Original Article

Evaluation of *in vitro* Antiviral Activity of Essential Oil Compounds Against Dengue Virus

María C. Flechas, Raquel E. Ocazionez*, Elena E. Stashenko

ABSTRACT

Introduction: There are not effective drugs available for treatment of dengue fever despite intensive research on synthetic inhibitors. The search for active phytochemicals could serve for the discovery of new drugs. This study aims to evaluate the antiviral activity *in vitro* of compounds found in essential oils from medicinal plants. **Materials and Methods:** Nine synthetic-derived essential oil compounds were evaluated. Antiviral effect was screened measuring the reduction of viral NS1 and E proteins in HepG-2 and Vero cells. **Results:** β-Caryophyllene was identified as the most active compound, it reduced the virus serotype-2 replication in HepG-2 cells at IC_{50} of 22 ± 5.6 µM, and blocked replication of all four serotypes in Vero cells at IC_{50} between 8.0 µM and 15.0 µM. The selectivity indexes were between 5.3 and 10. According to results from time-in-addition assays, the antiviral effect of β-caryophyllene appears to be associated with interruption of early steps of the virus life cycle. Citral revealed modest antiviral effect, it reduced the virus serotype-2 (IC_{50} of 31 ± 4.5 µM) replication but not the other three serotypes. Seven terpenes did not reveal antiviral activity at maximum concentration of 30 µM. **Conclusion:** Research on compounds found in essential oils can contribute to the drug discovery effort for dengue. β-Caryophyllene could serve as a starting point.

Key words: Terpene, Essential oil, Dengue, Antiviral, *Flavivirus*.

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INTRODUCTION

Dengue is caused by four antigenically related serotypes of dengue viruses (DENV-1, DENV-2, DENV-3 and DENV-4), which belong to the genus *Flavivirus*.[1] There were an estimated of 58.4 million symptomatic dengue infections in 141 endemic countries in 2013 (13,586 fatal cases), and the total cost of illness was estimated in US $ 8.9 billion.[2] No effective drugs exist to treat dengue infections, the current treatment is limited to fluid therapy and supportive care.[3] Treatment could be insufficient and lead to mortality in patients with severe disease. Drug discovery for dengue has focused on synthetic molecules targeting host and virus proteins. Numerous inhibitors with various modes of action have been discovered and several of them showed efficacy in rodent models.[4,5] A few molecules have advanced to a clinical trial but none so far has yielded encouraging results. Recent proof-of-concept clinical trials have been performed using approved drugs, they also showed lack of efficacy.[5,6] Search for effective and lower cost therapeutic agents remains of highest importance. Products obtained from medicinal plants represent a rich sources of molecules with therapeutic potential in the search for novel anti-DENV drugs.[7,8,9,10] Plants produce essential oils as secondary metabolites, which are known as natural antimicrobials and often cited as anti-inflammatories.[11] Several reports have demonstrated antiviral activity of essential oils against *Flavivirus*.[8,9,12,13,14,15] Essential oils are a mixture of high chemical diversity and biochemical specificity of compounds, which belong to different classes of chemicals. The main class include terpenes, phenylpropanoids, and short-chain aliphatic hydrocarbon derivatives, being responsible for their biological activities.[16]

The terpenes and terpenoids are the most representative components present in almost all essential oils and have a great variety of structures. There is an increasing interest on these compounds because of their antimicrobial, antitumoral and anti-inflammatory properties.[17,18,19] Thus, terpenes and terpenoids offer a great opportunity to find novel lead molecules with specific inhibitory mechanisms on DENV. Antiviral activity of terpenes against *Flavivirus* have been demonstrated: citral blocked the Yellow-Fever Virus replication *in vitro*,[15] and treatment with combined monoterpenes delayed morbidity of mice due to West Nile Virus infection.[20] There is no report about antiviral potential of essential oils compound on DENV.

The present study aimed to evaluate the antiviral activity of synthetic-derived compounds on DENV, which are main constituents of essential oils that revealed anti-dengue activity in previous studies.[8,12,13]

A cell-based assay-guided screening approach was followed to identify the strongest active compound.

MATERIALS AND METHODS

Compounds

Nine synthetic-derived essential oils compounds were selected: β-caryophyllene, citral, (R)- (-)-carvone, (S)-(+)-carvone, (R)-(+)-limonene, ρ-cymene, geranyl acetate, nerol, and α-phellandrene. Structural formulas are presented in Figure 1. Ribavirin was applied as reference antiviral drug. All compounds met high purity standards, they were purchased from Sigma-Aldrich (St. Louis, MO, USA). Compounds were dissolved in dimethyl sulfoxide and further diluted in medium for cell culture, resulting in dimethyl sulfoxide concentration below 1% which has no effect on cells and viruses' viability.

Cells and virus

HepG-2 cells (from human liver, hepatocellular carcinoma, ATCC® HB-8065™) were cultured in DMEM/F-12 medium (Gibco) supplemented with 10% (v/v) fetal calf serum (FCS) and 1% penicillin (100 U/ml) /streptomycin (100 mg/ml) solution (Gibco). Vero cells (from kidney of African green monkey; ATTCC® CCL-81™) were cultured in MEM medium (Gibco) supplemented with 8% (v/v) FCS and antibiotics. DENV-1 (Hawaii strain), DENV-2 (NGC strain), DENV-3 (H-87 strain), and DENV-4 (H-241 strain) viruses were propagated in *Aedes alboptictus* mosquito cells (clone C6/36).

Viral non-structural 1 (NS1) protein reduction assay

HepG-2 cells were used. Multiplicities of infection (MOI: 0.06, 0.13, 0.25, 0.5, and 1.0) were tested to find the optimal proportion of virus for cellular infection. MOI of 1.0 was used for subsequent experiments. DENV-2 was adsorbed 1h onto cells grown in 96 well plates, the virus inoculum was aspirated and the monolayer was rinsed. Culture medium containing the test compound (1, 5, 25, and 100 µM) was added and virus replication was allowed for 72h. Controls were run in parallel and included cells either uninfected or virus-infected in the absence of terpene. The IC_{50} value, which is defined as the compound concentration that is required to reduce viral NS1 protein by 50% relative to the untreated control cells (0% reduction), was determined using logarithmic interpolation.

In situ, cellular enzyme-linked immunosorbent (ELISA) assay

A modified protocol of the microneutralization assay described by Vorndam and Beltran was followed.[21] For optimization, MOIs of 1- 0.0003 for each virus serotype were tested using ribavirin. Briefly, DENV (MOI of 0.5) was adsorbed 1 h on Vero cells seeded into a 96-well plate, compound was added at non-toxic concentration range (1, 10, 30, and 50 µM) in triplicate, and the plate was incubated at 37°C. Controls (cells either uninfected or virus-infected in the absence of compound) were also run in parallel in every assay plate. After 5 days of incubation, the cells were fixed with a 1:1 methanol: ethanol solution for 30 min at -20°C, and the plates were washed five times with washing buffer. The ELISA was performed by adding anti-DENV MAB 4G2 antibody (CDC, Puerto Rico) 2h at 37°C, followed by the secondary antibody goat anti-mouse IgG HRP (Kirkegaard and Perry, Gaithersberg, USA) for 1h at the same temperature. Plates were washed, and ABTS substrate solution (Kirkegaard and Perry) was added (1h). The optical density (OD) was measured at 405 nm in a microplate reader (MultiskanGo, Thermo Fischer Scientific, USA). The IC_{50} value was determined using logarithmic interpolation.

MTT assay

Decrease of cell viability was examined on cell lines (HepG-2 and Vero) in which the antiviral activity was evaluated. Cells seeded in 96-well plates were exposed to a concentration range (10-1600 µM) of compound for 72 h at 37°C. The culture medium was removed and
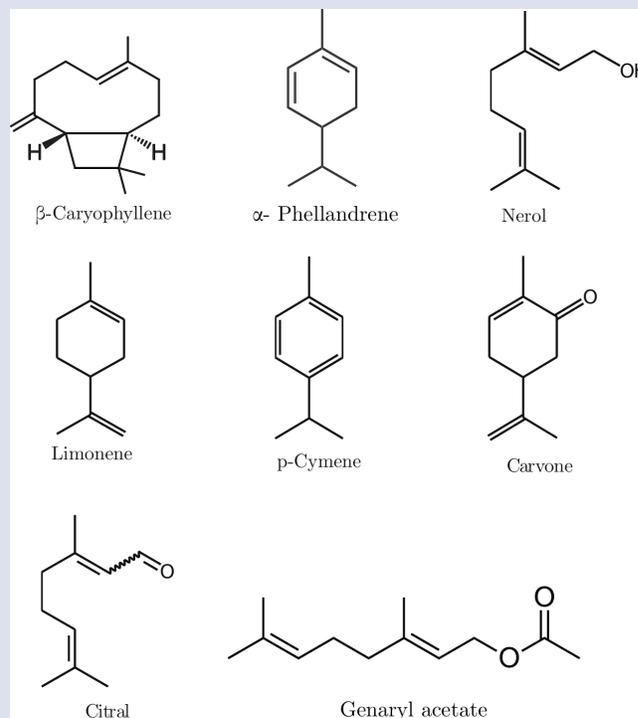

Figure 1: Structural formulas for the test essential oil compounds.
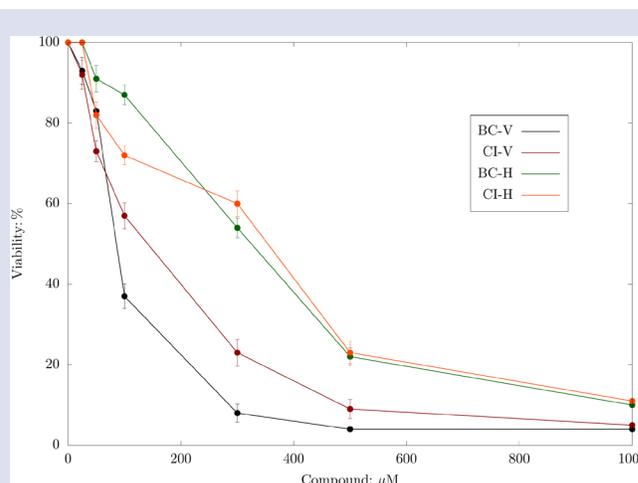

Figure 2: Representative dose response curves derived from MTT assays for evaluating of cytotoxicity. BC: β-caryophyllene. CI: citral. V: Vero cells. H: HepG-2 cells. Data are mean ± SD (n = 3).

MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] solution (5 mg/mL) was added to each well. The extent of MTT reduction to formazan was quantified by measuring the OD. at 580 nm in a microplate reader (Multiskan Go, Thermo Fischer Scientific, USA). The half-maximal cytotoxic concentration (CC_{50}) for the compound with reference to untreated control cells which represented 100% cell viability, was defined as the concentration (µM) that resulted in 50% cytotoxicity. Selectivity index (SI) is defined as the ratio of CC_{50} to IC_{50} values.
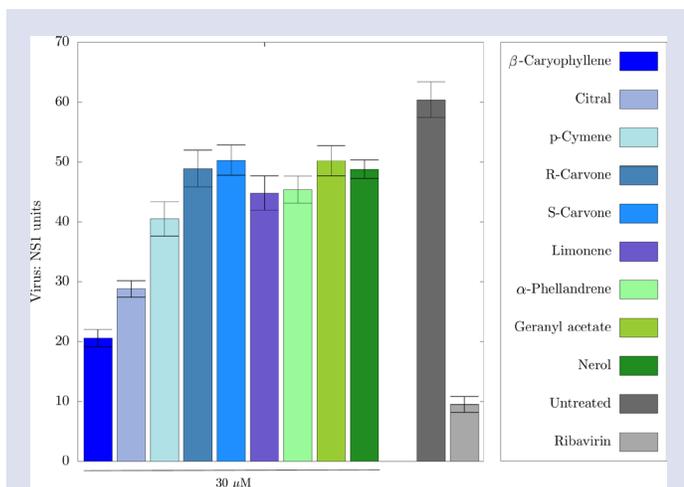
Figure 3: Antiviral activity of essential oil compounds against DENV-2 in the NS1 reduction assay. Ribavirin (100 μM) was used as reference antiviral. Data are mean ± SD (n = 2).
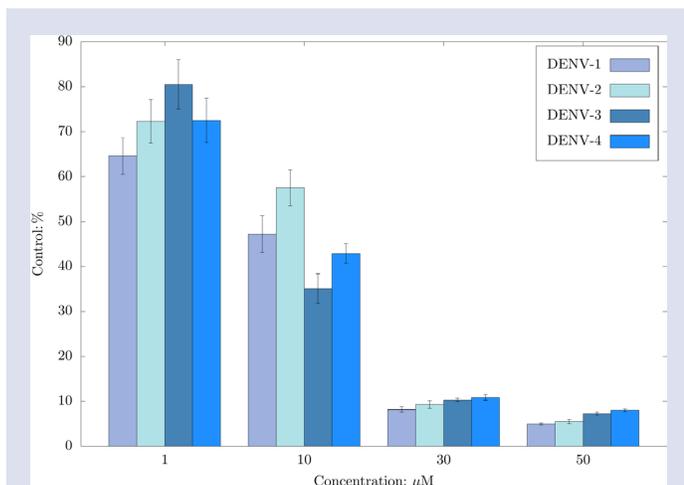

Figure 4: Antiviral activity of β-caryophyllene on dengue virus sero-types in the *in situ* cellular-ELISA assay. Data are mean ± SD (n = 3), and are presented relative to OD405nm (100%) for untreated control cells.

Table 1: Antiviral activity of active terpenes on DENV-2 in the NS1 reduction assay.

Terpene	CC_{50}: μM	IC_{50}: μM	IS
β-Caryophyllene	380 ± 25.4	22 ± 5.6	17.2
Citral	490 ± 10.2	31 ± 4.5	15.8

IS: selectivity index, Data are mean ± DS (n = 3).

Table 2: Antiviral activity of β-Caryophyllene on dengue virus in the *in-situ* ELISA assay.

Activity	Serotype			
	DENV-1	DENV-2	DENV-3	DENV-4
IC_{50}: μM	9 ± 1.5	15 ± 3.6	8 ± 3.4	11 ± 4.9
Selectivity index	8.8	5.3	10	7.3

CC_{50}: 80 ± 9.9 μM. Data are mean ± DS (n = 3).

Data analysis

All data were analysed using the R Project for Statistical Computing (R Development Core Team [2013]. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org). Data are shown as mean ± standard deviation of independent experiments performed as duplicates or triplicates and are presented relative to untreated control cells.

RESULTS

All test compounds were screened initially for cytotoxicity in the MTT assay. Figure 2 presents representative curves dose response. Results showed that the compounds have not discernible toxicity at the maximum concentration (100 μM or 50 μM) used for evaluating of antiviral activities in HepG-2 and Vero cells. CC_{50} values for all nine compounds were higher than 79 μM on both cell lines.

DENV directly invades and replicates in the liver during the acute course of natural infection.[22] HepG-2 cells were designed to evaluate antiviral effect on DENV-2 of all selected compounds in the NS1 reduction assay. In order to select a compound as good virus inhibitor, it was reasoned that it must reduce NS1 protein close to 50% respect to untreated control by using maximum concentration of 30 μM. This concentration is within the effective concentration range of antiviral ribavirin in cell-based assays.[23]. The test compound was added after virus adsorption; thus, the assay revealed if the compound possessed the ability to inhibit post-entry steps in the virus life cycle.

NS1 reduction assays revealed two of nine compounds to possess inhibitory effect on DENV-2 at concentration of 30 μM (Figure 3). β-caryophyllene reduced viral protein by 65% (21 ± 3.5 units) and citral by 50% (30 ± 3.4 units), relative to 100% (60 ± 3.2 units) in virus-infected untreated cells. Then, the terpenes were screened at concentration range, their IC_{50} values were less than 32 μM and selectivity indexes were between 15 and 18 (Table 1). The remaining seven compounds (*p*-cymene, limonene, α-phellandrene, nerol, R-carvone, S-carvone and geranyl acetate) did not reduce viral protein close to 50% at concentration of 30 μM. Thus, these compounds were scored as negative for antiviral effect and excluded from further analysis.

A good DENV inhibitor must be active against all four serotypes, and it should ideally have the potential to inhibit virus in any cell culture system.[24] β-Caryophyllene and citral were tested against all four serotypes at concentration range in the *in situ* cellular-ELISA assay. The antiviral action was based on reduction of cell-surface viral E protein expressed as OD_{405nm} value respect to 100% of virus-infected untreated cells. Vero cells not virus-infected that were exposed to the maximum concentration (50 μM) of terpene, showed 75-91% cell viability during entire time (five days) of experiments. β-Caryophyllene inhibited all four serotypes between 89-92% at 30 μM (Figure 4): their IC_{50} values were less than 16 μM and selectivity indexes were between 5 and 10 (Table 2). Citral did not reveal antiviral effect at maximum concentration of 50 μM: the reduction of OD_{405nm} values in DENV-1, -2, -3 and -4 infected-cells were, respectively, 24,3%, 9.8%, 2.4% and 45.0%.

In an attempt to determine how long the addition of β-caryophyllene could be postponed before it lost its antiviral effect, the NS1 protein reduction assay was performed as follows: DENV-2 (MOI of 0.5) was adsorbed 1h on cells, compound (100 μM) was added immediately (0h), 4h and 8h afterwards. The viral NS1 protein level was measured 72h after virus adsorption, in virus-infected untreated cells it was 45 ± 1.8 units. β-Caryophyllene reduced viral NS1 protein by 88.8% when it was added at 0h (5 ± 2.6 units) and 4h (5 ± 1.7 units) whereas 55.5% (20 ± 2.8 units)

at 8h. According to the results, the antiviral effect of the terpene appears to be associated with interruption of early steps of the virus life cycle.

DISCUSSION

Outcome of DENV infection is associated to both plasma viremia and proinflammatory cytokines levels.[25] It has suggested that reducing viral load and cytokines levels by therapy, could prevent or lessen the chances of patients progressing to severe disease.[3,4] Owing to their antiviral and anti-inflammatory properties,[17-19] terpenes could have strong potential for being used in the search of new drugs for dengue.

This study aimed to evaluate the antiviral activity *in vitro* of compounds found in essential oils with anti-dengue activity. It was began identifying the strongest compound against a single serotype, from nine selected for evaluation, by using a single concentration of 30 μM. At this concentration, it is expected that a lead compound for drug discovery should display an inhibition level of at least 50% without toxicity to cell.[24] β-Caryophyllene and citral were identified as inhibitors of the DENV-2 replication. Then, the terpenes were evaluated against all four-virus serotype, and β-caryophyllene was identified as a pan-DENV inhibitor.

Two cell-based assays were developed to identify essential oils compounds with potential DENV-inhibitory effect. Since these compounds may be toxic to cells, it is important to use non-toxic concentrations to preserve cell viability, thereby allowing extensive viral replication. As results of MTT assays indicated, the test compounds exhibited maximum non-toxic concentration in both cell lines at level higher than maximum concentration used for antiviral screening. Observations on uninfected cells treated with compound at 100 μM for three days (HepG-2 cells) or 50 μM for five days (Vero cells), showed no changes in the normal state of cell monolayers throughout the antiviral test period. Therefore, the antiviral activity of compounds did not result from general cellular toxicity.

The virus plaque assay, which is considered the reference test for screening,[26] was not used to evaluate the anti-DENV activity of test compounds. Instead of this assay, two assays based on detection of the viral NS1 and E proteins were used. It has been demonstrated that after DENV adsorption on HepG-2 and Vero cells, both viral protein (E and NS1) and virus progeny are detected.[27,28] Moreover, simultaneous reduction of NS1 protein secretion and progeny virus production has been demonstrated in assays evaluating anti-dengue activity *in vitro* of compounds.[29,30] Thus, the viral protein reduction caused by treatment with β-caryophyllene and citral indicated that viral progeny was also reduced. The cell-based assays were designed to identify compounds that had the ability to inhibit DENV within the cells. Therefore, virus adsorption preceded exposure to the compound. As virus was not incubated with compound before adsorption, virucidal activity was not evaluated.

An antiviral drug against dengue may be targeted against one or more of the key steps of the DENV life cycle to achieve effective reduction of viral progeny.[24] To understand the mode of antiviral action of β-caryophyllene, time of addition experiments were carried out. It has been shown that one round of DENV life cycle in HepG-2 cells encompassed between 12-17 h after viral adsorption.[27] More than one round of viral cycle was encompassed in the experiments testing β-caryophyllene, the NS1 protein was measured 72h after viral adsorption. Even so, β-caryophyllene almost completely reduced the virus replication when it was added at time (0h) and 4h after viral adsorption but far less at 8h afterwards. It is plausible that the terpene may interfere with virion envelope structures or mask membrane cellular components, which are required for adsorption and penetration of the virus into a host cell. It has been showed that β-caryophyllene and monoterpenes may inhibit enveloped viruses at early stage of the viral cycle.[20,31,32] Moreover, β-caryophyllene is able to interact with artificial lipid bilayer.[33] Citral reduced DENV-2 infection in the NS1 reduction assay in HepG-2 cells. Nevertheless, the same effect

was not observed against this serotype and the others in the *in-situ* ELISA assay in Vero cells. An explanation for these conflicting results might be related to different conditions for the virus to replicate. Increased viral replication for five days in Vero cells instead of three days in HepG-2 cells could result in a gradual loss in the antiviral effect. Antiviral effect of citral has been identified in cells infected with other enveloped viruses (Yellow-Fever Virus and Herpes Simplex Virus) for two or three days.[15,33]

CONCLUSION

Natural product-based drug discovery remains as alternative in the search for drugs to combat causing-diseases viruses. The interest of pharmaceutical companies in natural products as a source of drugs has grown notoriously, mostly in low income countries.[34] Essential oils can inhibit the DENV replication *in vitro*; thus, they offer a great opportunity to find novel compounds as starting points towards discovery of drug to treat dengue. This work has explored the potentials of synthetic-derived essential oils compounds to inhibit the DENV replication *in vitro*. β-Caryophyllene has demonstrated success in blocking replication of all four virus serotypes, making it a promising novel starting point for drugs discovery. β-Caryophyllene is a bicyclic sesquiterpene, it is a safe compound (GRAS) according to the Flavor and Extract Manufacturers Association (FEMA 2252) and the US Food and Drug Administration.[35] The results of this study encourage further research about the potentials of β-caryophyllene and other terpenes as lead molecules for discovering of antivirals and immunosuppressant drugs for dengue.

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CONFLICT OF INTEREST

No conflict of interest are declared.

ABBREVIATION USED

DENV: Dengue virus; **DMSO:** Dimethyl sulfoxide; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). CC_{50}**:** Cytotoxic concentration 50; IC_{50}**:** Inhibition concentration 50. **IS:** Index of selectivity.

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