

Virucidal activity of Colombian *Lippia* essential oils on dengue virus replication in vitro

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The inhibitory effect of Lippia alba and Lippia citriodora essential oils on dengue virus serotypes replication in vitro was investigated. The cytotoxicity (CC₅₀) was evaluated by the MTT assay and the mode of viral inhibitory effect was investigated with a plaque reduction assay. The virus was treated with the essential oil for 2 h at 37°C before cell adsorption and experiments were conducted to evaluate inhibition of untreated-virus replication in the presence of oil. Antiviral activity was defined as the concentration of essential oil that caused 50% reduction of the virus plaque number (IC₅₀). L. alba oil resulted in less cytotoxicity than L. citriodora oil (CC₅₀: 139.5 vs. 57.6 µg/mL). Virus plaque reduction for all four dengue serotypes was observed by treatment of the virus before adsorption on cell. The IC₅₀ values for L. alba oil were between 0.4-32.6 µg/mL and between 1.9-33.7 µg/mL for L. citriodora oil. No viral inhibitory effect was observed by addition of the essential oil after virus adsorption. The inhibitory effect of the essential oil seems to cause direct virus inactivation before adsorption on host cell.

Key words: essential oil - antiviral - virucidal activity - dengue virus - *Lippia* sp.

Dengue is an acute illness characterised by the hemorrhagic fever caused by dengue virus (DENV) that is transmitted to humans by *Aedes* mosquitoes. DENV infection results in a spectrum of disease ranging from a mild febrile illness or dengue fever to a severe disease or dengue hemorrhagic fever/dengue shock syndrome. Among the human arboviral diseases, dengue is responsible for causing most illness and death. It has been estimated that 50-100 million dengue infections occur each year in tropical urban areas around the world with 20,000-25,000 deaths (Gubler 2006).

DENV is a member of the Flaviviridae family and it exists as four related serotypes (DENV-1, 2, 3 and 4). The virus possesses an icosahedral nucleocapsid core surrounded by a host-derived lipid membrane (envelope), in which the envelope (E) protein and membrane (M) protein are embedded. The Flavivirus enters into the host cell by receptor-mediated endocytosis into coated vesicles, a process in which the E protein plays the major role. The E protein mediates interactions within the virus and the components of the host cell membrane involved in virus adsorption and the E protein interacts with the membrane cell during fusion (Lindenbach & Rice 2007). Compounds that interact with sites on the E protein that binds to structures on the cellular membrane affect the adsorption and fusion process. That interferes

with virus penetration into the cellular cytoplasm and consequently its subsequent replication (Leysen et al. 2000, Patkar & Kuhn 2006).

There is no antiviral drug for treatment for any of the Flavivirus and an effective vaccine for human use is not yet available to prevent dengue. Consequently, the development of antiviral drugs licensed for treatment of patients remains an urgent need to prevent dengue fatalities. Compounds obtained from traditional medicinal plants and herbs species have been reported to have antiviral activity and a wide variety of active phytochemicals have been identified (Jassim & Naji 2003). There is evidence that shows that essential oils from various herb species can directly inactivate the virus. Inhibition of enveloped virus subsequent replication in vitro (herpes simplex virus, hepatitis B virus and human immunodeficiency virus) is observed by incubation of the virus with essential oil before cell adsorption (Yamasaki et al. 1998, Zhang et al. 1998, Saddi et al. 2007).

To our knowledge, relatively little is known about antiviral activity of compounds obtained from plants on flavivirus. Recently, we reported the inhibitory effect of essential oils obtained from Colombian plants on yellow fever virus replication in vitro. Incubation of virus with *Lippia alba*, *Lippia organoides*, *Oreganium vulgare* and *Artemisia vulgaris* essential oils before cell adsorption inhibited more than 50% the subsequent extracellular virus progeny at concentrations between 3.7-11.1 µg/mL (Meneses et al. 2009).

The objective of this study was to evaluate the inhibitory effect of essential oils obtained from *L. alba* and *Lippia citriodora* grown in Colombia on DENV replication in vitro. Plants are widely used to alleviate almost any digestive, liver and respiratory diseases. It is suggested that some of the medicinal properties could be related to essential oil components (Pascual et al. 2001).

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SUBJECTS, MATERIALS AND METHODS

Plants - Propagation cuttings were obtained from *L. alba* and *L. citriodora* growing wild in Cundinamarca and Antioquia regions, Colombia. The cuttings were used to establish experimental contiguous plots at the Centro Nacional de Investigaciones para la Agroindustrialización de Especies Vegetales Aromáticas y Medicinales Tropicales Agroindustrial - Pilot Complex, located at the main campus of Universidad Industrial de Santander (Bucaramanga). Voucher specimens from each plant were deposited at the Colombian National Herbarium and the taxonomic identifications were performed by Dr. José Luis Fernández (National Herbarium, UN, Bogotá, Colombia).

Essential oil - The air-dried plant material was submitted to the microwave-assisted hydrodistillation in a Clevenger-type apparatus, as described elsewhere (Stashenko et al. 2004). To determine the identity of the essential oils tested, their chemical composition was analysed qualitatively and quantitatively using chromatographic (retention times, retention indices and standards) and spectroscopic (spectral interpretation, comparison with databases and standards) criteria (Stashenko et al. 2004, 2008). Two GC-MS systems were employed: an Agilent Technologies 6890 Plus gas chromatograph (Palo Alto, CA, USA) equipped with an Agilent Technologies 5973N mass selective detector and an Agilent Technologies 6890 gas chromatograph coupled to an Agilent Technologies 5975 mass selective detector. Both systems were equipped with a split/splitless injector (split ratio 1:50), a 7863 automatic injector and an MS-ChemStation G1701-DA data system that included the WILEY 138K, NIST 2002 and QUADLIB 2004 spectral libraries. A fused silica capillary column DB-5MS (J&W Scientific, Folsom, CA, USA) of 60 m x 0.25 mm ID x 0.25 μ m, d_f and a fused silica DB-WAX (J&W Scientific, Folsom, CA, USA) 60 m x 0.25 mm, ID x 0.25 μ m, d_f column were employed. The oven temperature was initially programmed from 45°C (5 min) to 150°C (2 min) at 4°C min⁻¹, followed by 250°C (5 min) at 5°C min⁻¹ and finally to 275°C (15 min) at 10°C min⁻¹. The ionisation chamber and transfer line temperatures were kept at 230°C and 285°C, respectively. Plant local names, voucher numbers and the main essential oil components are shown in Table I. For the antiviral assays, the essential oil was dissolved in a solution of 1% dimethyl sulfoxide in test medium.

Cell culture and virus - African green monkey kidney cells (Vero) were maintained in minimum essential medium (M-199) supplemented with 10% foetal calf serum (FCS) and 0.07% NaHCO₃ at 37°C in a humidified 5%-CO₂ atmosphere. DENV-1, DENV-2, DENV-3 and DENV-4 isolated from Colombian patients suffering dengue were included. The viruses were selected from the Arbovirus Laboratory collection at Laboratorio de Arbovirus, Centro de Investigaciones en Enfermedades Tropicales, Industrial University of Santander. YFV 17 DD was included in the study as positive control. Vero cell cultures were infected with the virus at one multiplicity of infection. Four to five days later, the viral supernatant consisting of culture medium was harvested and clarified by centrifugation at 400 g at 4°C. Virus

titer, expressed as plaque-formation unit per millilitre (PFU/mL), was determined by standard plaque assay on Vero cells grown in 24-well plates, as previously described (Meneses et al. 2009). DENV stocks were stored in suitable aliquots at -80°C until used.

Cytotoxicity assay - For cytotoxicity of the essential oils tested, the MTT assay was used following the protocol described in a previous study (Meneses et al. 2009). Briefly, Vero cells were seeded into 96-well plates at 1 x 10³ cells per well and incubated for 24 h at 37°C. The medium was removed and the M-199 medium containing the essential oil was added for 72 h at 37°C at concentrations of 900, 300, 100 and 3.7 μ g/mL. Subsequently, the culture medium was removed and the MTT solution (10 μ L, 5 mg/mL, Sigma Co) was added to each well. Cytotoxicity effect of the essential oil was defined as the 50% reduction of cell viability of treated culture cells with respect to untreated culture (CC₅₀). The CC₅₀ values were calculated using the xl-fit for Windows software, version 4.0. Each essential oil was tested in triplicate in two independent experiments.

Mode of antiviral activity - To determine the inhibitory effect of the selected essential oils on DENV replication in vitro, a plaque reduction assay was used with monolayer cultures of Vero cells grown in 24-well plates. A modified protocol described by Saddi et al. (2007) was followed.

The virus (80-100 PFU of DENV-1, 2, 3 or 4) was incubated before adsorption with varying concentrations of the essential oil (0.02, 0.1, 2.5, 12.5, 62.5 and 312.5 μ g/mL) for 2 h at 37°C in M-199 with 2% FCS. Vero cells were infected in duplicate with treated virus. After a 1 h period at 37°C, the cells were washed twice, followed by the incubation at 37°C in M-199 medium containing 5% FCS and 3% carboxymethyl-cellulose (0.5 mL/well plate). After six days of incubation at 37°C in a humidified 5%-CO₂ atmosphere, medium was removed and cell monolayers were fixed with formaldehyde (10%) for 1 h at RT, followed by staining (15-30 s) with a 1% crystal violet solution. The stain was removed and the plaques were counted. Two independent experiments were performed for each viral serotype and for each essential oil.

Experiments were also performed to determine the viral inhibitory effect of the selected essential oils after adsorption during the intracellular replication period. Vero cells grown in 24-well plates were infected in duplicate with 50-60 PFU of DENV (1, 2, 3 or 4). After 1 h adsorption at 37°C plates were washed and medium was replaced with MEM, 5% FCS, 3% carboxymethyl-cellulose containing test essential oil concentrations described above (0.5 mL/well plate). After six days of incubation, monolayers of cells were stained and the plaques were counted. Two independent experiments were performed for each viral serotype and for each of the test essential oil.

Infected cells with untreated virus were used as control in all experiments. Antiviral effect of the essential oil was defined as the 50% reduction of the plaque numbers formed by treated virus relative to the untreated virus (IC₅₀). The IC₅₀ values were calculated by regression analysis of the dose-response curves generated from the data.

TABLE I
Plants used in the study

Species	Local name	Voucher (COL)	Main essential oil components %
<i>Lippia alba</i>	<i>Pronto alivio</i>	480750	Carvone: 39.7 Limonene: 30.6 Bicyclosquiphellandrene: 8.9 Piperitenone: 4.5 Piperitone: 2.8 β -bourboneno: 1.7
<i>Lippia citriodora</i>	<i>Verbena olorosa</i>	480749	Geranial: 18.9 Neral: 15.6 Limonene: 10.7 1,8-Cineole: 5.0 Spathulenol: 4.7 Geraniol: 2.7 <i>trans</i> - β -cariofilene: 2.3 Nerol: 2.0 Geranyl acetate: 1.3

COL: Colombian National Herbarium.

RESULTS

The effect of the essential oils on the proliferation and viability of the mammalian cells was tested prior to the determination of their viral inhibitory effect, using the MTT reduction assay on Vero cell monolayers. The CC_{50} for *L. alba* oil was 139.5 μ g/mL and for *L. citriodora* oil it was 57.6 μ g/mL.

Antiviral activities of the selected essential oils were examined by using the plaque reduction assay. Relevant and selectivity activity was defined adopting standard criteria (Cos et al. 2006). Specifically, we have used the following criteria: CC_{50} value ≥ 100 μ g/mL, IC_{50} value ≤ 50 μ g/mL and selectivity index or ratio $CC_{50}/IC_{50} \geq 2.0$. The abilities of the essential oils to directly inactivate virus (virucidal action) were evaluated. Virus incubation with oil for 2 h at 37°C before adsorption on Vero cell inhibited the subsequent viral replication. The dose-response curves shown in Figs 1 and 2 demonstrate a dose-dependent virucidal action of the tested essential oils. A 50% inhibition of plaque formation was observed against all examined viruses.

Virucidal action, as measured by the IC_{50} and the selectivity index varied depending on the virus serotype and test essential oil used (Table II). For *L. alba*, IC_{50} values were between 0.4-33 μ g/mL with selectivity indices ranging from 4-349. According to the IC_{50} values, this essential oil was more active against DENV-2 than other serotypes, in particular DENV-3 ($p < 0.001$, ANOVA test). For *L. citriodora*, the IC_{50} values were between 1.9-33.7 μ g/mL with selectivity indices ranging from 2-30. The virucidal action against DENV-1, 2 and 3 of the *L. citriodora* essential oil was not different but it was lower against DENV-4 ($p < 0.001$, ANOVA test). As expected,

the examined essential oils were active against YFV ($IC_{50} < 6$ μ g/mL; selectivity index: 28).

No viral inhibition was observed by plaque reduction assay when the cells were infected with untreated DENV-1, 2, 3 or 4 and then incubated for virus replication in M-199 medium containing various concentrations of the *L. alba* essential oil. A dose-dependent antiviral action of the test essential oil was not observed. The IC_{50}

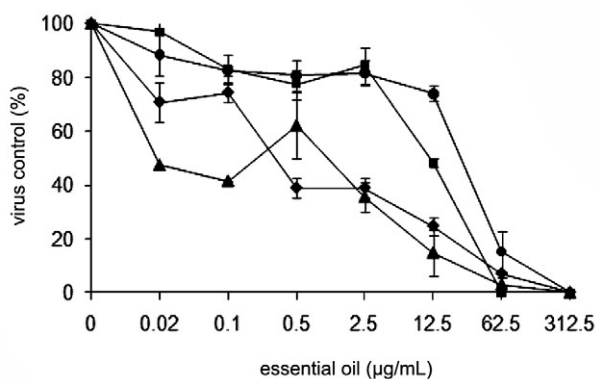


Fig. 1: virucidal activity of *Lippia* species essential oils from Colombia on dengue virus replication in vitro. Effect of *Lippia alba* essential oil on plaque formation by dengue virus (DENV)-1 (■), DENV-2 (◆), DENV-3 (▲) and DENV-4 (●) before adsorption on cell. Virus (80-100 plaque-formation unit) was pre-incubated for 2 h at 37°C in the presence of essential oil at varied concentrations and then adsorbed on Vero cells. Reduction of the viral plaque number was determined six days after cellular infection. The data represent the means for two replicate samples of two independent experiments.

TABLE II
Virucidal activity of the essential oil obtained from Colombian plants determined by plaque reduction assay

Virus	<i>Lippia alba</i> CC ₅₀ : 139.5 µg/mL		<i>Lippia citriodora</i> CC ₅₀ : 57.6 µg/mL	
	IC ₅₀ (µg/mL)	SI	IC ₅₀ (µg/mL)	SI
DENV-1	10.1 ± 1.5	14	1.9 ± 0.9	30
DENV-2	0.4 ± 0.2	349	2.9 ± 0.8	20
DENV-3	32.6 ± 4.3	4	2.6 ± 0.1	22
DENV-4	21.1 ± 3.2	7	33.7 ± 3.3	2
YFV 17 DD	4.9 ± 1.7	28	5.7 ± 0.8	10

virus was incubated for 2 h at 37°C with concentrations of essential oil and then was adsorbed on Vero cells. SI: selectivity index (CC₅₀/IC₅₀).

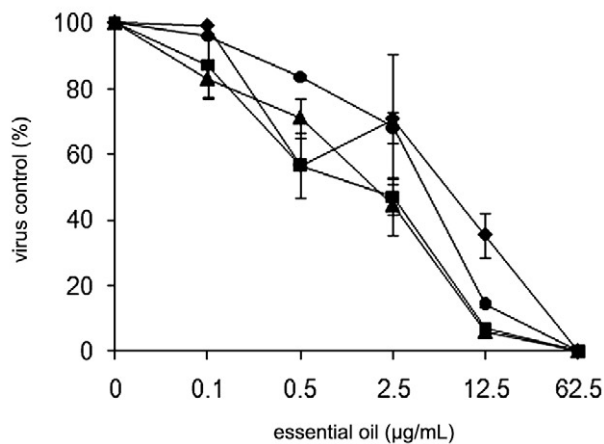


Fig. 2: virucidal activity of *Lippia* species essential oils from Colombia on dengue virus replication in vitro. Effect of *Lippia citriodora* essential oil on plaque formation by dengue virus (DENV)-1 (■), DENV-2 (◆), DENV-3 (▲) and DENV-4 (●) before adsorption on cell. Virus (80-100 plaque-formation unit) was pre-incubated for 2 h at 37°C in the presence of essential oil at varied concentrations and then adsorbed on Vero cells. Reduction of the viral plaque number was determined six days after cellular infection. The data represent the means for two replicate samples of two independent experiments.

values were estimated to be more than 312.5 µg/mL for all DENV serotypes (Fig. 3), with the selectivity indices less than 1.7. The results found with *L. alba* oil were also observed with the *L. citriodora* essential oil. At the maximum oil concentration (62.5 µg/mL) used in the antiviral assay, the reduction of the viral plaque number was 12, 22, 38 and 42% for DENV-1, 2, 3 and 4, respectively.

Taken together, the results suggest that the action of the essential oils as inhibitory agents of DENV infectivity is due to direct virus inactivation, preventing adsorption and subsequent cellular infection.

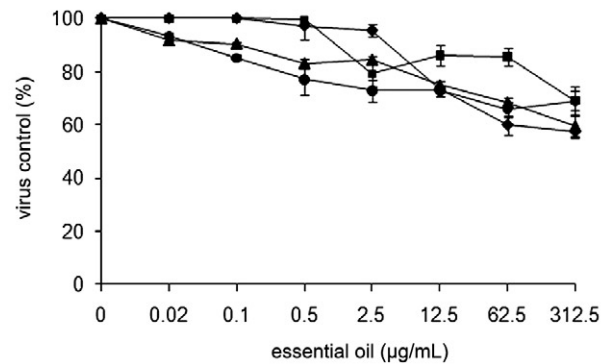


Fig. 3: virucidal activity of *Lippia* species essential oils from Colombia on dengue virus replication in vitro. Effect of *Lippia alba* essential oil on plaque formation by dengue virus (DENV)-1 (■), DENV-2 (◆), DENV-3 (▲) and DENV-4 (●) after adsorption on cell. Monolayers of Vero cell were infected with 50-60 plaque-formation unit of virus and then incubated at 37°C in the presence of cellular medium containing essential oil at varied concentrations. Reduction of the viral plaque number was determined six days after cellular infection. The data represent the means for two replicate samples of two independent experiments.

DISCUSSION

Various studies have been conducted exploring the antiviral activity of chemical compounds against DENV. Some compounds are small molecules that can inhibit specific steps of intracellular replication of the virus and other compounds are inhibitors of viral proteins (Leysen et al. 2000, Patkar & Kuhn 2006, Zhou et al. 2008, Wang et al. 2009). Although some compounds have shown promising results, none of these compounds has been approved for use in humans. Plant-derived drugs remain an important resource for treatment of tropical disease like dengue, especially in developing countries.

The *Lippia* species are mainly distributed throughout the South and Central America and African countries (Terblanché & Kornelius 1996). Infusions of *L. alba* and *L. citriodora* are used as a remedy for colds, influenza, bronchitis, coughs and asthma. These species are also considered very useful for treatment of stomach-ache, indigestion, hepatic disorders and to soothe vesicle pain. It is suggested that medicinal properties of the *Lippia* species can be due to the essential oils (Pascual et al. 2001).

In this study, the cytotoxicity of the *L. alba* and *L. citriodora* essential oils was tested on the Vero cells. Cytotoxicity of the essential oils examined can be rated as low to moderate based on the CC₅₀ classification system of Halle and Göres (1987). The results revealed *L. alba* as low cytotoxic and *L. citriodora* 2.4 times more cytotoxic. The essential oil of *L. citriodora* has geraniol and neral components (Table I) as well as a mixture of these results in a citral compound. We have observed that the incubation of Vero cells with citral at a concentration as low as 32 µg/mL for 24 h at 37°C inhibited the cellular proliferation and viability in MTT assays by 50% (unpublished data). It remains to be explored whether the higher cytotoxicity activity of the *L. citriodora* essential oil is due to the presence of geraniol and neral compounds.

To determine the antiviral action of the essential oils tested in this study, DENV was treated before adsorption on Vero cell without dilution of the virus-oil mixture to eliminate the oil. Under these conditions, the assay might not allow precise discrimination between antiviral and virucidal action because the oil could enter into the cells and interfere with the intracellular steps of the viral cycle. We can conclude presence of the virucidal effect and rule out antiviral activity of the essential oils. This is because the presence of tested essential oils in the culture medium during replication of untreated-DENV did not cause significant reduction of the viral plaque numbers six days after cellular virus infection (Fig. 3). In a previous study, we did not observe a decrease in viral progeny when Vero cells were exposed to 100 µg/mL of *L. alba* or *L. origanoides* essential oil for 24 h and then were infected with YFV (Meneses et al. 2009). These results suggest that changes in the plasma membrane cell or intracellular environment that could interfere with the adsorption or intracellular steps of the viral cycle were not induced by treatment of the cell with the essential oil.

The precise mechanism of the virucidal action of essential oils is still not fully understood. Results of the studies evaluating the interference of the essential oil on steps of the viral infection cycle indicate that the inhibitory effect occurs during adsorption and penetration steps but not after penetration of the virus into the cell (Schuhmacher et al. 2003, Saddi et al. 2007, Koch et al. 2008). One could speculate that components present in the oil interfere with the virion envelope structures masking the E and M viral proteins, which are necessary for adsorption and entry into the host cell. The main components of the essential oils tested in this study were monoterpenes, including carvone, geranial, neral and limonene. Several kinds of terpenes have been shown to inactivate human immunodeficiency virus by inhibiting virus adsorption to the target cell and causing inactivation of the virus reverse transcriptase (Pengsuparp et al. 1994, Sun et al. 1996, Xu et al. 1996). Studies on the inhibitory effect of citral and limonene on YFV and DENV replication *in vitro* are in progress in our laboratory.

The essential oils tested exhibited high virucidal action on DENVs according to the adopted criteria. Nonetheless, there were differences in the IC₅₀ values for *L. alba* oil with respect to other serotypes, albeit for *L. citriodora* it was observed for DENV-4 only. It is less plausible to be due to technical variations because the experiments simultaneously included all serotypes. It remains to be determined whether structural changes on the virion envelope due to essential oil components vary with respect to the virus serotype. Other studies have found variations in the antiviral activity with respect to dengue serotype when testing sulphated polysaccharide from the marine alga *Cladosiphon okamuranus* (Hidari et al. 2008), *Gymnogongrus griffithsiae* and *Cryptoneimia crenulata* (Talarico et al. 2005).

The results of the present paper showed that the essential oil obtained from *L. alba* and *L. citriodora* had virucidal action on all four DENV serotypes and experiments were conducted for evaluation of direct in-

activation on DENV-2 by incubation with the essential oils from other Colombian plants. *L. origanoides*, *Pimenta racemosa*, *Illicium verum* and *Zingiber officinalis* showed a 50% reduction of the viral plaque numbers at 100 µg/mL (unpublished data). In contrast, in studies evaluating the viral inhibitory effect of the essential oil from eight species of aromatic plants from Argentina, only *Artemisia douglasiana* and *Eucalyptus patens* had any discernible virucidal effect on DENV-2 replication *in vitro* (Garcia et al. 2003, Duschatzky et al. 2005).

Our results agree with other studies testing distinct natural products against DENV. Carbohydrate-binding agents originated from plant lectins can affect the binding of the virus to cell and subsequently inhibit viral replication (Talarico et al. 2007, Alen et al. 2009). Inhibitory activities by both extract and alkaloidal fraction of *Uncaria tomentosa* on infection rates and cytokine levels in treated monocytes-DENV infected have been reported (Reis et al. 2008). It seems that the zosteric acid derived from *Zostera marina* interferes with binding to the receptor required for DENV entry into the host cell (Rees et al. 2008). Anti-dengue activity of *Hippophae rhamnoides* leaf extract was demonstrated in DENV-2 infected blood-derived human macrophages (Jain et al. 2008). *In vitro* and *in vivo* antiviral properties of sulphated galactomannans obtained from the marine alga against DENV have been reported (Ono et al. 2003, Hidari et al. 2008). Moreover, enveloped viruses distinct to *flavivirus* were also inactivated by treatment with the essential oil obtained from medicinal plants. The *in vitro* replication abilities of the herpes simplex virus, hepatitis B virus and human immunodeficiency virus were suppressed by the previous exposure to essential oils but not by treatment of the cell with the essential oil before the adsorption of the virus (Zhang et al. 1988, Siddiqui et al. 1996, Yamasaki et al. 1998, Schuhmacher et al. 2003, Saddi et al. 2007, Koch et al. 2008).

The present paper has demonstrated the inhibitory effect of the essential oil obtained from *L. alba* and *L. citriodora* cultivated in Colombia on all four DENV serotypes. The essential oil seems to penetrate the skin and might act inside the cell to block viral replication (Carson et al. 2001). The topical use of the essential oil might be explored for prevention of virus infection through the bite of the vector mosquitoes.

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