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# Anti-influenza virus activity of essential oils and vapors

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## Abstract
Few satisfactory therapeutic agents are available for the control of Influenza virus, largely because of the continual emergence of drug-resistant mutants. Some essential oils (EOs) have demonstrated effective antimicrobial and antiviral properties in experimental conditions, but most of these studies tested the liquid oil phases, which are generally less practical and are potentially toxic for oral applications. In the present study, we evaluated several EOs and some of their major constituents for their possible anti-influenza virus properties in both liquid and vapor phases. In vapor phase *Citrus bergamia*, *Eucalyptus globulus*, and the isolated compounds citronellol and eugenol were very active against influenza virus following exposures of only 10 minutes. *Pelargonium graveolens*, *Cinnamomum zeylanicum*, *Cymbopogon flexuosus* were also very active with 30 minutes exposure. In liquid phase, *Cinnamomum zeylanicum, Citrus bergamia, Cymbopogon flexuosus* and *Thymus vulgaris* displayed 100% inhibitory activity at 3.1 µL/mL concentration. Under these conditions the vapors showed no measurable adverse effect on epithelial cell monolayers. This suggests that these oils in their vapor phases could be potentially useful in influenza therapy. The oil vapors were also evaluated for possible direct effects on the principal external proteins of the influenza virus, namely the HA (hemagglutinin) and NA (Neuraminidase). Several of the vapors inhibited the HA activity, but not the NA activity, suggesting that interaction with HA is a possible mechanism for the antiviral activity. Thus some of these oil vapors could have therapeutic benefits for people suffering from influenza, and possibly other membrane containing respiratory viruses.

**Keywords:** Essential oil, Vapor phase, antiviral activity, Hemagglutination (HA) Inhibition

## 1. Introduction
Influenza viruses continue to pose threats of epidemics, resulting from mutated viruses, to which we have inadequate therapeutic remedies, largely because of the continuing emergence of drug-resistance. Thus alternative therapies, targeting the viruses themselves rather than their individual genes, could be useful.

Essential oils (EOs) of plants have been used traditionally for numerous applications in health-related areas, and in foods and commercial uses [1, 2]. In most medical applications the oils were applied directly to the skin, although the potential cytotoxicity of EOs precluded internal consumption [3]. This problem could, at least in theory, be avoided by inhalation of the vapors of EOs, as practiced in aromatherapy. Furthermore in many traditional remedies for colds and respiratory disorders, formulations often included plant EOs to provide relief through inhalation of the vapors [3].

Recently a number of studies reported the presence of antimicrobial and antiviral activities in certain EOs and their components, such as monoterpenes. However, those studies were carried out with the liquid phases of the oils and their components [3, 4, 5, 6, 7, 8, 9, 10, 11].

In contrast, Inouye *et al*. [12] Thyagi and Malik [13], Hudson *et al*. [14] and Vimalanathan and Hudson [15] demonstrated that the vapor or "gaseous" phase of certain EOs showed good antibacterial, antifungal and antiviral activity, sometimes better than the corresponding liquid phase of the oil.

Some studies also indicated that the whole unfractionated oil was as potent as any of the individual components, suggesting synergism [3, 10].These observations clearly indicate that, while there is great potential for the use of EOs as antimicrobials and antivirals, there is still scope for further evaluation of the optimal methods for their applications.

In order to address these issues, we compared the anti-influenza virus activities, and relative cytotoxic potentials, of a number of commercial EOs, and some of their pure compounds in their liquid and vapor phases. In addition we also examined their effects on the influenza neuraminidase and hemagglutinin, the major viral antigens.

## 2. Materials and methods

### 2.1 Test materials

All the essential oils (EOs) were standard commercial brands purchased from local suppliers (Table 1).

**Table 1:** Essential oils used and their suppliers, major constituents and references.

| Scientific name and family | Common name<br>Supplier and origin | Major components (%) | References |
|---|---|---|---|
| *Lavandula officinalis*<br>Lamiaceae | Lavender<br>Fresh flowering heads<br>Julia lawless<br>Aqua Oleum<br>Bulgaria | Linalyl acetate<br>Linalool | [24] |
| *Pelargonium graveolens*<br>Geraniaceae | Geranium<br>Leaves and flowering branchlets<br>Aura Cacia<br>China | Citronellol<br>Geraniol | [25, 26] |
| *Cinnamomum zeylanicum*<br>Lauraceae | Cinnamon leaf oil<br>Leaves Aura Cacia<br>Sri Lanka | Eugenol | [23] |
| *Salvia officinalis*<br>Lamiaceae | Sage<br>Partially dried leaves<br>Aura Cacia<br>Austria, Croatia | 1,8 –Cineole<br>α-Thujone | [7, 22] |
| *Eucalyptus globulus*<br>Myrtaceae | Eucalyptus<br>Leaves and twigs<br>Julia lawless<br>Aqua Oleum<br>South Africa | 1,8-Cineole<br>α-Pinene | [13] |
| *Cymbopogon flexuosus*<br>Poaceae | Lemongrass<br>Freshly cut grass<br>Aura Cacia<br>India | Geranial<br>Neral | [27, 28] |
| *Thymus vulgaris*<br>Lamiaceae | Red Thyme<br>Partially dried above ground plant parts.<br>Aura Cacia<br>Spain | 1,8-cineole<br>Terpenyl acetate<br>Borneol | [21] |
| *Citrus bergamia*<br>Rutaceae | Bergamot<br>Fruit Peel<br>Aura Cacia<br>Italy | (–)-linalyl acetate<br>(–)-linalool<br>(+)-limonene<br>γ-Terpinene,<br>β-Pinene<br>α-pinene<br>α-terpinene | [29, 30] |
| *Cupressus sempervirens*<br>Cupressaceae | Cypress oil<br>Needles & Twigs<br>Aura Cacia<br>Morocco | α-pinene<br>α-terpinene | [7] |

### 2.2 Cells and virus

Madin-Darby canine kidney cells (MDCK) and A549 human lung epithelial cells were acquired originally from ATCC (American Type Culture Collection, Rockville, MD), and were passaged 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 A1/Denver/1/57 (H1N1) was acquired from BC Centre for Disease Control, Vancouver, and was grown in MDCK cells with TPCK (L-1-Tosylamide-2-phenylethyl chloromethyl ketone; Sigma Chemical co.) treated trypsin (2 µg/mL) and assayed by plaque formation. The following pure compounds citronellol and eugenol were kindly supplied to us by Dr. Murray Isman, University of British Columbia. The infectious titer of stock virus varied from $10^5$-$10^6$ PFU/mL.

### 2.3 Virucidal activity (liquid phase)

The assay technique was based on our standard techniques for the evaluation of plant extracts for antiviral activity [16, 17]. The experimental procedure consisted of incubating two-fold dilutions of the test oil or compound in phosphate buffered saline, in 96-well trays, with 20 µL of virus containing 800 plaque forming unit of virus. The mixtures, in triplicate, were incubated for 60 min at 22 ºC. The total volume of 120 µL from each mixture was then transferred into confluent MDCK cells containing 1mL PBS, and incubated at 37 ºC to allow adsorption of remaining virus. After 60 min the inocula were removed and replaced with 0.5% agarose in MEM and 2 µg/mL trypsin. Monolayers were fixed with 3.6% formalin after 48 h and stained with 0.1% crystal violet. Virus plaques were counted. Inhibitory concentration was calculated as $MIC_{100}$ by comparison with untreated virus controls.

### 2.4 Virucidal activity (vapor phase)

The method used was a modification of the standard plaque reduction assay described above. Aliquots (20 µL) of virus (10000 pfu) were individually dried on the underside of the caps from sterile Eppendorf tubes, within the biosafety cabinet (10 min). Test oils (250 µL) were carefully added to each tube, the caps were replaced with caps containing dried virus film and exposure to oil allowed for 10 or 30 min, at 37 ºC. Caps were removed again and each dried exposed film was reconstituted in 1 mL of PBS. All samples (in triplicate) were then assayed for virus plaque formation in MDCK cells as described above. Canola oil, which does not inhibit influenza virus, was used as a negative control. The reduction of viral titer was quantified and viral infectivity loss due to drying was determined to be $\leq$ 1.0 $\log_{10}$. Starting from a titer of 10,000 PFUs, the virus titer was reduced to ± 1,000 PFUs after drying, before Eos vapor exposure.

### 2.5 Neuraminidase Assay (liquid phase)

The Amplex Red Neuraminidase (Sialidase) Assay Kit from Invitrogen (Ontario) was used. Briefly, equal volumes (25 µL) of two fold dilutions of EOs and virus (1:4 dilution of stock virus) were mixed and incubated for at 37 °C with continuous shaking. After 60 min, 50 µL of 2X working solution of 100 µM Amplex Red reagent containing 0.2 U/mL HRP, 4 U/mL galactose oxidase and 500 µg/mL fetuin was added and the mixtures incubated overnight. Absorbance (A) was measured at 550 nm in a plate reader. The percentage of Neuraminidase inhibition was calculated by the following formula: $A_{virus}$-$A_{test}/A_{virus}\times100$.

### 2.6 Hemagglutination (HA) Inhibition Assay (Liquid and Vapor phases)

Since all the liquid EOs were toxic to erythrocytes on direct exposure, HA inhibition was measured only in dried films of virus exposed to the vapor phases, as described above for antiviral activity of EO vapors in Eppendorf tubes. Reconstituted exposed virus (50 µL) were mixed with 50 µL of 0.75% suspension of human type O Rh+ erythrocytes and incubated at 22 ºC for 60 min [18]. The hemagglutination reaction was observed after 60 min incubation.

### 2.7 Cytotoxicity assay (Liquid and Vapor phases)

The Cell Proliferation Assay Kit (XTT) (ATCC, Manassas, VA) was used according to the manufacturer's instructions. Human lung epithelial cells (A549 cell line) were used as the indicator cells. The cells ($5\times 10^3$) were seeded in each well containing 100 µL of the MEM medium supplemented with 5% FBS in a 96-well plate. Cells were grown for 48 h and the test materials, prepared as a series of two-fold dilutions in MEM, without phenol red, were added to the cells and incubated for 15 min, followed by removal of test material and incubation for a further 24 h in MEM, followed by measurement of cell viability. For cytotoxicity of EOs vapor, monolayers of human lung A549 cells grown to confluence in 6-well trays for 48 hour. For EOs exposure, the media were removed by aspiration, and the moist cells were exposed to EOs vapor. Following further 24 h incubation in normal medium, cell viability was measured. The results were measured as absorbance at 490 nm in a plate reader, in comparison with similar cells exposed to medium only. Cytotoxicity is expressed as the concentration of test sample inhibiting cell growth by 50% ($TC_{50}$). All tests were run in triplicate and mean values recorded.

## 3. Results

### 3.1 Antiviral Activities of Liquid Phase EOs

Three of the oils, *Cinnamomum zeylanicum*, *Citrus bergamia* and *Thymus vulgaris* (Figure 1, 2), were able to completely inactivate ($IC_{100}$) the virus at high dilutions, down to <3.1 µL per mL or less (Figure 3). *Lavandula officinalis* and *Eucalyptus globulus* also showed excellent activity at higher concentrations, but were much less effective at the lower concentrations. *Salvia* was only partly active at the concentrations tested. In similar experiments liquid phase *Pelargonium graveolens* (Geranium) oil also showed good antiviral activity. *Cupressus sempervirens* and its main constituent α-pinene did not show antiviral activity even at very low dilutions. Among the pure components tested, only eugenol showed 100% plaque reduction (Figure 3) but citronellol displayed partial inactivation. Liquid canola oil, used as a negative control, had no activity even at very high concentration (100%)

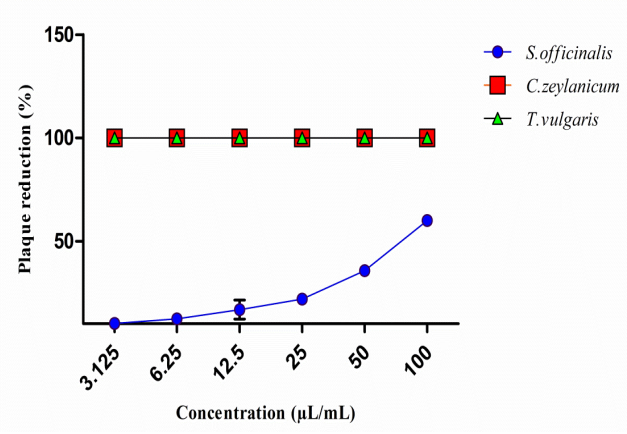


**Fig 1:** Antiviral Activities of serial dilutions of EOs. Each sample, in triplicate, was serially diluted 2x and incubated with a standard amount of H1N1 virus (800 pfu per reaction). Remaining infectious viruses were measured by plaque assay on MDCK cells.

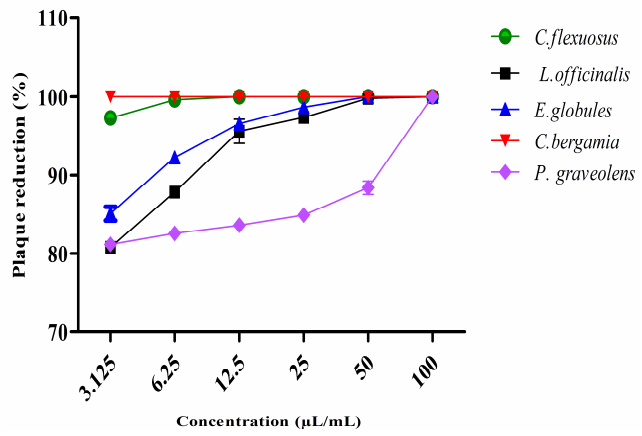


**Fig 2:** Antiviral Activities of serial dilutions of EOs. Each sample, in triplicate, was serially diluted 2x and incubated with a standard amount of H1N1 virus (800 pfu per reaction). Remaining infectious viruses were measured by plaque assay on MDCK cells.

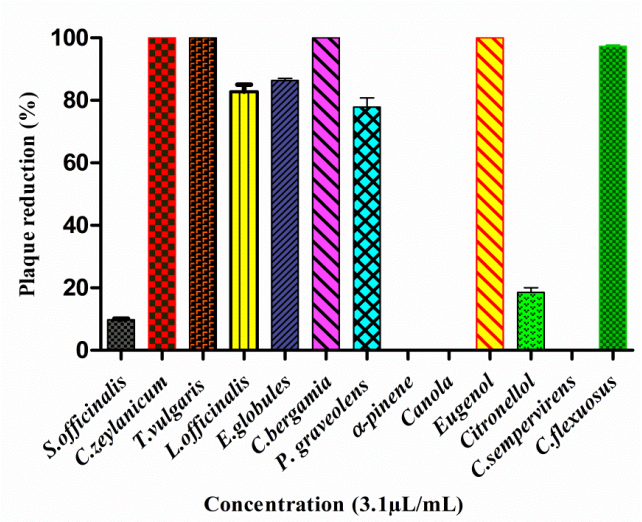


**Fig 3:** Antiviral Activities of EOs and selected monoterpenes against H1N1 virus with 3.1 µL/mL. Each sample, in triplicate, was diluted to 3.1 µL/mL and incubated with a standard amount of H1N1 virus (800 pfu per reaction). Remaining infectious viruses were measured by plaque assay on MDCK cells. Results are expressed as percentage of plaque reduction.

### 3.2 Antiviral Activities of EO Vapor Phases

Two EOs, *C. bergamia, E. globulus*, and the tested pure compounds citronellol and eugenol showed significant activity against influenza virus following exposures of only 10 minutes.

Several of the oil vapor phases were able to completely inactivate influenza virus following exposures of 30 minutes, as shown in Figure 4. These were, *C. zeylanicum, C. flexuosus, L. officinalis* and *P. graveolens*. *T. vulgaris* and *S. officinalis* showed only partial activity, and the two negative controls, olive oil and canola oil, showed no antiviral activity. Thus the relative activities did not reflect the corresponding activities of the liquid phases.

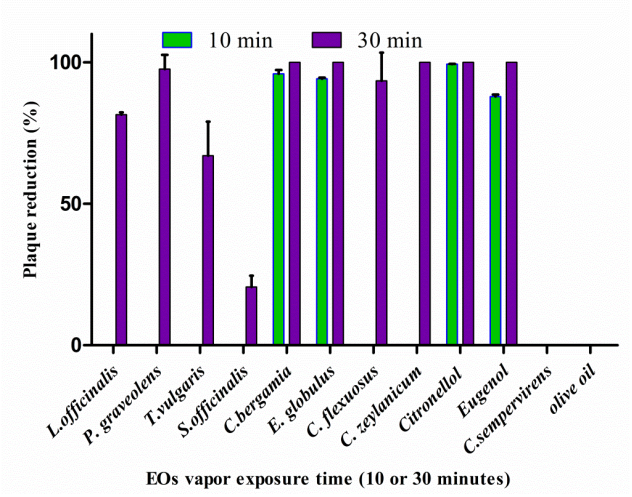


**Fig 4:** Antiviral Activities of EOs in vapor phase. Aliquots of influenza virus, in triplicate dried films of H1N1 virus (1000 pfu of H1N1 per reaction) were exposed to EO vapors for 10 and 30 minutes, reconstituted in PBS, and remaining infectious viruses measured by plaque assays on MDCK cells.

### 3.3 Activities Against Viral HA (Hemagglutinin) and NA (Neuraminidase)

HA and NA are the two most important proteins of the influenza virus which together determine successful infection and dissemination of the virus.

Most of the oil vapors tested showed anti-hemagglutination activity against the indicator human erythrocytes type O Rh+, as shown in Table 2 and Figure 5. Since most of the tested Eos in liquid phase had hemolytic effect, that disabled further testing of HAI assay with liquid oils.

In contrast to the anti-HA activities, most of the oils, even in liquid phase, were not able to inhibit NA activity, as shown in Figure 6. The exception was *Cinnamomum zeylanicum*, which showed complete inhibition at least down to 1.5 µL/mL. The positive control anti-influenza compound zanamivir was effective as expected.

Human lung epithelial cells (A549) were exposed to EOs for 15 min, followed by incubation in normal medium for 24 h. They were then assayed for cell viability. Results are shown in Table 3. All the EOs showed some toxicity at the high concentrations but were non-toxic at concentrations less than 10 µL/mL. The 24 hour exposures showed greater degrees of cytotoxicity, except for olive oil, which remained non-cytotoxic.

In contrast to the results with the liquid phases, when cell monolayers were exposed to each of the oil vapors for 10 or 30 min there were no adverse effects on cell appearance or viability, as determined by XTT measurements.

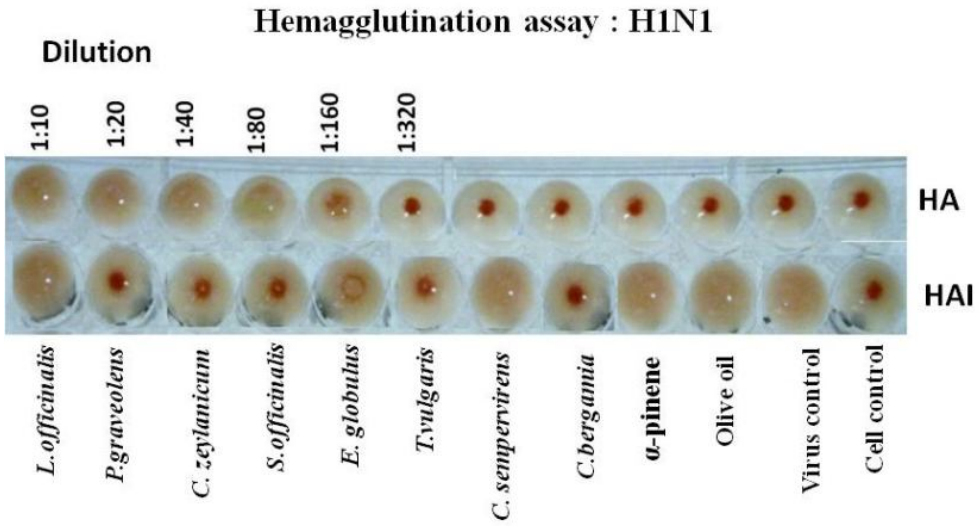


**Fig 5:** Viral HAI Assay plate. Inhibitory activity of EOs' vapor on agglutination with viral hemagglutinin and human type O Rh+ erythrocytes, as described in Methods (and in WHO Manual, 2011). The presence of conspicuous red buttons in the well indicates absence of agglutination, i.e. inhibition of HA activity. The other wells show normal hemagglutination.

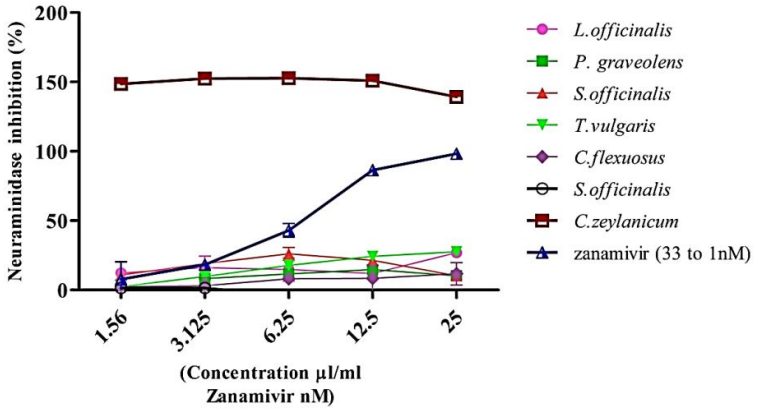


**Fig 6:** Effect EOs and zanamivir on the influenza virus Neuraminidase activity. Only the Cinnamon and the positive control zanamivir were active.

**Table 2:** Inhibitory activity of EO vapors on agglutination with viral hemagglutinin and human RBC O Rh+.

| EOs vapor | Hemagglutination inhibition activity (HAI) |
|---|---|
| *C. bergamia* | + |
| *C. flexuosus* | + |
| *C. sempervirens* | - |
| *C. zeylanicum* | + |
| *E. globulus* | + |
| *L. officinalis* | - |
| *P. graveolens* | + |
| *S. officinalis* | + |
| *T. vulgaris* | + |
| Olive oil | - |
| Virus control | - |
| Cell control | + |

**Table 3:** TC50 values of EOs in human lung epithelial cells.TC50 = 50% tissue culture cytotoxicity, or concentration giving 50% reduction in cell viability

| Eos liquid | $TC_{50}$ Viability (µL/mL) |
|---|---|
| *Citrus bergamia* | 15.98±6.92 |
| *Cymbopogon flexuosus* | 12.21±2.64 |
| *Cupressus sempervirens* | 75±7.98 |
| *Cinnamomum zeylanicum* | 12.3± 2.9 |
| *Eucalyptus globulus* | 26.51± 4.9 |
| *Lavandula officinalis* | 26.46± 3.9 |
| *Pelargonium graveolens* | 67.46± 6.85 |
| *Salvia officinalis* | 26.85± 7.2 |
| *Thymus vulgaris* | 14.34± 3.32 |
| Olive oil | >100±0 |
| Cell control | 100 |
| All EO vapors | No cytotoxicity |

## 4. Discussion

Respiratory viruses continue to cause problems within the general population, as a result of frequent acute and chronic infections, including occasional epidemics. Few satisfactory therapeutic agents are available, in part because of the diversity of replication schemes among these viruses, and consequently the lack of a generic molecular target; and partly because of the continual emergence of drug-resistant mutants in the viral populations. These problems are well illustrated by influenza viruses.

Some essential oils have demonstrated effective antimicrobial and antiviral properties, and in a few cases beneficial anti-inflammatory properties [4, 8, 19, 6, 3, 20]. However, these studies tested the liquid oil phases, which are generally less practical and potentially toxic for nasopharyngeal or oral applications. A few reports have indicated that the vapors of some oils might be useful for this purpose [3, 13, 14], and this type of application would be in accord with anecdotal reports of the usefulness of inhaled vapors [3, 14].

In the present study we evaluated several essential oils (EOs) for their possible anti-influenza virus properties under conditions that are relevant to potential applications. Four of the EOs, *Cinnamomum zeylanicum*, *Citrus bergamia* and *Thymus vulgaris* were very active against influenza virus at relatively low concentrations ($MIC_{100}$ 3.1 µL/mL) in the liquid phase, but only *Citrus bergamia* showed prominent activity (95% ± 2) in its vapor phase (10 min exposure), the rest showed activity only after 30 min exposure. On the other hand, *Eucalyptus globulus* had less activity ($MIC_{100}$ 50 µL/mL) in liquid phase but showed prominent activity in 10 min vapor phase (94% ± 3). Interestingly, although 1,8-cineole is the major component [13, 21, 7, 22] of *Eucalyptus globulus*, *Thymus vulgaris* and *Salvia officinalis*, only *Eucalyptus globulus* and *Thymus vulgaris* exhibited antiviral activity, suggesting that 1,8- cineole might not be responsible for the antiviral property of *Eucalyptus globulus* and *Thymus vulgaris*.

Under these conditions the vapor showed no measurable adverse effect on epithelial cell monolayers. This suggests that these oils in their vapor phases could be potentially useful in influenza therapy. In addition *Cinnamomum zeylanicum* and *Thymus vulgaris* were also very active in the liquid phase, although they were only partially effective in the vapor phase. However eugenol, the major component [23] of *Cinnamomum zeylanicum* possessed the most potent anti-influenza activity in both liquid and vapor phases. This suggests that eugenol might be one of the major components responsible for antiviral property of *Cinnamomum zeylanicum*.

Conceivably a longer exposure to these oil vapors could result in complete virus inactivation. In contrast *Salvia officinalis* displayed only slight antiviral activity, only 20% plaque reduction following 30 minutes exposure. *Cupressus sempervirens* oil did not show any activity, this may be correlated to the inactivity of the main component α-pinene in both phases. Liquid canola oil, included as a negative control [19] showed no activity.

The oils were also evaluated for possible direct effects on the principal external proteins of the influenza virus, namely the membrane proteins HA (hemagglutinin) and NA (Neuraminidase). It was not possible to test the liquid oils against HA because all of them lysed the indicator erythrocytes. However, we were able to evaluate the vapors, which did not affect the integrity of the test erythrocytes, and in this situation most of the oil vapors inhibited viral HA activity, with the exception of *Lavandula officinalis*.

In contrast none of the liquid oils, except Cinnamon, were able inhibit NA activity. This suggests that a primary target for most of the oils is the viral HA, and this activity was demonstrated with the EO vapors. However Cinnamon may be able to target both external proteins. Since the HA and NA proteins of influenza virus are responsible for virus entry and exit into and from cells respectively, then inhibition of either of these viral functions would decrease the growth and dissemination of the virus.

Most of the liquid phase oils showed cytotoxic effects in human lung epithelial cells, but in contrast the vapor phases did not appear to show adverse effects following exposures of at least 10 minutes.

## 5. Conclusion

Several of the essential oil vapors evaluated possess potent anti-influenza virus activity, under conditions that did not adversely affect cultured epithelial cells. The hemagglutinin protein of the virus appeared to be a major target. Thus some of these oil vapors could have therapeutic benefits for people suffering from influenza, and possibly other respiratory viruses.

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