in neighboring cells amplify this response, and the result is a condition of inflammation, which could spread throughout the body by the manifold pro-inflammatory mediators, including additional cytokines and chemokines. Other tissues and organs may be involved, including the brain, by virtue of interactive neurotransmitter and endocrine signaling pathways in various cells. In this way, cellular stress can be translated into physiological and even psychological stress [21, 60-63]. Recent studies have illustrated how inflammatory cytokines can lead to numerous disturbances and pathology in the CNS including brain [61, 62].

In view of the ubiquitous nature of the inflammasome and its capacity to produce large amounts of pro-inflammatory cytokines and consequent stressful conditions, a potentially useful form of treatment could involve the use of inhibitory molecules capable of reversing the maturation or function of inflammasomes. Although some recent studies have found that certain plant derived compounds or extracts can inhibit the production of the pro-inflammatory inhibitors, terpenes have not yet been examined in this regard [63]. Nevertheless the studies referred to above in connection with cedar oils and anti-inflammatory activities suggest that terpenes might explain how forest-bathing/nature therapy, as well as CLO vapor in HVAC systems, may work by alleviating stress at cellular and body levels, and restore and maintain optimal health.

According to this concept, a stressed individual would inhale or absorb the terpene mixture, which would then penetrate the skin and mucosal layers, where they would encounter NK cells, which need to be activated, and numerous epithelial cells, which would be stressed from the various endogenous and exogenous triggers and consequently be in a state of inflammation resulting from the inflammasome pathways. Some terpenes could then inhibit the inflammasomes and consequently stop the

production and excretion of excess IL-1 beta, IL-6, and other inflammatory mediators. This could then result in cross-talk with other signaling pathways, including those involved in cortisol, adrenaline and noradrenaline production, and dispersal throughout the body. They could also affect the production and fate of exosomes (terpenes are lipophilic) responsible for communicating with other cells. The outcome would be restoration of non-stressed organs and tissues, including the brain, i.e. homeostasis, at least temporarily.

According to this scheme the terpenes may not have to gain direct access to all the tissues, only sites where inflammasomes are produced and excreted, for example in the epidermal tissues that are rich in many cells of the immune system. The networks of signaling pathways would do the rest. In addition the circulation transports exosomes around the body. These are small extracellular vesicles that are under intensive study from the aspect of informational or messenger molecules that are excreted and received by and from tumor cells, and possibly the products of the inflammasomes [60].

Thus in contrast to acute inflammation, in which the various inflammatory mediators would find their way to the site of the initial injury or infection and attract a variety of healing factors (such as anti-inflammatory cytokines) to control the situation, chronic inflammation could result from an inability to remove the initial trigger, or inability to dampen the level of inflammation produced. In practice many of us are probably in a state of low level inflammation, resulting in stress somewhere in the body, including the brain, and this could in turn result in damage to certain tissues (Alzheimer's or Parkinson's disease), or clinical states like depression [20-23].

Conclusions

Many recent studies have shown that the terpenes in cedar-leaf, or similar conifer leaf oils, can inactivate viruses and microbes, and activate NK (natural killer) cell activities. These bioactivities could be beneficial in counteracting the symptoms often found in people suffering from SBS, or inadequate HVAC systems. In addition terpenes were found to reduce levels of pro-inflammatory cytokines, in various model systems, thus providing evidence to corroborate the field studies indicating health benefits of forest bathing.

These results can be explained by a model in which the terpene mixtures interact with specific cellular proteins and consequently control cytokine levels and reduce inflammation at the cellular and tissue levels. In turn this could result in a significant reduction in stress and its many psychological and clinical manifestations.

Conflicts of interest

There were no conflicts of interest.

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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	-	-

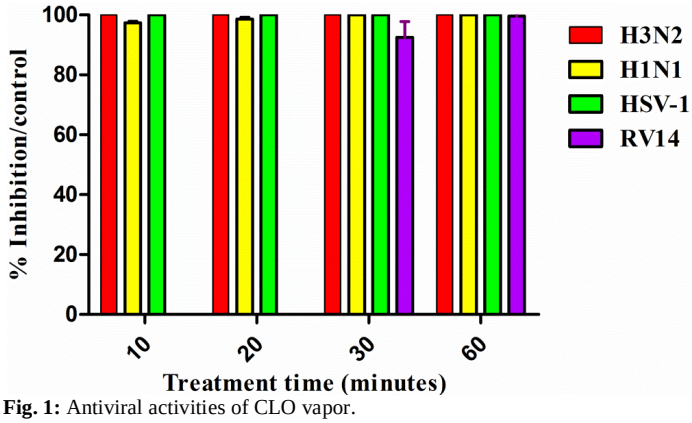

Fig. 1: Antiviral activities of CLO vapor.
Dried films of each virus were exposed to CLO vapor for the time periods indicated, as described in Methods. The films were then reconstituted in PBS and assayed by plaque formation in the appropriate cells. Controls consisted of identical dried films without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

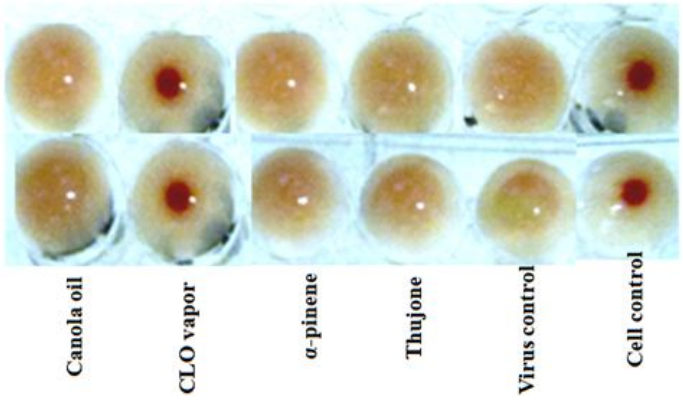

Fig. 2: Viral HA Assay plate.
Viral hemagglutination assays were conducted with human type O Rh+ erythrocytes, as described in Methods (and in WHO Manual, 2011). The presence of conspicuous red buttons in the well indicates absence of agglutination, ie. inhibition of HA activity. The other wells show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

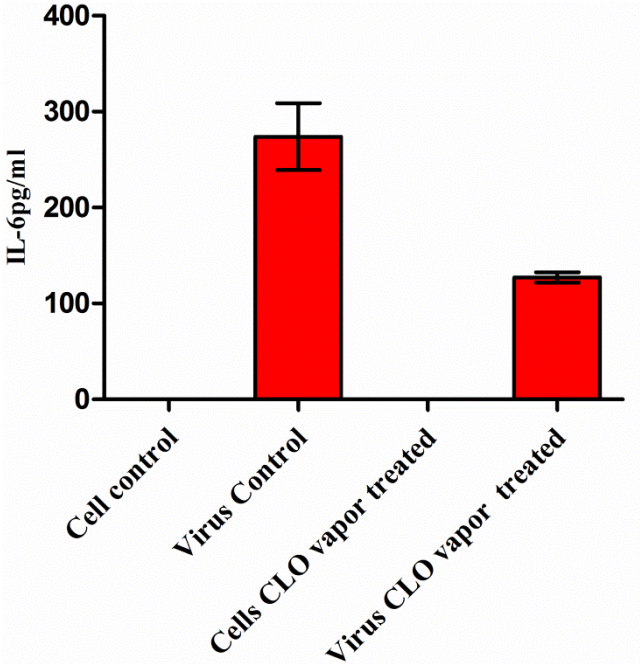

Fig. 3: Anti-inflammatory activity of CLO vapor.
Human A 549 epithelial cells were inoculated with rhinovirus 14 (1 pfu/cell), or control medium, for 60 min, followed by CLO vapor treatment for 60 min. After a further 24 h in normal medium, supernatants were removed for assay of IL-6 content, as described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

ACKNOWLEDGEMENT

Partial funding for this research was provided by Bio Aerosol Technologies Inc. Vancouver.

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How to cite this article:

Vimalanathan Selvarani, Hudson James. The Activity of Cedar Leaf oil Vapor Against Respiratory Viruses: Practical Applications. J App Pharm Sci. 2013; 3 (11): 011-015.

May 12, 2014

RE: Western Red Cedar Leaf Oil

We performed a mould and bacteria analysis of an 11- story co-op apartment building to determine the fungal and bacterial status of the air as well as on HVAC surfaces.

The location was an 11-story residential high-rise building in mid-town Toronto, ON.

Initial results showed extensive airborne bacillus bacteria in unit #511 as well as bacillus and E.coli surface bacteria on the HVAC diffuser on the 5th floor. In both cases, bacteria levels were TNTC or Too Numerous To Count.

Escherichia coli, usually called *E. coli*, refers to a large group of bacteria that is commonly found in the intestines of humans and animals. There was a large scale construction site to the West of the building where sewers were being replaced. The E.coli and bacillus may have come through the ventilation system from the construction project.

WRC Oil was diffused through the roof top air handling system into the building for an initial 4 hours and then 8 hours the following day. Air and surface testing was performed approximately 24 hours after the last WRC Oil treatment of the air.

Laboratory results show no bacillus counts in the air in unit #511 with only a few bacteria counts found also in the outdoor sample. Surface sampling also found no E.coli on the HVAC diffusers with only a few counts of bacteria found in our outdoor sample.

No topical cleaning of any kind was performed during this test.

HAVERKATE & ASSOCIATES Inc.
Frank H. Haverkate, BA, BB, BBEI, CMC, CMRS, CIHM
Certified Indoor Environmental Inspector
Council Certified Microbial Investigator
Council Certified Mould Remediation Supervisor
Certified Industrial Hygiene Manager
Master Trainer – Approved Certification Course Provider - ACAC
Past Founding Director –Indoor Air Quality Association – Toronto Chapter

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LAB # 172958

Project: 100 Merton **Project # 022714-B** **Lab # E60096**

Report Date: 03/12/2014 **Sampled: 02/28/2014** **Received: 03/04/2014** **Analyzed: 03/12/2014**

Table of Contents

IMS Laboratory

Report Prepared For: Haverkate & Associates Inc
Project Name: 100 Merton
Project Number: 022714-B
Report Date: 03/12/2014
Lab Number: E60096

1 - Laboratory Results

Location: Outside

Sample # E60096 - 1

Medium Type: Bioaerosol
Serial # 100
Exposure: 28.30 l/min. for 3.00 min.
Reporting Limit: 12 CFUs/cu. m

Sample Identification	Raw Count	CFUs/cu. m	Percent(%)
- Fungi -			
No Fungal Growth	0	0	N/A%

Background Item	Level
(None)	N/A

Location: Floor 11

Sample # E60096 - 2

Medium Type: Bioaerosol
Serial # 100 M-1
Exposure: 28.30 l/min. for 3.00 min.
Reporting Limit: 12 CFUs/cu. m

Sample Identification	Raw Count	CFUs/cu. m	Percent(%)
- Fungi -			
Aspergillus	1	12	100.00%
TOTALS:	1	12	100.00%

Background Item	Level
(None)	N/A

Location: Floor 5

Sample # E60096 - 3

Medium Type: Bioaerosol
Serial # 100 M-2
Exposure: 28.30 l/min. for 3.00 min.
Reporting Limit: 12 CFUs/cu. m

Sample Identification	Raw Count	CFUs/cu. m	Percent(%)
- Fungi -			
Yeast	1	12	100.00%
TOTALS:	1	12	100.00%

Background Item	Level
(None)	N/A

Location: Floor 5 Unit 511

Sample # E60096 - 4

Medium Type: Bioaerosol
Serial # 100 M-3
Exposure: 28.30 l/min. for 3.00 min.
Reporting Limit: 12 CFUs/cu. m

Sample Identification	Raw Count	CFUs/cu. m	Percent(%)
- Fungi -			
Cladosporium	1	12	50.00%
Yeast	1	12	50.00%
TOTALS:	2	24	100.00%

Background Item	Level
(None)	N/A

IMS Laboratory

Report Prepared For: Haverkate & Associates Inc
Project Name: 100 Merton
Project Number: 022714-B
Report Date: 03/12/2014
Lab Number: E60096

Location: Floor 1

Sample # E60096 - 5

Medium Type: Bioaerosol
Serial # 100 M-4
Exposure: 28.30 l/min. for 3.00 min.
Reporting Limit: 12 CFUs/cu. m

Sample Identification	Raw Count	CFUs/cu. m	Percent(%)
- Fungi -			
No Fungal Growth	0	0	N/A%

Background Item	Level
(None)	N/A

Location: Floor 1 Unit 104

Sample # E60096 - 6

Medium Type: Bioaerosol
Serial # 100 M-5
Exposure: 28.30 l/min. for 3.00 min.
Reporting Limit: 12 CFUs/cu. m

Sample Identification	Raw Count	CFUs/cu. m	Percent(%)
- Fungi -			
No Fungal Growth	0	0	N/A%

Background Item	Level
(None)	N/A

Location: Outside

Sample # E60096 - 7

Medium Type: Bioaerosol
Serial # 100
Exposure: 28.30 l/min. for 3.00 min.
Reporting Limit: 12 CFUs/cu. m

Sample Identification	Raw Count	CFUs/cu. m	Percent(%)
- Bacteria -			
Escherichia coli (E. coli)	1	12	50.00%
Pseudomonas	1	12	50.00%
TOTALS:	2	24	100.00%

Background Item	Level
(None)	N/A

Location: Floor 11

Sample # E60096 - 8

Medium Type: Bioaerosol
Serial # 100 B-1
Exposure: 28.30 l/min. for 3.00 min.
Reporting Limit: 12 CFUs/cu. m

Sample Identification	Raw Count	CFUs/cu. m	Percent(%)
- Bacteria -			
Corynebacterium	1	12	33.33%
Kurthia	1	12	33.33%
Micrococcus roseus	1	12	33.33%
TOTALS:	3	36	100.00%

Background Item	Level
(None)	N/A

IMS Laboratory

Report Prepared For: Haverkate & Associates Inc
Project Name: 100 Merton
Project Number: 022714-B
Report Date: 03/12/2014
Lab Number: E60096

Location: Floor 5

Sample # E60096 - 9

Medium Type: Bioaerosol
Serial # 100 B-2
Exposure: 28.30 l/min. for 3.00 min.
Reporting Limit: 12 CFUs/cu. m

Sample Identification	Raw Count	CFUs/cu. m	Percent(%)
- Bacteria -			
Micrococcus lylae	2	24	40.00%
Staphylococcus hominis	2	24	40.00%
Kurthia	1	12	20.00%
TOTALS:	5	60	100.00%

Background Item	Level
(None)	N/A

Location: Floor 5 Unit 511

Sample # E60096 - 10

Medium Type: Bioaerosol
Serial # 100 B-3
Exposure: 28.30 l/min. for 3.00 min.
Reporting Limit: 12 CFUs/cu. m

Sample Identification	Raw Count	CFUs/cu. m	Percent(%)
- Bacteria -			
Corynebacterium	1	12	N/A%
Bacillus	TNTC	TNTC	N/A%

Background Item	Level
(None)	N/A

Location: Floor 1

Sample # E60096 - 11

Medium Type: Bioaerosol
Serial # 100 B-4
Exposure: 28.30 l/min. for 3.00 min.
Reporting Limit: 12 CFUs/cu. m

Sample Identification	Raw Count	CFUs/cu. m	Percent(%)
- Bacteria -			
Staphylococcus sciuri	2	24	50.00%
Micrococcus lylae	1	12	25.00%
Staphylococcus haemolyticus	1	12	25.00%
TOTALS:	4	48	100.00%

Background Item	Level
(None)	N/A

Location: Floor 1 Unit 104

Sample # E60096 - 12

Medium Type: Bioaerosol
Serial # 100 B-5
Exposure: 28.30 l/min. for 3.00 min.
Reporting Limit: 12 CFUs/cu. m

Sample Identification	Raw Count	CFUs/cu. m	Percent(%)
- Bacteria -			
Streptococcus	1	12	100.00%
TOTALS:	1	12	100.00%

Background Item	Level
(None)	N/A

IMS Laboratory

Report Prepared For: Haverkate & Associates Inc
Project Name: 100 Merton
Project Number: 022714-B
Report Date: 03/12/2014
Lab Number: E60096

Location: Flr 5

Sample # E60096 - 13

Medium Type: Swab
Serial # 100 - S1
Reporting Limit: 1 CFU

Sample Identification	Raw Count
- Bacteria -	
Bacillus	TNTC
Enterobacter	TNTC
Escherichia coli (E. coli)	TNTC

Background Item	Level
(None)	N/A

Analytic Methods and Formulas:

Colony Forming Units per cubic meter is determined by: Total Colony Count x (1000/(sampling rate x sampling time))
IMS Laboratory Analytical Method: 2.1 (method for analyzing cellophane tape lift)
IMS Laboratory Analytical Method: 2.1 (method for analyzing cellophane tape lift) & 2.4 (method for analyzing viable air samples)
Positive Hole Correction used for Bioaerosol sampling
Results are rounded to 3 significant figures per AIHA policy module 2A.5.10.6

Note that this report may use mold-specific units, such as Spores/cu. m and CFU/cu. m for Sample Identifications such as pollen, fiberglass fibers, and bacteria, which are not molds.

IMS Laboratory, LLC is accredited through the American Industrial Hygiene Association (AIHA) and participates in Environmental Microbiology Proficiency Testing, EMPAT #172958. Data is provided in compliance with AIHA policy modules and ISO 17025 guidelines.

Analyst

03/12/2014

Brittney Holtz, Lab Analyst

AIHA LAP, LLC
ACCREDITED LABORATORY
ENVIRONMENTAL MICROBIOLOGY
ISO/IEC 17025:2005
LAB # 172958

Reviewer

03/12/2014

Bethany Sisco, Lab Analyst

2 - Sample Comparison Graph

Bioaerosol Samples - Colony Forming Units per Cubic Meter

Outside
NO FUNGI FOUND

Floor 11

Floor 5

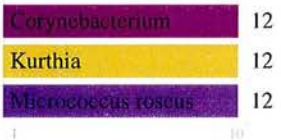
Floor 5 Unit 511

Floor 1
NO FUNGI FOUND

Floor 1 Unit 104
NO FUNGI FOUND

Outside

Floor 11

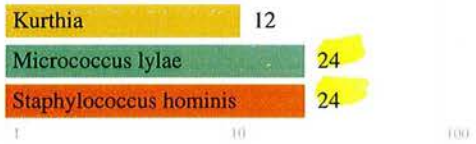


IMS Laboratory

Report Prepared For: Haverkate & Associates Inc
Project Name: 100 Merton
Project Number: 022714-B
Report Date: 03/12/2014
Lab Number: E60096

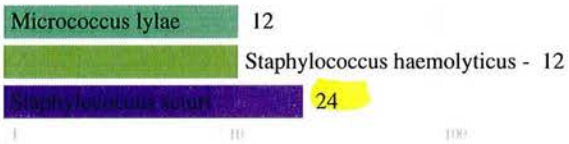
Bioaerosol Samples - Colony Forming Units per Cubic Meter

Floor 5



Floor 5 Unit 511

Floor 1



Floor 1 Unit 104

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Project: 100 Merton - Hospital Workers **Project # 032114-X** **Lab # E60637**

Report Date: 03/27/2014 **Sampled: 03/21/2014** **Received: 03/24/2014** **Analyzed: 03/27/2014**

Table of Contents

IMS Laboratory

Report Prepared For: Haverkate & Associates Inc
Project Name: 100 Merton - Hospital Workers
Project Number: 032114-X
Report Date: 03/27/2014
Lab Number: E60637

1 - Laboratory Results

Location: Outside

Sample # E60637 - 1

Medium Type: Bioaerosol
Serial # 100-1
Exposure: 28.30 l/min. for 3.00 min.
Reporting Limit: 12 CFUs/cu. m

Sample Identification	Raw Count	CFUs/cu. m	Percent(%)
- Bacteria -			
Corynebacterium	1	12	100.00%
TOTALS:	1	12	100.00%

Background Item	Level
(None)	N/A

Location: Unit 511

Sample # E60637 - 2

Medium Type: Bioaerosol
Serial # 100-2
Exposure: 28.30 l/min. for 3.00 min.
Reporting Limit: 12 CFUs/cu. m

Sample Identification	Raw Count	CFUs/cu. m	Percent(%)
- Bacteria -			
Corynebacterium	1	12	50.00%
Enterobacter	1	12	50.00%
TOTALS:	2	24	100.00%

Background Item	Level
(None)	N/A

Location: Blank

Sample # E60637 - 3

Medium Type: Bioaerosol
Serial # 100-3
Exposure: 0.00 l/min. for 0.00 min.
Reporting Limit: 1 CFU/cu. m

Sample Identification	Raw Count	CFUs/cu. m	Percent(%)
- Bacteria -			
No Bacterial Growth	0	0	N/A%

Background Item	Level
(None)	N/A

Location: Floor 5 Vent

Sample # E60637 - 4

Medium Type: Swab
Serial # 100-1S
Reporting Limit: 1 CFU

Sample Identification	Raw Count
- Bacteria -	
Corynebacterium	13
Bacillus	1

Background Item	Level
(None)	N/A

IMS Laboratory

Report Prepared For: Haverkate & Associates Inc
Project Name: 100 Merton - Hospital Workers
Project Number: 032114-X
Report Date: 03/27/2014
Lab Number: E60637

Location: Blank

Sample # E60637 - 5

Medium Type: Swab
Serial # 100-2S
Reporting Limit: 1 CFU

Sample Identification	Raw Count
- Bacteria -	
No Bacterial Growth	0

Background Item	Level
(None)	N/A

Analytic Methods and Formulas:

Colony Forming Units per cubic meter is determined by: Total Colony Count x (1000/(sampling rate x sampling time))
IMS Laboratory Analytical Method: 2.1 (method for analyzing cellophane tape lift)
IMS Laboratory Analytical Method: 2.1 (method for analyzing cellophane tape lift) & 2.4 (method for analyzing viable air samples)
Positive Hole Correction used for Bioaerosol sampling
Results are rounded to 3 significant figures per AIHA policy module 2A.5.10.6

Note that this report may use mold-specific units, such as Spores/cu. m and CFU/cu. m for Sample Identifications such as pollen, fiberglass fibers, and bacteria, which are not molds.

IMS Laboratory, LLC is accredited through the American Industrial Hygiene Association (AIHA) and participates in Environmental Microbiology Proficiency Testing, EMPAT #172958. Data is provided in compliance with AIHA policy modules and ISO 17025 guidelines.

Analyst

Amy Lebeis 03/27/2014

Amy Lebeis, Lab Analyst

AIHA LAP, LLC
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ISO/IEC 17025:2005
LAB # 172958

Reviewer

Kathryn C. Langley 03/27/2014

Kathryn C. Langley, Lab Analyst

IMS Laboratory

Report Prepared For: Haverkate & Associates Inc
Project Name: 100 Merton - Hospital Workers
Project Number: 032114-X
Report Date: 03/27/2014
Lab Number: E60637

2 - Sample Comparison Graph

Bioaerosol Samples - Colony Forming Units per Cubic Meter

Outside

Unit 511

Blank

NO FUNGI FOUND

CERTIFIED (*Thuja plicata*) CEDAR LEAF OIL PROJECT—PHASE 1: ADDENDUM

(Published APRIL 2011)

Dr. James Hudson (Behalf of Cedar Biotech Inc.)

Additional Studies on Antiviral Activities of CLO Vapor:

Results of additional tests of CLO vapor against human pathogenic viruses are shown in Table App-1. In addition to Herpes simplex viruses types 1 and 2, the relatively more robust viruses, such as common cold virus, rhinovirus type 14 (RV14), and the respiratory virus adenovirus type 4, were also found to be susceptible to CLO vapor although, as expected, the rate of killing was significantly slower than observed previously in direct contact tests (oil plus virus in liquid). Under similar conditions of exposure to CLO vapor, the cells themselves, in the absence of virus, were assessed for possible cytotoxic effects, but no significant cell killing was observed microscopically or by LDH cell viability assay, reinforcing the conclusion that CLO vapor can kill viruses selectively without adversely affecting human lung cells.

Table App-1. Antiviral effects of CLO Vapor

Time of exposure to CLO vapor (mins)	HSV-1 % kill	HSV-2 % kill	RV-14 % kill	Adeno-4 % kill	Cell survival % of control
10	> 99			~ 0	97.5 ± 13.7
20	> 99				
30	> 99	91	93		96.7 ± 7.6
60					97.0 ± 8.3
90		> 99	> 99	> 99	

Infectious viruses were measured by plaque assays, cell viability by the LDH (lactate dehydrogenase) assay.

Anti-inflammatory Effects of CLO.

We have previously established a model system for the measurement of virus-induced pro-inflammatory responses in human lung epithelial cell cultures. This system can be used to evaluate the potential of prospective anti-inflammatory agents. In the test shown in Table 2, CLO vapor was able to partially reverse the influenza virus-induced secretion of the pro-inflammatory cytokine IL-6, ie. the CLO 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. In addition the CLO did not produce IL-6 in uninfected control cells. This result complements the lack of cytotoxicity of CLO vapor.

Table App-2. Anti-inflammatory Effect of CLO Vapor

Treatment of cells	**IL-6 secretion (pg/ml)**
Control uninfected	0 (not detected)
Control + CLO	0 (not detected)
Influenza virus infected	27.98 ± 4.7
Infected + CLO	13.03 ± 0.22 (54 % inhibition)

CONCLUSIONS:

These additional data confirm and expand the broad spectrum of antiviral activity of CLO Vapor, and also provide further evidence for the absence of cytotoxic effects, thus supporting the claim that CLO Vapor is safe to use in occupied buildings.

Dr. James Hudson - 2011.

CEDAR LEAF OIL PROJECT—PHASE 1: FINAL REPORT

(DEC 2010)

Dr. James Hudson (JB Hudson Consulting Ltd)

1. Objectives:

1. To establish laboratory conditions appropriate for the evaluation of cedar leaf oil (CLO) as an antibacterial, antifungal (anti-mold) and antiviral agent.
2. To evaluate the efficacy of CLO and CLO-vapor against selected bacteria/molds/spores.
3. To evaluate the efficacy of CLO and vapor against selected viruses.
4. To determine that cultured human cells do not suffer cytotoxic effects from exposure to CLO vapor.

2. Summary:

Laboratory tests were designed to simulate real-life situations and potential applications of CLO in the field. A number of human bacteria, fungi, and viruses, known to be common environmental sources of potential infection, were selected and tested by means of quantitative assay methods, and all of them were found to be susceptible to CLO liquid and vapor. In other words, CLO was able to kill them efficiently. Bacterial spores however were significantly more resistant, and required prolonged exposure. Similar tests with cultured human lung cells showed that continuous exposure to CLO vapor for at least 30 minutes was not toxic to the cells.

3. Conclusions:

1. All the organisms selected for testing (five bacteria, bacterial spores, two fungi, and four viruses), representing human pathogens commonly encountered in the environment, were susceptible to CLO and CLO vapor. None were resistant.
2. The five bacteria consisted of three Gram-positive organisms, *Bacillus subtilis*, *Streptococcus pyogenes*, and *Enterococcus fecalis*, and two Gram-negative organisms, *Acinetobacter baumanii* and *Hemophilus influenzae*. They were all readily killed by CLO, although their relative sensitivities were somewhat different.
3. Two fungi, the filamentous mold *Aspergillus niger*, and the medically important yeast *Candida albicans*, were also readily inactivated by CLO, although the *A. niger* appeared to recover to some degree after several days.

4. Purified spores of *Bacillus subtilis* were also sensitive to CLO, but they were relatively resistant in comparison to the corresponding vegetative (growing) cells.
5. Influenza viruses (type A/H1N1 and type B), and herpes simplex viruses (types 1 & 2), were readily killed by CLO and CLO vapor.
6. Human lung cell cultures did not show any signs of cytotoxicity following short-term exposure to CLO vapor, under conditions similar to those that killed viruses.
7. Additional laboratory tests should be conducted to confirm the efficacy of CLO vapor against other important organisms, to examine the apparent relative resistance of bacterial spores in more detail, and to investigate the importance of certain parameters that could influence the efficacy of the CLO-V.

4. Results:

4.1. Antibacterial Activities:

A total of 5 bacteria, known to be potentially pathogenic to humans and recognized as environmental contaminants, were selected and evaluated. Each one was propagated and assayed (as colony-forming units, CFU, equivalent to viable organisms) according to standard bacteriological methods. These are described in the Methods section. The test protocols were designed on the basis of their relevance to applications of CLO in the field.

Bacillus subtilis (*B. subtilis*, sub sp. *spizizenii*) is an environmental Gram-positive bacterium which grows in suspension or on agar plates as individual cells (cocci), and also persists in the environment in a spore form (endospores). The *B. subtilis* spores are often used in laboratory tests as surrogates for the anthrax organism (*Bacillus anthracis*). This organism therefore can be tested in both the vegetative (growing) and spore forms. Both forms are likely to be encountered in buildings, but the spores would be expected to be more stable.

Streptococcus pyogenes (*S. pyogenes*), a Gram-positive coccus, is a part of the normal human flora, but can often cause diseases such as “sore throats” and more serious respiratory problems.

Hemophilus influenzae (*H. influenzae*) is a Gram-negative rod-shaped bacterium, often responsible for upper respiratory infections, especially as secondary infections following influenza infection.

Acinetobacter baumanii (*A. baumanii*) an environmental Gram-negative bacterium, was recently identified as an important cause of serious infections in wounded personnel returning from war zones, and is increasingly associated with outbreaks of disease in hospitals. Antibiotic-resistant forms are continually emerging.

Enterococcus fecalis (*E. fecalis*) is a Gram-positive coccus associated with gastro-intestinal disease, and is increasingly presenting in hospitals as life-threatening antibiotic resistance forms.

4.1.1. Antibacterial tests with liquid CLO:

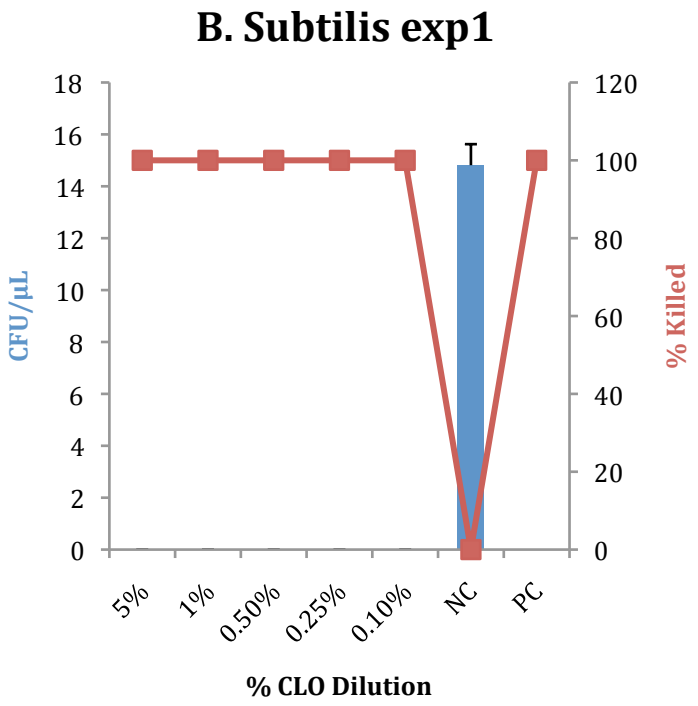
Initially *Bacillus subtilis* was used to determine whether or not CLO possessed antibacterial activity, and to examine various parameters in the reaction of CLO liquid with the test bacterium, in different vessels. It was found that most of the materials used routinely in the laboratory were appropriate (ie. they were resistant to CLO liquid and vapor), with the exception of polystyrene culture vessels (trays, tubes, flasks), which were degraded by direct contact with CLO. The resulting degradation product was toxic to bacteria and to cultured cells. Consequently polypropylene and glass vessels were used in the tests. Other materials tested, such as metals, wood, hard plastic, glass and fabrics, appeared to be resistant to CLO. All the bacterial assays were carried out by mean of conventional colony counts (colony forming units per unit volume, CFU) on the appropriate type of agar plate (details given in the Methods section, below).

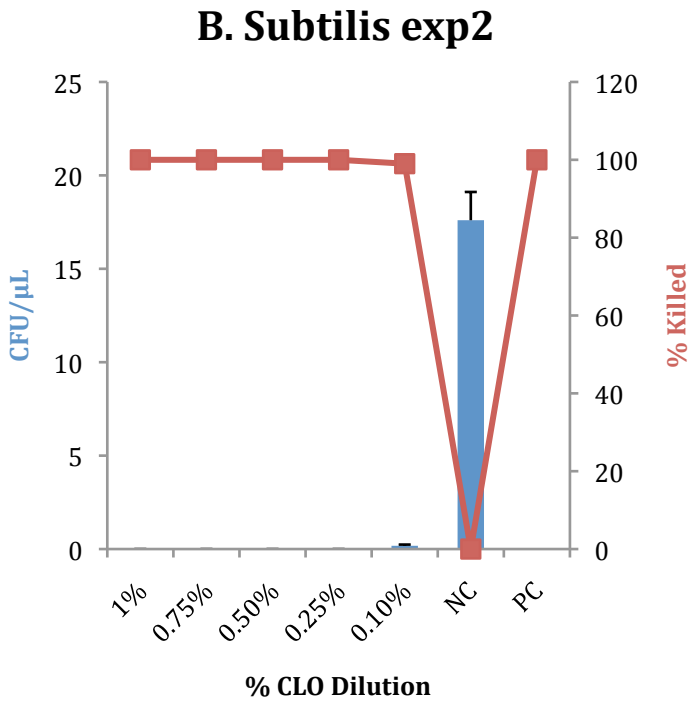
B. subtilis was readily killed by CLO after several minutes contact with the oil, as shown in Fig 1. The dose response tests showed that concentrations of CLO down to 0.1 % (in saline) completely killed the bacterial inoculum within one hour. Further dilutions showed successively less efficacy. Figure 2 shows examples of agar plates with bacterial colonies from treated and untreated samples.

In these and subsequent tests, Tea Tree oil (TTO), which is recognized as a powerful antibacterial oil on direct contact with contaminated surfaces, was used routinely as a “positive control”. Untreated bacteria (ie, the same number of bacteria incubated without exposure to oil or vapor) represented the “negative control”.

FIGURE 1: Efficacy of diluted CLO against *Bacillus subtilis*

These experiments show that CLO in dilutions down to less than 0.1% are very effective as bactericidal agents. NC = negative control (untreated bacteria). PC = positive control (bacteria treated by 10% TTO, tea tree oil)





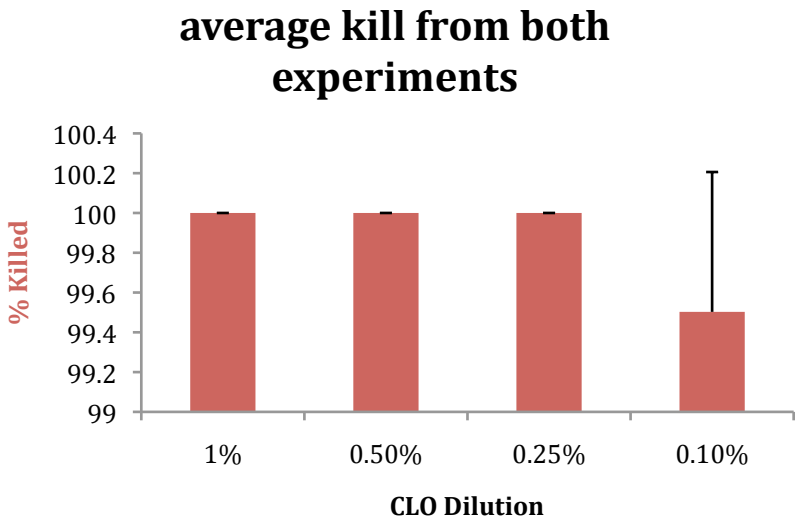
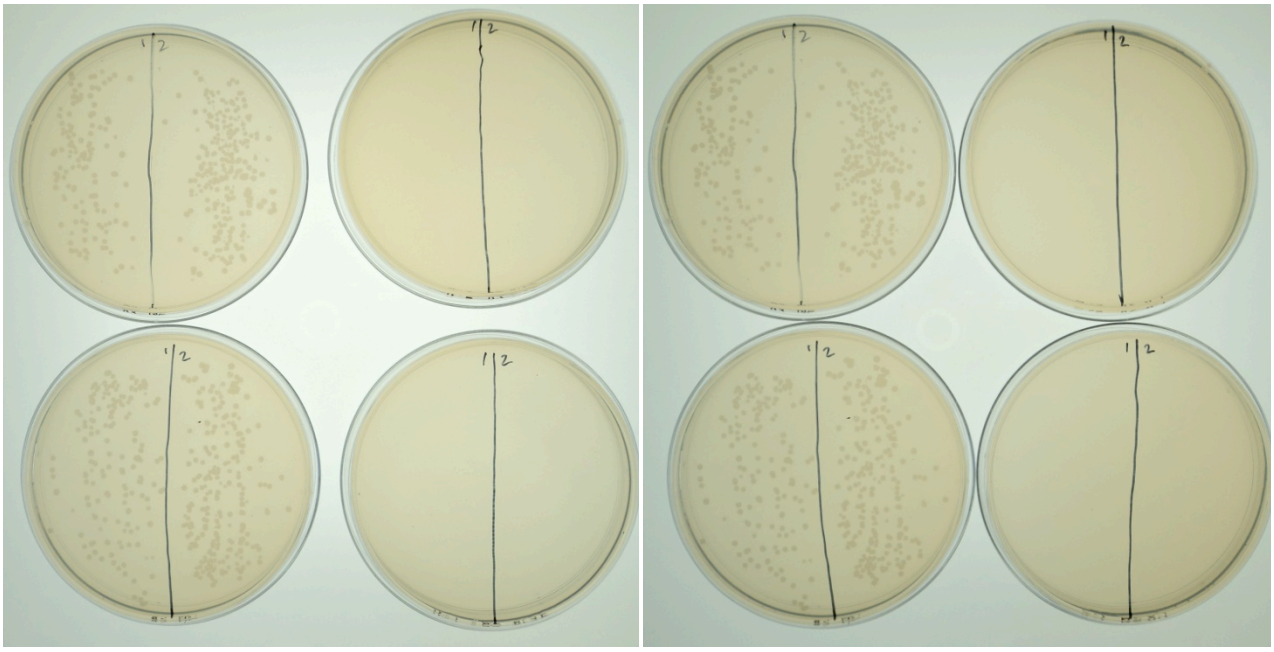


FIGURE 2: *B. subtilis* colonies on agar plates

Duplicate plates on left of each panel represent untreated (unexposed) bacteria, plates on right side are CLO-exposed bacteria (no colonies visible). After an additional incubation of treated bacterial plates for 7 days (no further exposure to CLO), there were still no colonies, indicating that the treated bacteria did not recover and therefore the antibacterial effect was bactericidal rather than bacteriostatic.

Panel A, control 0.5% CLO Panel B, control 0.1% CLO

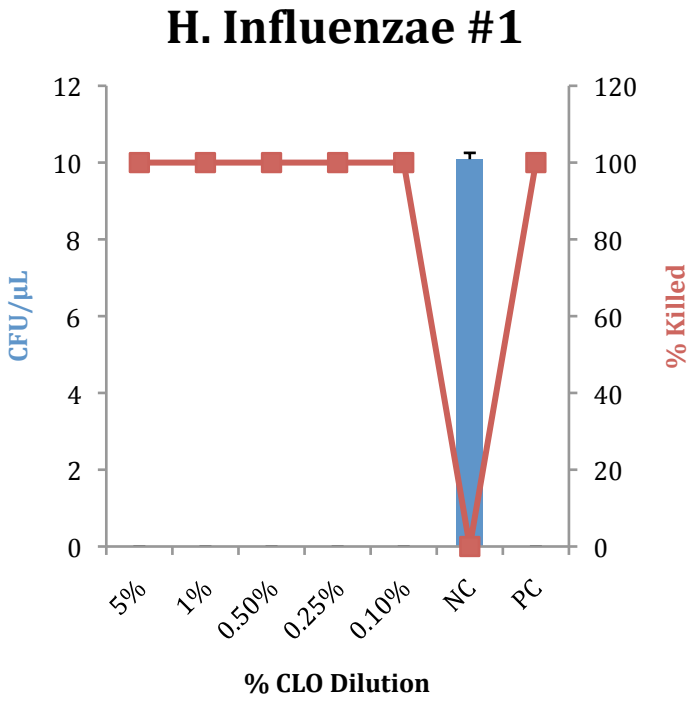


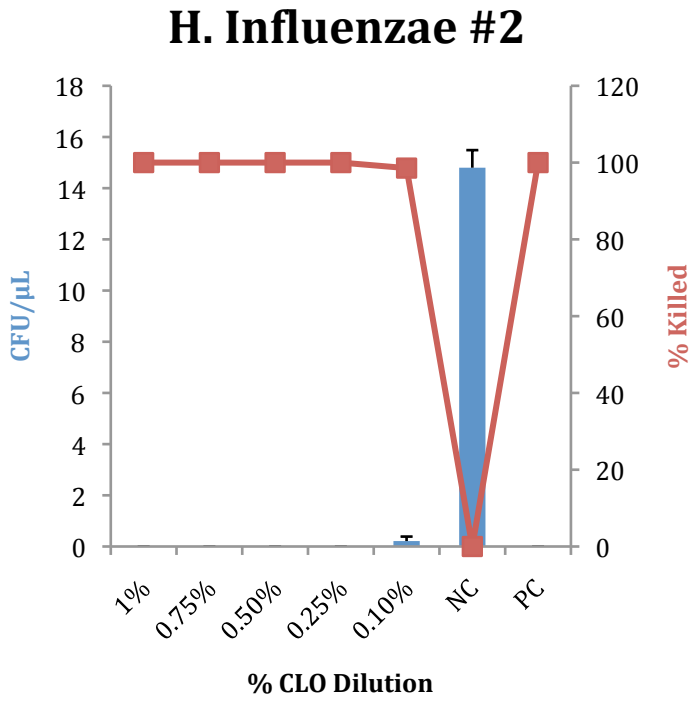
Since CLO is immiscible with aqueous solutions, incubations of CLO with bacterial suspensions required frequent agitation or mixing, although efficient bacterial killing was achieved. Attempts were made to overcome this limitation by incorporating small amounts of the neutral detergents Tween 20 or Tween 80 into the oil, as recommended by the Carson group for their studies on Tea Tree oil (TTO). However the addition of Tween did not improve the antibacterial efficiency of CLO any further. Consequently Tween was omitted in subsequent tests.

Following the successful elimination of *B. subtilis*, the other four bacteria were tested by the same protocols. All of them were readily killed by CLO down to high dilutions, as shown by the charts in Figures 3-8, although the relative sensitivities varied somewhat. *H. influenzae* and *S. pyogenes* were as susceptible as *B. subtilis*, whereas *A. baumanii* was slightly more resistant, and *E. fecalis* was considerably more resistant. However even the latter was completely killed by exposure to 1% CLO. Figures 4 and 6 show examples of colonies on agar plates.

FIGURE 3; Antibacterial effect of CLO against *Hemophilus influenzae*

These experiments show that CLO in dilutions down to less than 0.1% are very effective as bactericidal agents. NC = negative control (untreated bacteria). PC = positive control (bacteria treated by 10% TTO, tea tree oil)





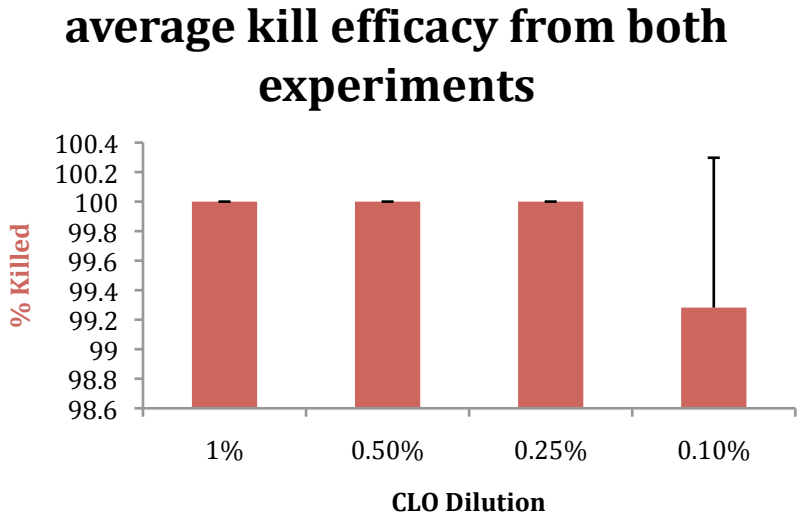


FIGURE 4: Colonies of *H. influenzae*, unexposed (agar plates on left side) and exposed to 1% CLO (right side; no recovery of growth after further 7 days incubation without CLO).

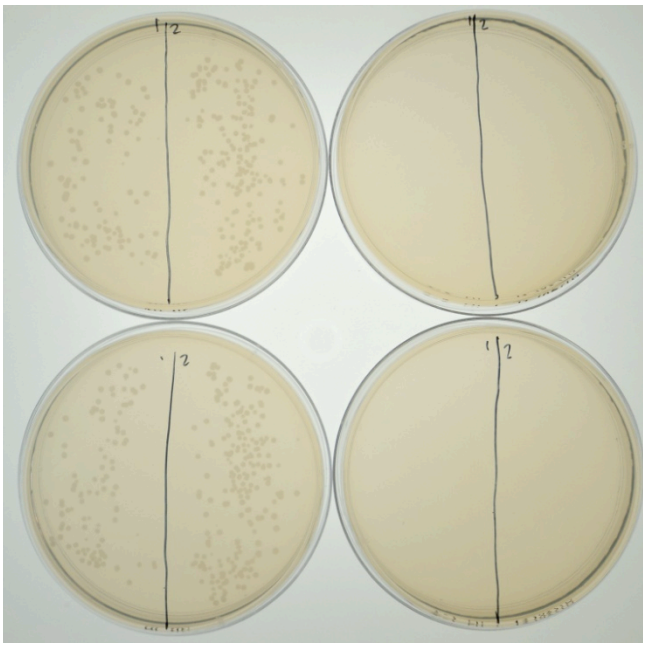


FIGURE 5: Antibacterial efficacy of CLO against *Streptococcus pyogenes*

These experiments show that CLO in dilutions down to less than 0.1% are very effective as bactericidal agents. NC = negative control (untreated bacteria). PC = positive control (bacteria treated by 10% TTO, tea tree oil)

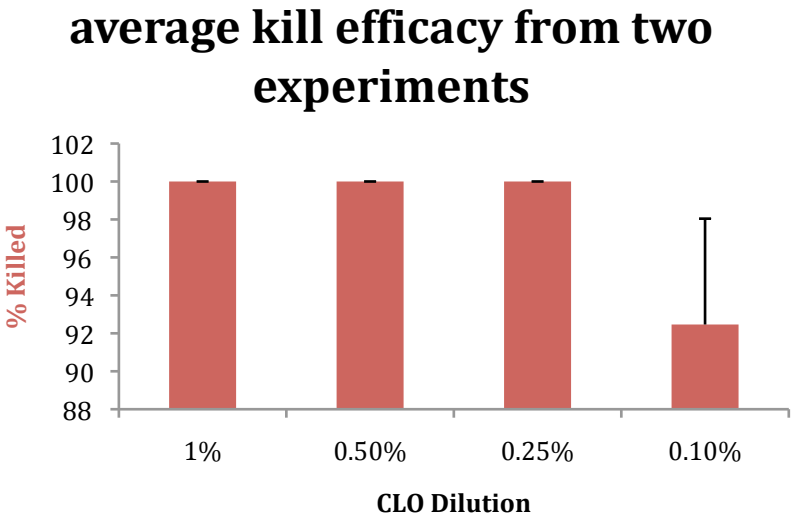


FIGURE 6: Antibacterial efficacy against *S. pyogenes*.

Plates on left, untreated. Right side, exposed to 0.5% CLO

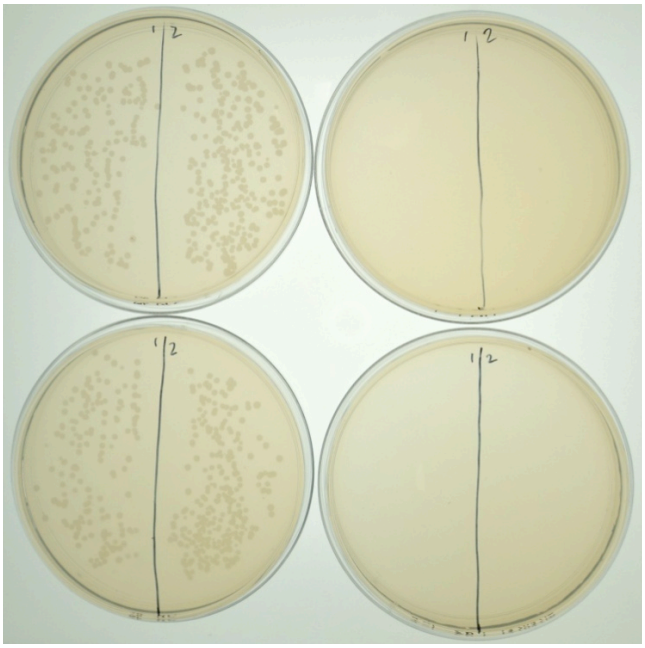


FIGURE 7: Antibacterial efficacy against *Acinetobacter baumanii*

These experiments show that CLO in dilutions down to less than 0.25% are very effective as bactericidal agents.

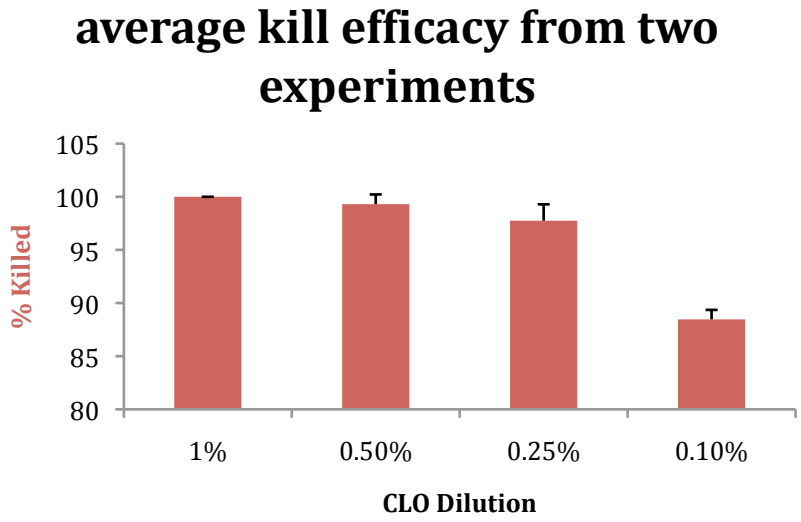
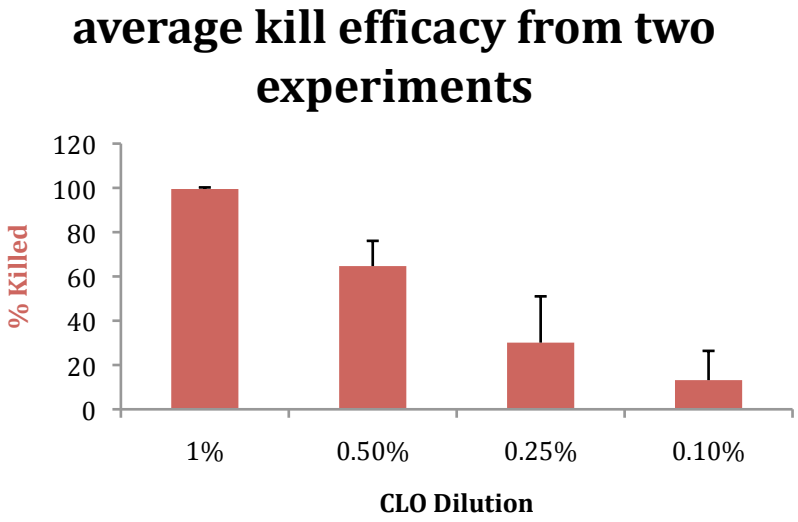


FIGURE 8: Antibacterial efficacy against *Enterococcus fecalis*:

These experiments show that CLO in dilutions down to less than 1.0% are very effective as bactericidal agents, although this organism is relatively more resistant than the others

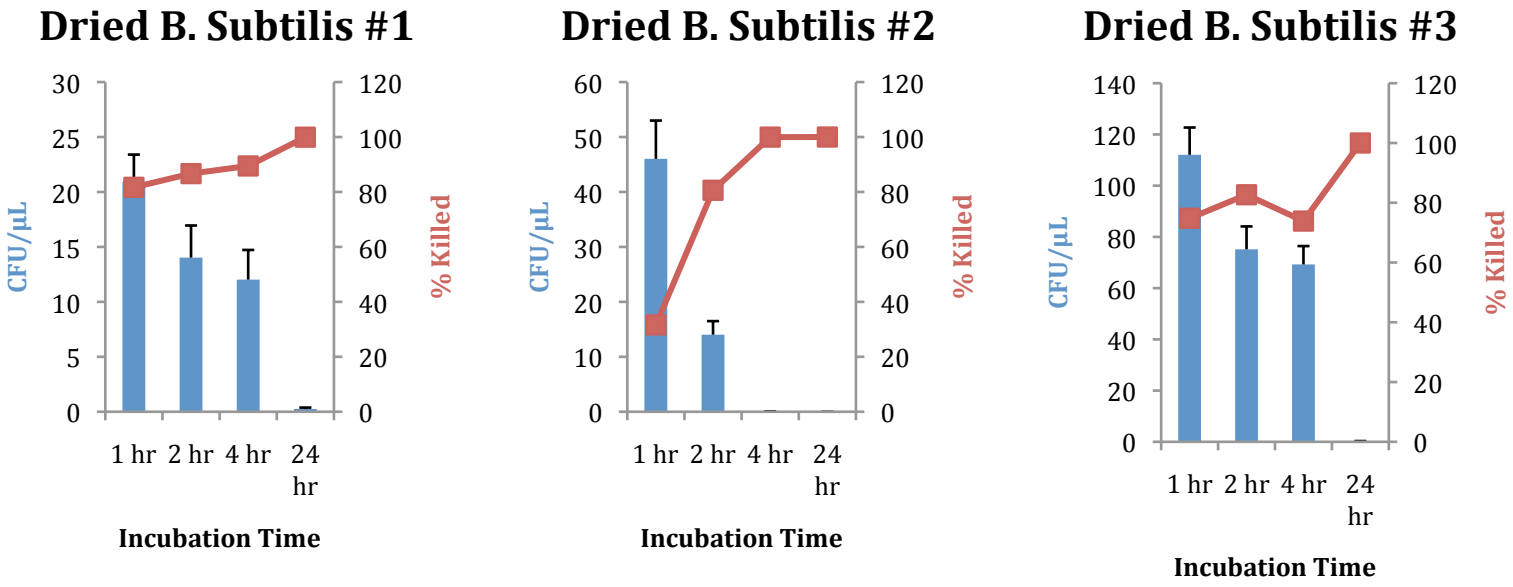


4.1.2. Antibacterial effects of CLO vapor on dried films

The experiments described up to this point indicated that CLO possesses potent antibacterial activity.
In subsequent experiments the bacteria were used as dried films on glass slides, to mimic real life conditions. The treated films were reconstituted in saline solution after exposure, followed by serial dilution and measurement of CFUs on agar plates. The films, containing different numbers of viable bacteria, were found to be just as vulnerable to liquid CLO.
The next series of experiments consisted of exposing various dried films of bacteria to the vapor of CLO. This was accomplished by drying the bacterial samples onto a glass or plastic surface, which was then exposed to CLO vapor within a confined space. This treatment was also very effective, although the time of exposure required for efficient killing was significantly longer than with the liquid oil. Results are shown in Figures 9-11.

FIGURE 9: CLO vapor on dried films of *Bacillus subtilis*

The vapor was a very effective bactericidal agent against dried bacteria, with increasing efficacy over increasing time of exposure



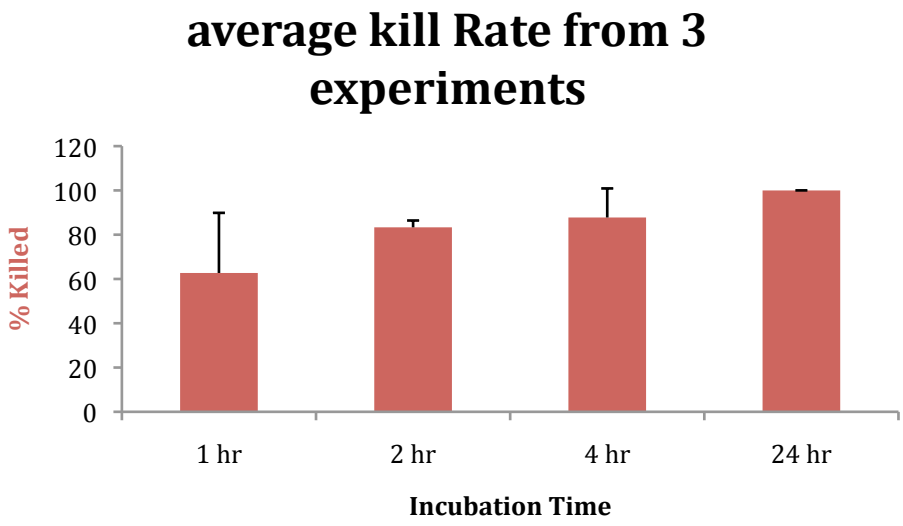
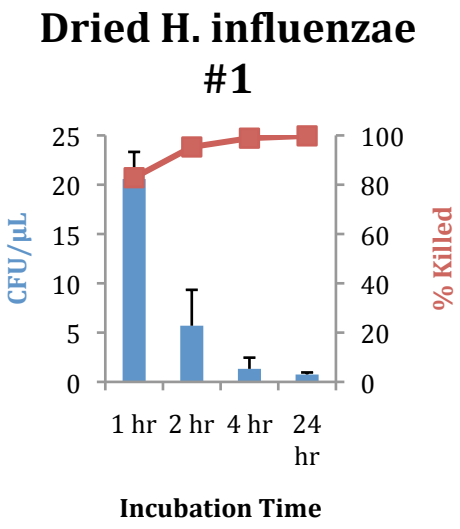
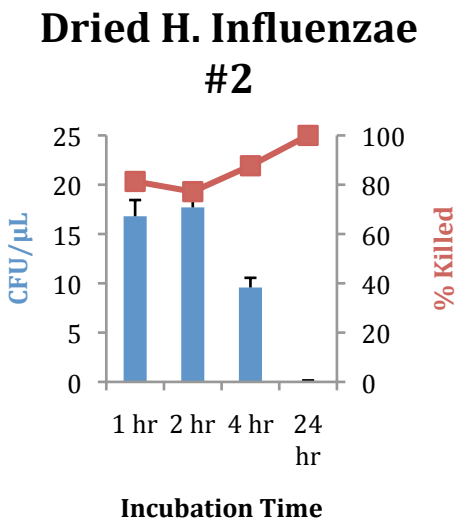
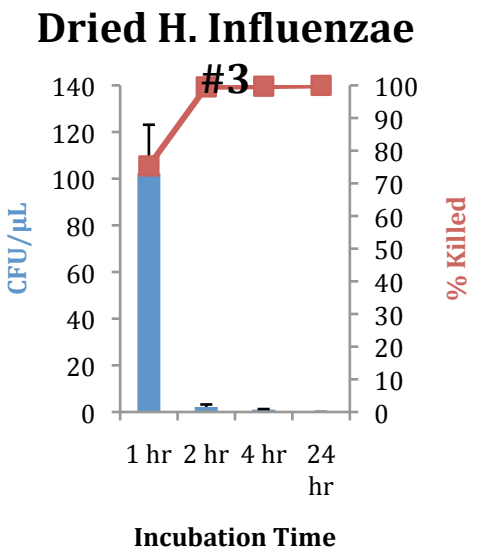


FIGURE 10: CLO vapor on dried *H.influenzae*:

The vapor was a very effective bactericidal agent against dried bacteria, with increasing efficacy over increasing time of exposure







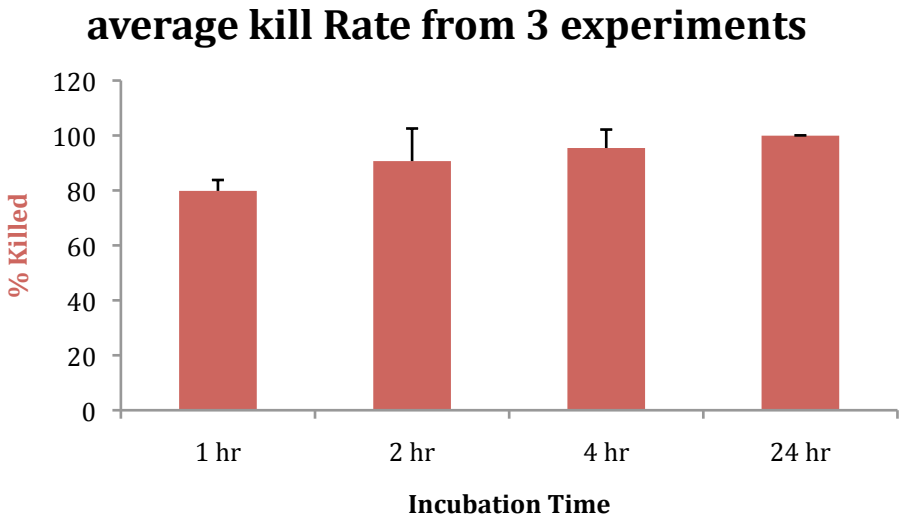
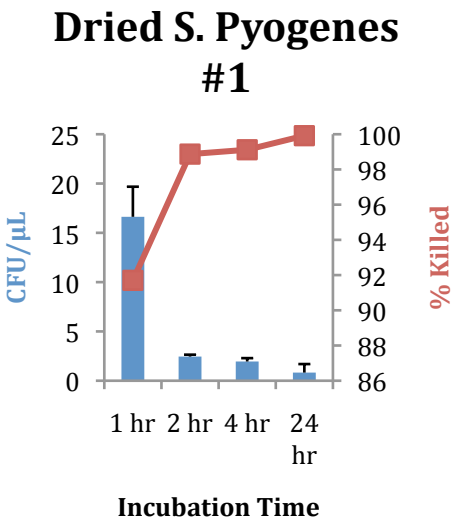
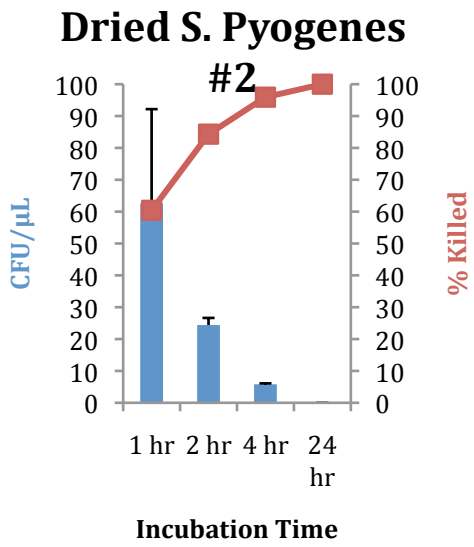
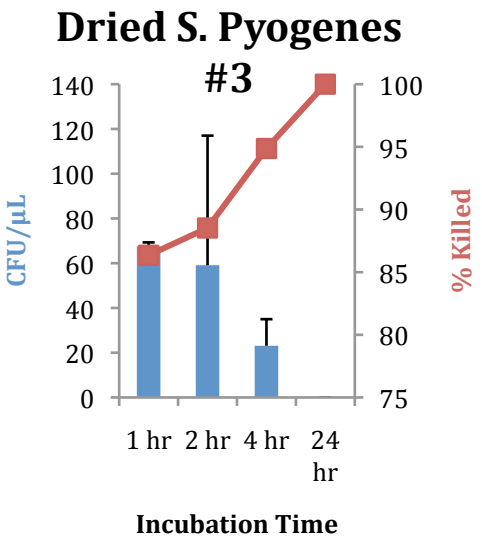


FIGURE 11: CLO vapor on dried *S. pyogenes*

The vapor was a very effective bactericidal agent against dried bacteria, with increasing efficacy over increasing time of exposure. Similar results were obtained with *A. baumanii* and *E. fecalis*, although these organisms were again relatively more resistant.







4.1.3. Effect of Bacterial dose.

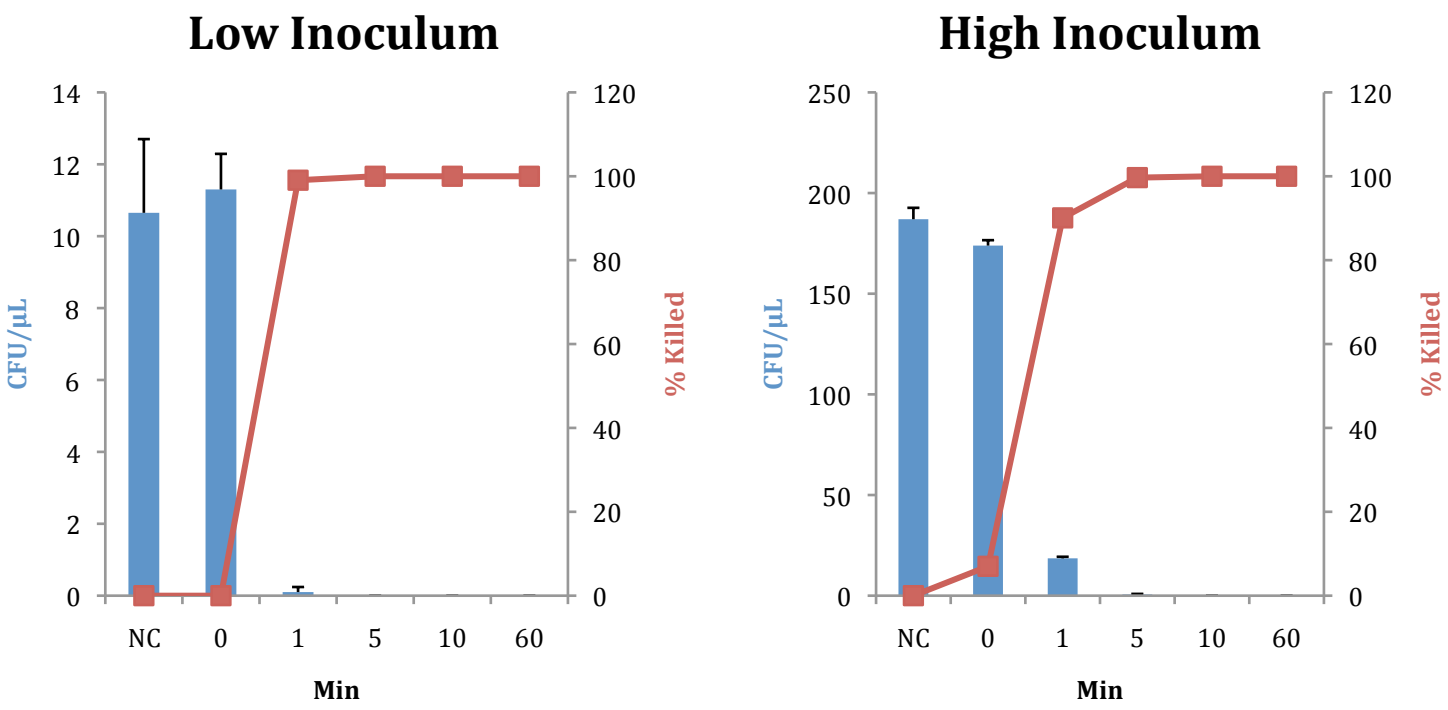
Since the number of viable organisms in dried samples of bacteria in the field are likely to vary considerably from a few bacteria to many thousands, it was important to establish that the efficacy of CLO could not be influenced detrimentally by the presence of large numbers of organisms. The results shown in Fig. 12 show that inoculum size (number of bacteria) is not an important factor.

FIGURE 12: Effect of bacterial inoculum size (*B. subtilis*)

Low inoculum = 1500 CFU/100 µL, high inoculum = 150,000 CFU/100 µL. 1% CLO liquid.

These experiments show that inoculum size is not a significant factor in determining efficacy of bactericidal effect.

As little as one minute of exposure to the oil is effective.



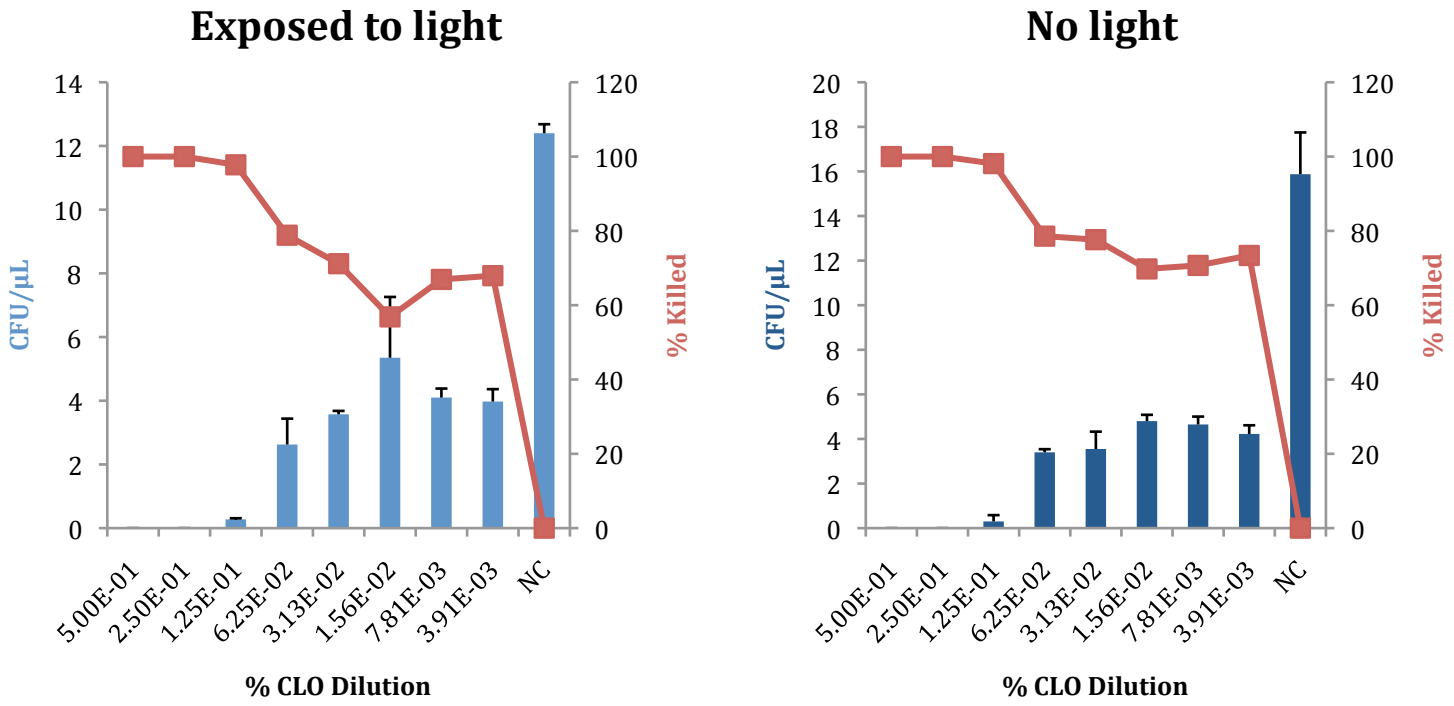
4.1.4. Possible effect of light:

Some plant-based antimicrobials are influenced by ambient light. In theory the antibacterial activity could be enhanced by light (due to the presence of photosensitizers), or inhibited, or reduced as a result of photo-degrdadation. Since the CLO applications could take place in either the presence or absence of ambient light, it was important to establish that the efficacy of the CLO would not affected significantly. Results are shown in Fig. 13.

FIGURE 13: Possible effect of light on bactericidal activity (*B. subtilis*)

These experiments show that light has no significant effect (enhancement or inhibition or photo-degradation) on the activity of CLO.

Dilutions of CLO were two-fold, starting at 0.5%



4.1.5. Bacterial spores:

Some environmental bacteria, including various species of Bacillus (such as *B. subtilis*, and *B. anthracis*, anthrax) produce endospores, which usually survive environmental conditions not conducive to bacterial growth. Under more appropriate conditions, these spores can then germinate to produce the vegetative cells. In general spores are more resistant than vegetative bacteria to antimicrobial treatments, and consequently present a potential hazard in buildings.

Conventional methods of producing spores for study often contain mixtures of spores and vegetative cells. Therefore in order to ensure that prospective antimicrobials are really effective against spores, it is necessary to experiment with spore preparations that are relatively free of dead and vegetative forms. Recently a method of purifying spores was reported for *Clostridium difficile* (an anaerobic intestinal pathogen). This involved centrifugation of partly purified spores through gradients of Histodenz. The spores of *C. difficile* sedimented through 50% Histodenz, leaving them free from the vegetative cells and dead organisms, which were found in the layers of less dense Histodenz.

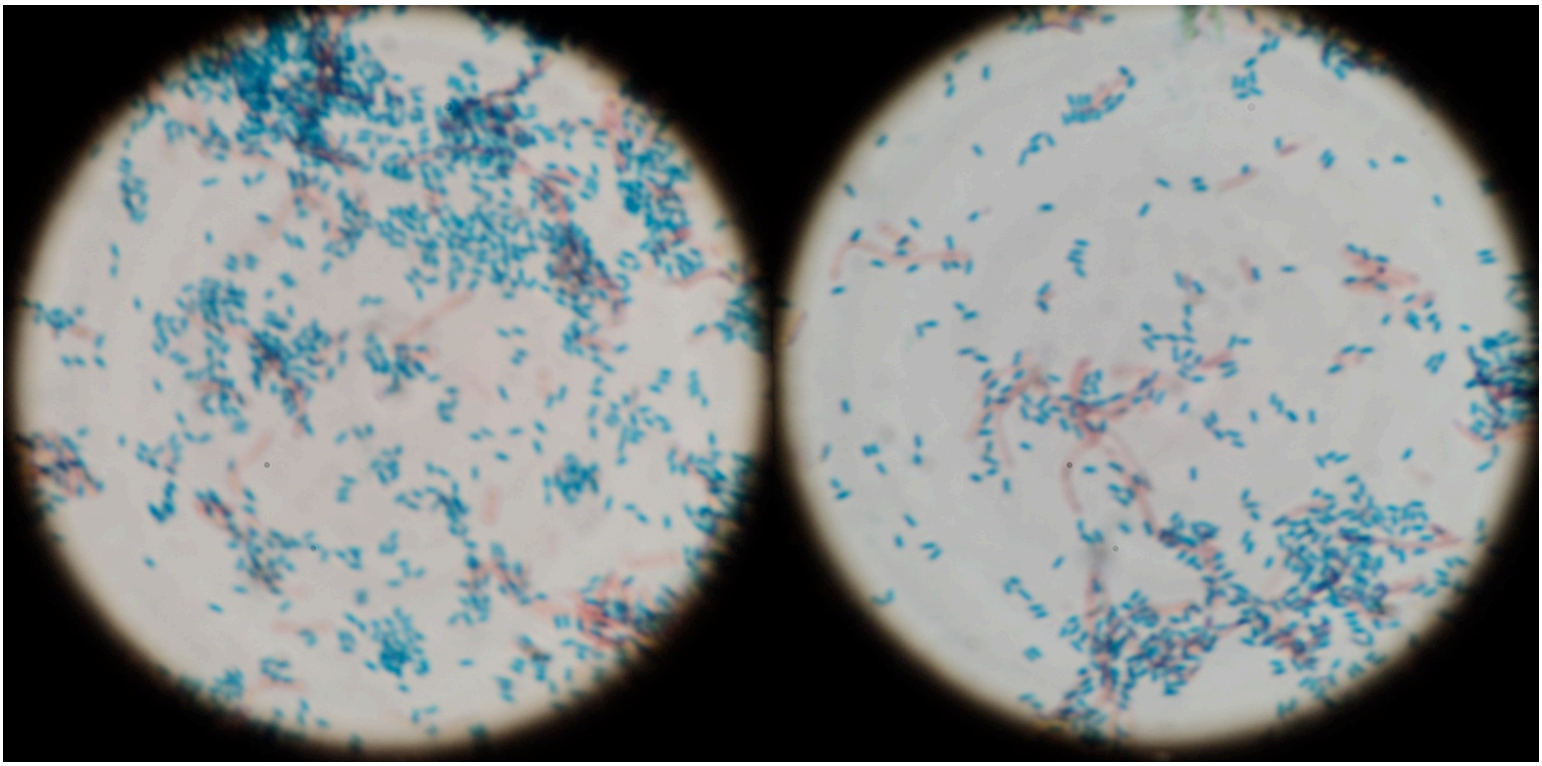
In our experiments with different concentrations of Histodenz, it was shown that purification of *B. subtilis* spores worked best by sedimentation through 45% Histodenz (they did not sediment through 50%). Fig 14 shows typical stained preparations under the microscope, with the standard malachite green-saffranin staining technique.

FIGURE 14: purification of *B. subtilis* spores.

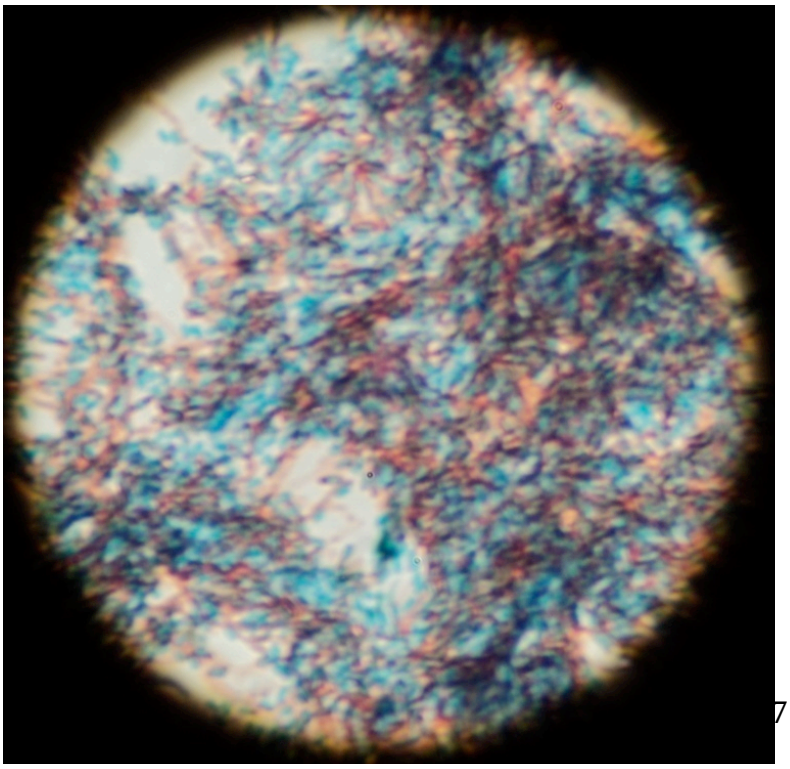
Microscopic examination of stained material following purification on Histodenz.

Spores stain blue/green, vegetative bacteria stain orange. These results show that centrifugation of crude *B. subtilis* spores through gradients of Histodenz produces a preparation highly enriched in viable endospores, with little contamination by vegetative bacteria and dead organisms. Consequently the efficacy of CLO against these spores would not be confused by the presence of contaminating non-spore elements.

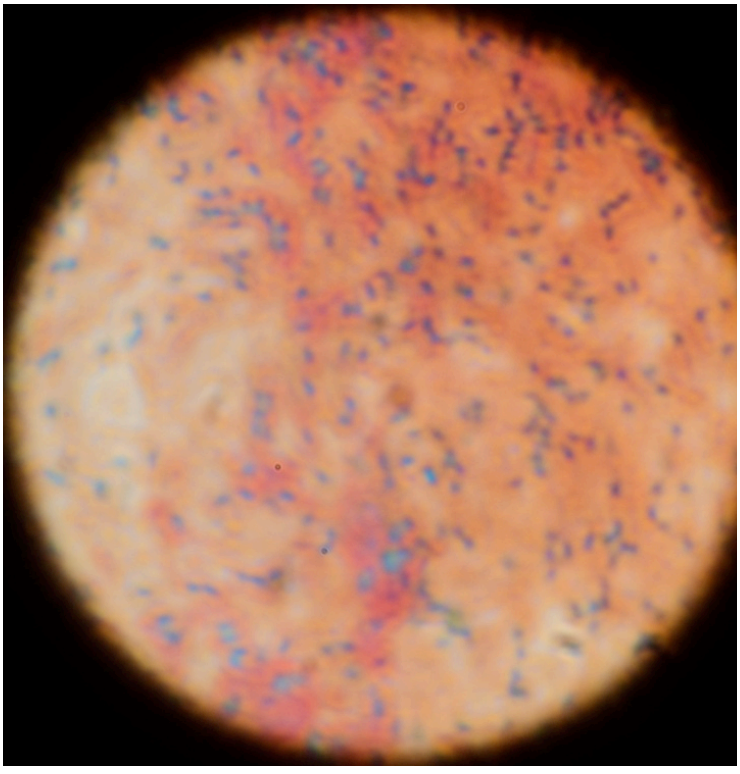
Purified spores from 45% histodenz



Spores from 30% histodenz



unpurified spores



4.1.6. Sporicidal activity of CLO

Preliminary tests on partly purified spores of *B. subtilis* indicated that spores could be killed by contact with CLO, but that they were significantly more resistant than the vegetative bacteria discussed above. The purified spore preparation, shown in Fig. 14, was tested in a time course experiment, and the results are summarised in Table 1. Increasing exposure times and concentrations resulted in greater killing of the spores, although the level of killing achieved was not as great as that observed previously with the vegetative *B. subtilis*.

Further tests need to be carried out with CLO vapor, and spore preparations that have been stored or dried for various times to mimic field situations. There have been reports in the literature indicating that certain oil vapors (eg Eucalyptus oil) are more bactericidal than liquid oil, and sensitivities of spores to antibacterial agents can be influenced significantly by spore age and storage conditions.

Table 1. Sporicidal activity of CLO; % kill (CFU)

Exposure time hours	1% CLO	5% CLO	10% CLO
24	30.5	48.0	63.6
48	41.2	50.0	88.0
72	64.5	70.2	93.4

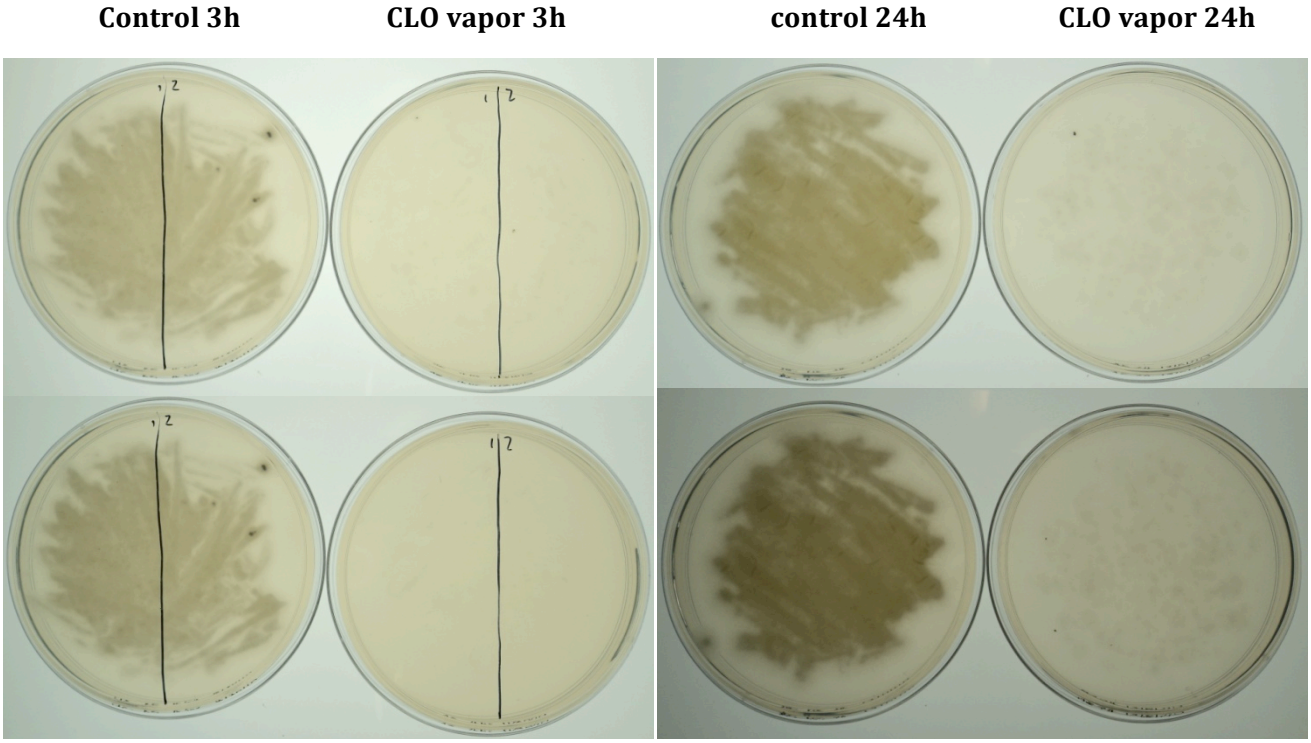
4.2. Effect of CLO-vapor on *Aspergillus niger*

The fungus (mold) *A. niger* is a common environmental organism which can cause serious disease in humans with compromised immune systems. It can be grown and assayed conveniently on appropriate agar plates, although it tends to produce filamentous growth (Fig. 14, below) rather than discrete colonies. Quantitative measurements are therefore performed by serial dilutions and determination of growth end – point. The organism is evidently very susceptible to CLO-V, although at this time we cannot be sure if it is irreversibly killed (fungicidal, analogous to the bacteria) or just growth-inhibited (fungistatic). Nevertheless even if eradication could not be achieved, the prospect of its control by CLO vapor is realistic. Further experimental study is needed.

Other environmental fungi, which grow in a similar manner to *A. niger,* could also be vulnerable.

FIGURE 14: Effect of CLO vapor on the fungus *Aspergillus niger*.

Like most fungi, *A. niger* grows in agar with a filamentous morphology rather than distinct colonies. The results show substantial inhibition of growth of *A. niger* (agar plates on right side of each panel). However some of the treated organisms recovered after subsequent incubation without CLO, indicating either incomplete killing of the fungi or a fungistatic effect (growth inhibition) rather than fungicidal (killing).



4.3. Effect of CLO vapor on *Candida albicans*

Candida species are dimorphic fungi; they can grow in suspension and on surfaces as individual yeast cells, and they can also grow in a filamentous form (like *Aspergillus*). They are often part of the human flora, eg as yeasts in the oral cavity; but they can also colonize tissues in their filamentous form and produce potentially life-threatening diseases, especially in immune-compromised individuals. *C. albicans* is difficult to eradicate in human infections because the filamentous form may be relatively inaccessible and resistant to anti-mycotic drugs.

In the experiments performed, *C. albicans* was exposed to CLO liquid or vapor and assayed on agar plates as CFU (analogous to bacterial CFU). As shown in Tables 2 & 3, *C. albicans* in its yeast form is very sensitive to CLO. Further studies should be performed with the filamentous form, which can be produced in the laboratory under artificial culture conditions.

Table 2. Activity of CLO liquid against *Candida albicans*

This result shows that the pathogenic yeast is readily killed by contact with CLO

% CLO	Average CFU of *C. albicans*	% kill
0	1300	--
0.5	2	~ 99.9
0.75	0	> 99.9
1.0	0	> 99.9

Table 3. Activity of CLO vapor against *Candida albicans*

This result shows that *C albicans* is readily killed by CLO vapor, in a time-dependent manner

Exposure time hours	Average CFU of *C. albicans*	% kill
0	1270	
4	67	95
24	0	> 99.9

4.5. Antiviral Activities:

The methods developed for the antibacterial tests were also used for the evaluation of antiviral efficacy. Viruses were quantified by plaque assay on cultured cells, giving rise to plaque-forming units, PFUs, analogous to bacterial CFUs.

Influenza viruses (strains A/H1N1, the so-called "pandemic strain", and influenza B virus), and herpes viruses (HSV type 1, responsible for recurrent cold sores and mouth ulcers; HSV type 2, responsible for recurring genital sores) were very susceptible to CLO liquid and CLO vapor, within 30 minutes or less of exposure. Further studies should be conducted with additional human pathogenic viruses.

Table 4. Activity of CLO vapor against Influenza virus H1N1 (PFU assay)

This result shows that exposure of infectious virus to vapor for as little as 10 minutes can kill more than 98%. Similar results were obtained for influenza B and herpes simplex viruses (HSV-1 & 2).

Taking into account the dilutions of the untreated virus controls, it was concluded that 30 min exposure to CLO-V could kill more than 10^5 infectious virus particles.

Time of exposure min.	PFU untreated	Average PFU + CLO-V	% kill
10	> 350	4.5	> 98
20	> 350	1.5	> 99.4
30	> 350	0	> 99.8

4.6. Cytotoxicity of CLO vapor.

It is important to establish experimentally that short-term exposure to CLO vapor is harmless to building occupants and operators. This was evaluated by exposing cultures of human lung cells (A549 epithelial cell line), with culture medium removed to permit exposure of the monolayer of cells, to CLO vapor for different times, followed by microscopic examination of the cells for signs of cell toxicity or death, and assays for cell viability (by conventional XTT and LDH methods).

The results showed that exposures up to 30 minutes had no deleterious effects on the cells. However a continuous exposure of one hour at body temperature (36 C, in contrast to the normal ambient temperature of 22 C) did show some signs of toxicity in the cells, probably because at this temperature the evaporation rate of CLO from the liquid was much greater. This was noticed in the form of a marked increase in the aroma from the warm CLO. Further studies are needed to verify the safety under normal conditions of use, which would utilize the CLO at ambient temperature, preferably in the absence of people. A slight lingering of vapor in the treated building after application would not likely pose a danger.

4.7. Further Experiments.

Some additional tests are planned for Jan-Feb 2011, after the compilation of this report. The results will be available subsequently as an appendix.

5. Summary of Methods:

The methods used for the enumeration of viable bacteria, fungi, and viruses are standardized methods developed in our laboratory for the evaluation of plant-based materials with antimicrobial properties. Details can be found in our recent publications.

Bacteria were measured as colony forming units CFU), ie. viable organisms, by performing serial dilutions of the treated bacteria and counting the numbers of growing colonies on appropriate nutritive agar plates. Controls consisted of similar measurements of untreated bacteria. Spores were purified by the novel technique of Histodenz centrifugation described in the Results section.

Fungi were measured either in a manner analogous to bacterial CFU, in the case of *Candida* yeast cells, or as limiting dilution end point assays, also on appropriate nutritive agar plates, for filamentous fungi (*Aspergillus*).

Viruses were measured by standard plaque assays, comprising serially diluted virus preparations, with or without treatment, inoculated into the appropriate cell cultures and overlaid with medium-containing agarose. Virus plaques were subsequently counted and expressed as plaque-forming units (PFU), equivalent to viable/infectious viruses.

All assays were performed in triplicate and the results expressed as means ± standard deviations.

For the assessment of cytotoxicity, cell cultures in multi-well trays, with the media removed, were exposed to CLO vapor for various times and fresh medium added to permit subsequent microscopic observations. In addition the colorimetric cell viability assays (XTT and LDH techniques) were used for confirmation of microscopic examination.

CLO reactions were generally carried out, with the organisms in saline or as dried films, in various types of sterilized glass and plastic (polypropylene) vessels with covers or lids to confine the vapor when required. Eppendorf tubes were convenient reaction vessels, since they could be easily manipulated and centrifuged when required to separate liquid CLO from the organisms following treatment.

All reactions were carried out at ambient temperature, usually 22±1 C.