

## Potential interaction of components from essential oils with dengue virus proteins

[Potencial interacción de componentes de aceites esenciales con proteínas del virus del dengue]

Nerlis PÁJARO-CASTRO<sup>1</sup>, María C. FLECHAS<sup>2</sup>, Raquel OCAZIONEZ<sup>2</sup>,  
Elena STASHENKO<sup>3</sup> & Jesús OLIVERO-VERBEL<sup>1</sup>

*Centro Nacional de Investigaciones para la Agroindustrialización de Especies Vegetales Aromáticas y Medicinales Tropicales (CENIVAM).*

<sup>1</sup>*Grupo de Química Ambiental y Computacional, Facultad de Ciencias Farmacéuticas, Universidad de Cartagena, Cartagena, Colombia.*

<sup>2</sup>*Centro de Investigaciones en Enfermedades Tropicales (CINTROP).*

<sup>3</sup>*Centro de Investigación en Biomoléculas (CIBIMOL), Universidad Industrial de Santander, Bucaramanga, Colombia.*

*Contactos / Contacts: Jesús OLIVERO-VERBEL - E-mail address: [joliverov@unicartagena.edu.co](mailto:joliverov@unicartagena.edu.co)*

**Abstract:** An antiviral drug for treatment of dengue is an urgent necessity. In this study *in silico* activities of essential oils components on dengue virus (DENV) were evaluated, and  $\beta$ -caryophyllene was subjected to biological examination to assess inhibition of DENV-2 *in vitro* replication. Components previously optimized were coupled with viral proteins prepared, using AutoDock Vina. Theoretical affinity values varied between -4.0 and -7.3 kcal/mol.  $\alpha$ -copaene,  $\beta$ -bourbonene, germacrene D, spathulenol,  $\beta$ -caryophyllene, caryophyllene oxide and (+)-epi-bicyclosesquiphellandrene showed the greatest interaction with viral proteins.  $\beta$ -caryophyllene inhibits DENV-2 *in vitro* (50% inhibitory concentration [IC<sub>50</sub>] = 22.5  $\pm$  5.6  $\mu$ M [4.6  $\pm$  1.1  $\mu$ g/mL] and resulted non-cytostatic with a selectivity index value of 71.1. The *in silico* results permit infer that DENV proteins are potential targets for the concomitant docking of various essential oils components. Biological examination suggest that  $\beta$ -caryophyllene acts on very early steps of the viral replication cycle and it might prove virucidal.

**Keywords:** Dengue, antiviral, autodock vina, sesquiterpenes

**Resumen:** Una droga antiviral para tratamiento del dengue es una necesidad urgente. En este estudio se evaluó la actividad *in silico* de componentes de aceites esenciales sobre el virus del dengue (VDEN) y el  $\beta$ -cariofileno se seleccionó para evaluar la inhibición sobre la replicación *in vitro* del VDEN-2. Los componentes previamente optimizados fueron acoplados con proteínas virales preparadas, utilizando AutoDock Vina. Los valores de afinidad variaron entre -4.0 y -7.3 kcal/mol.  $\alpha$ -copaeno,  $\beta$ -bourboneno, germacreno D, spatulenol,  $\beta$ -cariofileno, óxido de cariofileno y (+)-epi-biciclosesquifellandreno presentaron la mayor interacción con las proteínas virales.  $\beta$ -cariofileno inhibió VDEN-2 *in vitro* (concentración inhibitoria 50 [IC<sub>50</sub>] = 22.5  $\pm$  5.6  $\mu$ M [4.6  $\pm$  1.1  $\mu$ g/mL] y resultó no-citostático con índice de selectividad de 71.1. Los resultados *in silico* indican que proteínas del VDEN son blancos potenciales para varios componentes. El análisis biológico sugiere que el  $\beta$ -cariofileno actúa en etapas tempranas de la replicación viral y podría ser virucida.

**Palabras clave:** Dengue, antiviral, autodock vina, sesquiterpenos

**Recibido | Received:** October 11, 2013

**Aceptado | Accepted:** March 3, 2013

**Aceptado en versión corregida | Accepted in revised form:** January 23, 2015

**Publicado en línea | Published online:** May 30, 2015.

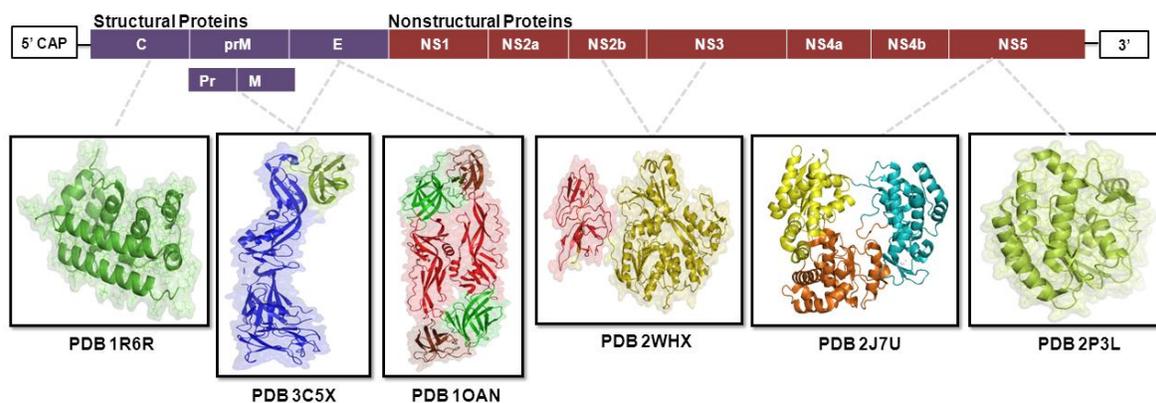
**Declaración de intereses | Declaration of interests:** This research was carried out thanks to financial support received from the Colombian government - Grants RC-0572-2012 and 11075192058-2010 (Patrimonio Autónomo del Fondo Nacional de Financiamiento para la Ciencia, la Tecnología y la Innovación, Francisco José de Caldas). The authors thank to the University of Cartagena, Cartagena (Colombia), Program to Support Research Groups, sponsored by the Vice-Rector for research (2010–2012).

**Este artículo puede ser citado como / This article must be cited as:** N Pajaro-Castro, MC. Flechas, R Ocazonez, E Stashenko, J Olivero-Verbel. 2015. Potential interaction of components from essential oils with dengue virus proteins. *Bol Latinoam Caribe Plant Med Aromat* 14 (3): 141 – 155.

## INTRODUCTION

Dengue viral infection has become an increasing global health concern with over two-fifths of the world's population at risk of infection. Dengue virus (DENV) is a vector-borne virus, transmitted to humans via infected *Aedes* mosquitoes in tropical and sub-tropical areas (Murrell *et al.*, 2011). There are four antigenically distinct, closely related serotypes of the dengue virus (DENV1–4), exhibiting a 65–70% sequence homology (Noble *et al.*, 2010). The DENV genome is comprised of approximately 10.7

kilobases (kb) of single stranded RNA of positive polarity (Rossi *et al.*, 2012). This RNA encodes three structural proteins (C, prM and E) that form the components of the virion, and seven nonstructural proteins (NS1, NS2A/B, NS3, NS4A/B, NS5) (Perera & Kuhn, 2008; Herrero *et al.*, 2013), (Figure 1). The DENV capsid (C) is a small, positively charged protein containing membrane-associated alpha-helical structures that form the inner core of the virion (Ma *et al.*, 2004; Rossi *et al.*, 2012).



**Figure 1**  
Dengue Virus proteins

The prM protein is the immature precursor to the membrane (M) protein found in the virions. It forms a scaffold over the viral envelope (E) protein to prevent premature fusion during virion maturation (Perera & Kuhn, 2008; Rossi *et al.*, 2012). The E protein comprises the majority of the surface area on the mature virion and is responsible for receptor binding and host cell surface fusion (Stevens *et al.*, 2009; Rossi *et al.*, 2012). The functions of NS1 are not completely known. Like the E protein, NS1 contains 12 disulfide bridge-forming cysteine residues and encodes N-linked glycosylation sites at N130 and N207 (Rossi *et al.*, 2012). NS2A is a small, hydrophobic protein that in several flaviviruses plays a role in modulating the type I interferon (IFN) response (Rossi *et al.*, 2012). NS2B is another small, hydrophobic protein that acts as a cofactor for the enzymatic activity of NS3. NS3, along with NS2B, has multiple functions including protease and helicase (Sampath & Padmanabhan, 2009; Rossi *et al.*, 2012). NS4A and NS4B are small, multifunctional, hydrophobic proteins that have

recently been shown to play a role in modulating the host type I IFN responses (Rossi *et al.*, 2012). NS5 acts as the RNA-dependent RNA-polymerase (RdRp) and is responsible for RNA cap processing (Noble *et al.*, 2010; Rossi *et al.*, 2012).

Four strategies have been pursued to identify inhibitors of DENV through targeting both viral and host proteins: (i) HTS (high-throughput screening) using virus replication assays; (ii) HTS using viral enzyme assays; (iii) structure-based *in silico* docking and rational design; (iv) repurposing hepatitis C virus inhibitors for DENV (Lim *et al.*, 2013). A number of inhibitory agents for the flaviviruses, directed against a wide range of both cellular and viral targets have already been reported. These include those that suppress viral RNA synthesis (Puig-Basagoiti *et al.*, 2006), inhibitors of NS3 protein helicase and protease activities (Knox *et al.*, 2006), suppressors of the assembly and maturation of the DENV (Chu and Yang, 2007), peptides targeting the E protein (Bai *et al.*, 2007), and others that produce interference with the structural changes of the envelope protein during

viral fusion (Li *et al.*, 2008). However, none of these small molecule inhibitors have yet entered clinical trials (Kampmann *et al.*, 2009).

Numerous reports describe antiviral activities of plant essential oils on enveloped viruses (Duschatzky *et al.*, 2005; Saggi *et al.*, 2007; Schnitzler *et al.*, 2008; Koch *et al.*, 2008; Bicchi *et al.*, 2009; Wu *et al.*, 2010; Garozzo *et al.*, 2011; Wu *et al.*, 2012;). In previous studies we have demonstrated inhibitory effects *in vitro* of the oil from *Lippia* species on DENV (Meneses *et al.*, 2009a; Meneses *et al.*, 2009b; Ocazonez *et al.*, 2010). Antiviral properties of essential oils have been attributed to their chemical constituents: sesquiterpenes and monoterpenes are active on enveloped viruses (Armaka *et al.*, 1999; Astani *et al.*, 2010; Astani *et al.*, 2011).

To our knowledge, there is no reports about the inhibitory effect of essential-oils-derived constituents on DENV. In this study, we used *in silico* screening to evaluate the theoretical binding affinity of components present in some essential oils that have been reported to have inhibitory actions.  $\beta$ -caryophyllene was subjected to a biological examination to assess inhibition *in vitro* of the DENV-2 replication by using cell-based assays.

## MATERIALS AND METHODS

### Software

AutoDock Vina was the primary docking program used in this work (Trott & Olson, 2010). The geometries of bioactive compounds were obtained utilizing Gaussian 03 package (Frisch *et al.*, 2003). A molecular format conversion program, Open BabelGUI, was used to transform geometries to Mol2 format for their subsequent processing (O'Boyle *et al.*, 2011). The preparation of the pdbqt files and determination of the grid box size were carried out using Auto-Dock Tools version 1.1. SYBYL 8.1.1 was utilized to prepare dengue virus protein structures (SYBYL, 2007). PyMol (DeLano Scientific LLC, USA) was employed to visualize protein-ligand complex structures (DeLano, 1998-2003; Lim *et al.*, 2011). The identification of protein residues that interact with the essential oils (EOs) components was carried out employing LigandScout 3.0 (Wolber & Langer, 2005).

### Proteins and ligand structure preparation

The three-dimensional structure of dengue virus proteins: capsid, premembrane, envelope, protease/serine/NTPase, NS5 polimerase and NS5

methyltransferase were downloaded from Protein Data Bank (PDB: 1R6R, 3C5X, 1OAN, 2WHX, 2J7U and 2P3L, respectively) and prepared with SYBYL 8.1.1 package (Maldonado & Olivero, 2011a). Proteins were minimized using atomic partial charges by Kollman method, which describes the potential of the system in terms of the energy positions of the atoms, and is parameterized for proteins and nucleic acids (SYBYL, 2007; Zaheer-ul-Haq *et al.*, 2010). MGLTools 1.5.0 software was utilized to convert structures from PDB to PDBQT format, adding polar hydrogens and assigning Kollman partial charges (Lauro *et al.*, 2011). Ligands chosen to carry out the docking simulation were selected based on reports of anti-dengue activity for essential oils (Duschatzky *et al.*, 2005; Meneses *et al.*, 2009a; Meneses *et al.*, 2009b; Ocazonez *et al.*, 2010). These molecules (55) were downloaded from Pubchem or drawn using Gaussian03 program, saved in mol2 format, and optimized by quantum chemical calculations, based in Density Functional Theory (DFT) at the 6-311G level, using Gaussian 03 as previously described (Maldonado *et al.*, 2011b; Balachandran *et al.*, 2012).

### Protein-ligand docking

Molecular docking was performed using AutoDock Vina. The docking site for ligands on evaluated proteins was defined by establishing a cube with a sufficient dimension to cover the complete protein, with a grid point spacing of 1 Å. One hundred (100) runs were carried out by ligand, and for each run the best pose was saved. Finally, the average binding affinity for best poses was accepted as the binding affinity value for a particular complex (Maldonado & Olivero, 2011a). The identification of protein residues that interact with the essential oils components having the greatest affinities was carried out with LigandScout 3.0. This program develops pharmacophores to propose the number and type of primary existing ligand-residue interactions on the protein active site (Wolber & Langer, 2005; Maldonado & Olivero, 2012).

### Docking validation

The validation of the docking process was carried out by redocking with RNA cap site and the S-adenosyl-L-methionine (SAM) binding site of NS5 MTase (2P3L) and the ligands S-adenosyl-L-homocysteine (SAH) y ribavirin triphosphate (RVP) into your binding site. To examine the broader predictability of the docking, we compared the affinity values

reported and ours for SAH and RAV. The program of identification of protein residues LigandScout 3.0 was used to do post-docking analyses. Validation of the accuracy and performance of AutoDock Vina was evaluated by calculating RMSD of the best docked conformation of the native ligand and the experimental one.

### ***Inhibitory effect of $\beta$ -Caryophyllene***

#### ***Synthetic compounds***

$\beta$ -Caryophyllene (> 80% purity) was purchased from Sigma Aldrich Co. (USA) and it was dissolved in solution of 1% DMSO. Ribavirin (Sigma) was included as control drug dissolved in test medium.

#### ***Cytotoxicity assay***

Cytotoxicity to human cells used for antiviral assays was evaluated by the MTT assay as described in a previous study (Gómez *et al.*, 2013). Briefly, HepG-2 cells (from human liver, hepatocellular carcinoma; American Type Culture Collection (ATCC) HB-8065) or HEK-293 cells (from human normal embryonic kidney, ATCC CRL-1573) were seeded into 96-well plates at  $1 \times 10^4$  cells per well and incubated for 24 h at 37° C. The medium (DMEM/F-12) was removed and fresh medium containing compound at concentrations of 100, 200, 400, 800 and 1600  $\mu$ M was added for 72 h at 37° C. After that, medium was removed and MTT solution (20  $\mu$ L, 5 mg/mL, Sigma Co.) was added to each well. The extent of MTT reduction to formazan was quantified by measuring the optical density (OD) at 580 nm. Cytotoxicity was expressed as the concentration of compound that decreased by 50% (CC<sub>50</sub>) cell viability compared to control (CC<sub>50</sub>).

#### ***Virus yield reduction assay***

A previously described protocol was followed with minor modifications (Meneses *et al.*, 2009c). Briefly, DENV-2 (local strain G025) was added (31.6 CCID<sub>50</sub>/well) onto HepG-2 cells grown in 96-well plates. After 1 h, culture medium was removed, the cells were washed to remove non-adsorbed virus and then incubated in DMEM/F-12 medium containing concentrations of compound. At 72 h after incubation, the clarified supernatant was collected and pooled and stored at -20° C, for later to be tested for the presence of DENV-2/NS1 protein using PanBio Dengue NS1 Antigen Capture ELISA following the manufacture's instructions. Assays were done in duplicate in at least two independent experiments, and virus-infected cells in medium

containing ribavirin or medium free of compound were included as controls. The concentration of  $\beta$ -Caryophyllene that reduced viral NS1 protein (PBU.:PanBio units) by 50% (IC<sub>50</sub>) with respect to untreated control was calculated.

#### ***Time-of-drug-addition assay***

HepG-2 cells were seeded into 96-well plates at  $1 \times 10^4$  cells per well and incubated for 24 h at 37° C. The next day, DENV-2 (31.6 CCD<sub>50</sub>/well) was adsorbed for 1 h, the cells were washed to remove non-adsorbed virus and then incubated in DMEM/F-12 medium.  $\beta$ -Caryophyllene (100  $\mu$ M/well) was added to the assay medium at 0 (immediately), 4, 8, 12 y 24 h after virus adsorption. At 72 h postinfection (p.i.) the clarified supernatant was collected to be tested for the presence of NS1 protein. To detect infectious virus, supernatants were used to infect mosquito cells (*Aedes albopictus*, clone C6/36HT) and the presence of DENV-2 was confirmed by indirect fluorescent antibody test on virus-infected cell as previously described (Ocazonez *et al.*, 2006).

#### ***Statistical analysis***

The differences between the means of the variables for different groups were evaluated by analysis of variance (ANOVA). Results were expressed as mean  $\pm$  standard deviation (SD). For all statistical analyzes the significance criterion was set at  $P < 0.05$ . Comparisons between the treated group ( $\beta$ -caryophyllene) and untreated (control) were performed.

## **RESULTS AND DISCUSSION**

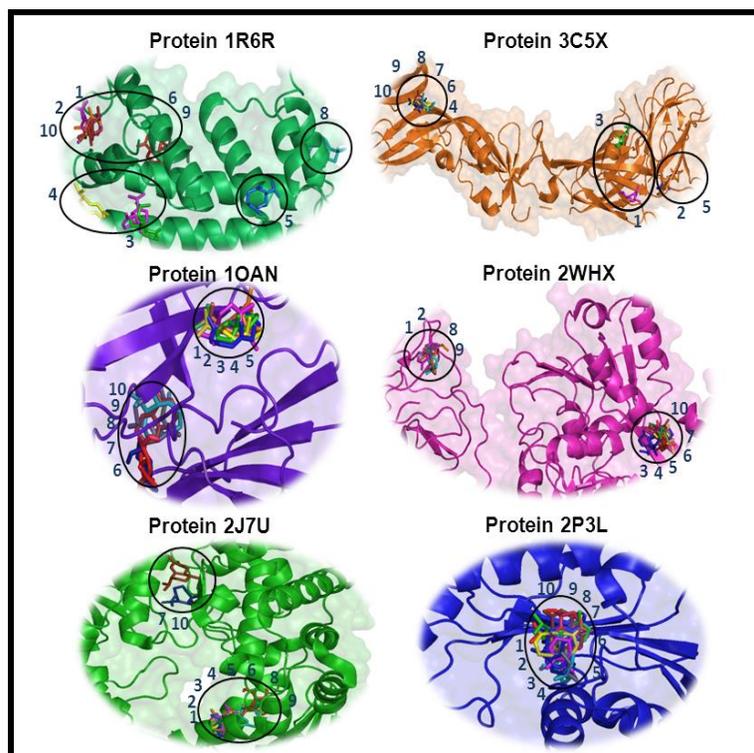
### ***Molecular docking of major compounds of essential oils with dengue virus proteins***

AutoDock Vina-calculated affinity scores obtained for compounds from EOs on examined dengue virus proteins structures (PDB ID: 1R6R, 3C5X, 1OAN, 2WHX, 2J7U and 2P3L) are presented in Table 1. This software was utilized due to its accuracy and speed, and is approximately two orders of magnitude faster than its predecessor, AutoDock 4 (Trott and Olson, 2010). Among the evaluated compounds, (-)-germacrene D, acting on 2WHX protein, had the highest affinity (-7.3 kcal/mol), followed by (-)- $\alpha$ -copaene, Z- $\beta$ -caryophyllene, -7.2 and -7.2 kcal/mol in the same protein, respectively. These chemicals are the major constituents of the EOs of *Heterothalamus alienus* (6.8%), *Buddleja cordobensis* (8.53%) and *B. cordobensis* (16.54%), respectively, for which antiviral activity toward dengue virus has been

demonstrated (IC<sub>50</sub> 122.3 y 86.4 ppm, respectively) (Duschatzky *et al.*, 2005; Meneses *et al.*, 2009b). However, until now no *in silico* activity has been reported for the individual essential oil component or their isomers.

The affinity scores for the selected components on viral proteins varied between -4.0 and -7.3.  $\alpha$ -Copaene, germacrene D,  $\beta$ -caryophyllene, spathulenol, caryophyllene oxide,  $\beta$ -bourbonene and (+)-epi-biciclosesquifellandrene showed the greatest theoretical interactions (Figure 2). All of these compounds belong to the sesquiterpenes family, and they are major constituents of the essential oil from *B. cordobensis* (8.53%), *H. alienus* (6.8%), *Lippia*

*citriodora* (2.3% - 4.7%), *B. cordobensis* (32.09% ) and *Lippia alba* (1.7% -8.9%), respectively (Duschatzky *et al.*, 2005; Meneses *et al.*, 2009b; Ocazonez *et al.*, 2010). Enantiomeric ratios for these compounds has not yet been determined. These results provide special interest, because it could explain the synergistic action of several constituents of essential oils, as a requirement to exert their antiviral activity. However, experimental studies are needed to corroborate this hypothesis. Since  $\beta$ -caryophyllene showed promising *in silico* activity, it was selected for biological evaluation in order to assess its inhibitory effect on DENV-2 replication *in vitro*.



**Figure 2**

**Essential oils components with greatest affinities for dengue virus proteins. 1. (-)- $\alpha$ -Copaene. 2. (+)- $\alpha$ -Copaene. 3.  $\beta$ -Bourbonene. 4. (-)-Germacrene D. 5. (+)-Germacrene D. 6. (+)-Spathulenol. 7. (-)-Spathulenol. 8.  $\beta$ -Caryophyllene. 9. Caryophyllene oxide. 10. (+)-Epi-biciclosesquifellandrene.**

**Table 1**

**AutoDock Vina-calculated affinities obtained for docking of major components  
of essential oils on DENV proteins**

Componentes de los AEs	Viral protein / affinity*					
	C	PrM-E	E	NS3-NS2B	NS5	
	1R6R	3C5X	1OAN	2WHX	2J7U	2P3L
1,8-Cineole	-4.3 ± 0.0	-4.9 ± 0.0	-4.4 ± 0.0	-5.9 ± 0.1	-5.1 ± 0.1	-5.1 ± 0.0
(+)- $\alpha$ -Copaene	-5.3 ± 0.0	-5.7 ± 0.1	-5.7 ± 0.1	-6.8 ± 0.0	-6.3 ± 0.0	-6.8 ± 0.0
(-)- $\alpha$ -Copaene	-5.3 ± 0.1	-5.9 ± 0.0	-6.0 ± 0.2	-7.2 ± 0.0	-6.2 ± 0.0	-6.5 ± 0.0
S-Camphor	-4.4 ± 0.0	-5.2 ± 0.1	-4.3 ± 0.0	-6.2 ± 0.0	-5.0 ± 0.0	-5.3 ± 0.0
D- Camphor	-4.4 ± 0.0	-5.1 ± 0.1	-4.3 ± 0.0	-6.1 ± 0.0	-5.1 ± 0.1	-5.1 ± 0.0
(+)-3-Carene	-5.2 ± 0.5	-4.9 ± 0.0	-4.9 ± 0.2	-5.7 ± 0.1	-5.4 ± 0.0	-5.3 ± 0.0
(-)-3-Carene	-5.5 ± 0.8	-4.9 ± 0.0	-5.0 ± 0.0	-5.8 ± 0.1	-5.6 ± 0.0	-5.4 ± 0.0
3-Octanol	-4.3 ± 0.1	-4.1 ± 0.1	-4.0 ± 0.2	-4.5 ± 0.1	-4.3 ± 0.2	-4.5 ± 0.2
3-Octanone	-4.4 ± 0.1	-4.0 ± 0.1	-4.0 ± 0.1	-4.5 ± 0.2	-4.3 ± 0.2	-4.3 ± 0.1
6-Metil-5-hepten-2-one	-4.6 ± 0.1	-4.2 ± 0.1	-4.5 ± 0.1	-4.9 ± 0.1	-4.8 ± 0.2	-4.8 ± 0.0
$\alpha$ -Pinene	-5.3 ± 0.4	-4.9 ± 0.1	-4.6 ± 0.0	-5.8 ± 0.1	-5.0 ± 0.0	-5.0 ± 0.0
$\beta$ -Pinene	-4.9 ± 0.4	-4.7 ± 0.0	-4.4 ± 0.0	-5.7 ± 0.1	-4.9 ± 0.0	-5.0 ± 0.1
Artemisia alcohol	-4.5 ± 0.2	-4.7 ± 0.1	-4.5 ± 0.0	-5.2 ± 0.2	-4.9 ± 0.2	-5.1 ± 0.0
$\beta$ -Bisabolene	-5.7 ± 0.2	-5.9 ± 0.2	-5.3 ± 0.2	-6.2 ± 0.2	-5.9 ± 0.1	-6.5 ± 0.2
$\beta$ -Bourbonene	-5.5 ± 0.1	-6.1 ± 0.0	-5.8 ± 0.2	-7.0 ± 0.0	-6.5 ± 0.0	-6.8 ± 0.0
Z- $\beta$ - Caryophyllene	-5.0 ± 0.0	-5.7 ± 0.1	-5.6 ± 0.0	-7.2 ± 0.0	-6.7 ± 0.0	-6.3 ± 0.0
E- $\beta$ - Caryophyllene	-5.0 ± 0.0	-5.8 ± 0.0	-5.5 ± 0.0	-6.9 ± 0.0	-6.4 ± 0.0	-6.2 ± 0.0
Caryophyllene oxide	-5.2 ± 0.0	-6.4 ± 0.4	-5.7 ± 0.0	-7.1 ± 0.0	-6.3 ± 0.1	-6.4 ± 0.0
$\beta$ -Mircene	-5.0 ± 0.2	-4.2 ± 0.2	-4.8 ± 0.1	-4.7 ± 0.2	-5.0 ± 0.2	-4.8 ± 0.1
Trans- $\beta$ -Ocimene	-5.0 ± 0.1	-4.6 ± 0.1	-4.8 ± 0.1	-5.1 ± 0.3	-5.1 ± 0.2	-5.2 ± 0.0
cis- $\beta$ -Ocimene	-4.9 ± 0.1	-4.2 ± 0.1	-4.9 ± 0.2	-5.0 ± 0.3	-5.0 ± 0.2	-4.9 ± 0.1
$\beta$ -Sesquiphellandrene	-5.6 ± 0.1	-5.3 ± 0.2	-5.7 ± 0.2	-5.9 ± 0.1	-5.8 ± 0.2	-6.3 ± 0.1
(+)-Epi-Bicyclosquifellandrene	-5.0 ± 0.0	-5.9 ± 0.0	-5.7 ± 0.0	-6.8 ± 0.0	-6.3 ± 0.0	-7.0 ± 0.0
(+)-Borneol	-4.4 ± 0.0	-5.2 ± 0.0	-4.4 ± 0.0	-5.9 ± 0.0	-5.0 ± 0.0	-5.2 ± 0.0
(-)-Borneol	-4.5 ± 0.0	-5.1 ± 0.0	-4.3 ± 0.1	-6.1 ± 0.1	-5.1 ± 0.0	-5.2 ± 0.0
Carvacril methyl éter	-5.3 ± 0.2	-4.9 ± 0.0	-4.9 ± 0.0	-6.1 ± 0.2	-5.9 ± 0.3	-5.7 ± 0.0
Carvacrol	-5.4 ± 0.0	-5.0 ± 0.0	-5.0 ± 0.0	-6.1 ± 0.1	-5.9 ± 0.3	-6.1 ± 0.1
(+)-Carvone	-5.1 ± 0.2	-5.2 ± 0.0	-4.8 ± 0.0	-5.7 ± 0.2	-5.3 ± 0.0	-5.7 ± 0.0
(-)-Carvone	-5.2 ± 0.1	-5.0 ± 0.0	-4.9 ± 0.1	-5.8 ± 0.3	-5.4 ± 0.3	-5.7 ± 0.0
Chavicol	-5.1 ± 0.2	-4.7 ± 0.0	-5.0 ± 0.0	-5.5 ± 0.1	-5.2 ± 0.1	-5.2 ± 0.1
Geranial	-4.6 ± 0.2	-4.5 ± 0.1	-4.8 ± 0.1	-5.2 ± 0.1	-5.1 ± 0.2	-5.4 ± 0.0
Geraniol	-4.5 ± 0.2	-4.5 ± 0.1	-4.9 ± 0.2	-5.3 ± 0.1	-5.1 ± 0.2	-5.6 ± 0.1
(+)-Limonene	-5.3 ± 0.0	-4.9 ± 0.0	-4.7 ± 0.0	-5.5 ± 0.0	-5.5 ± 0.2	-5.4 ± 0.0
(-)-Limonene	-5.3 ± 0.0	-4.9 ± 0.0	-4.8 ± 0.0	-5.6 ± 0.1	-5.6 ± 0.2	-5.3 ± 0.0
$\gamma$ -Terpinene	-5.5 ± 0.0	-4.8 ± 0.0	-4.7 ± 0.0	-5.5 ± 0.0	-5.6 ± 0.2	-5.5 ± 0.0
(+)-Piperitone	-4.3 ± 0.1	-5.4 ± 0.1	-4.7 ± 0.2	-5.4 ± 0.1	-5.0 ± 0.1	-5.7 ± 0.0

(-)-Piperitone	-4.6 ± 0.3	-5.4 ± 0.1	-4.9 ± 0.2	-5.8 ± 0.1	-5.4 ± 0.2	-5.5 ± 0.0
Piperitenone	-5.2 ± 0.2	-5.4 ± 0.0	-5.0 ± 0.0	-5.7 ± 0.1	-5.6 ± 0.1	-6.1 ± 0.0
Estragole	-5.1 ± 0.0	-4.7 ± 0.1	-4.7 ± 0.0	-5.4 ± 0.1	-5.4 ± 0.1	-5.3 ± 0.0
Eugenol	-5.1 ± 0.1	-5.1 ± 0.1	-5.2 ± 0.1	-6.1 ± 0.1	-5.4 ± 0.0	-5.5 ± 0.0
Geranyl acetate	-4.9 ± 0.1	-5.0 ± 0.2	-5.0 ± 0.2	-6.0 ± 0.5	-5.4 ± 0.2	-5.6 ± 0.1
(+)-Germacrene D	-5.5 ± 0.4	-5.9 ± 0.0	-5.8 ± 0.1	-6.8 ± 0.2	-6.8 ± 0.0	-6.4 ± 0.0
(-)-Germacrene D	-5.4 ± 0.1	-5.9 ± 0.1	-6.3 ± 0.4	-7.3 ± 0.0	-6.5 ± 0.0	-6.5 ± 0.0
(+)-Linalool	-4.7 ± 0.4	-4.6 ± 0.1	-4.5 ± 0.2	-5.0 ± 0.2	-5.0 ± 0.1	-5.2 ± 0.1
(-)-Linalool	-4.8 ± 0.4	-4.7 ± 0.2	-4.5 ± 0.2	-5.0 ± 0.2	-5.0 ± 0.2	-5.0 ± 0.2
R- $\alpha$ -Zingiberene	-5.8 ± 0.1	-5.8 ± 0.1	-5.3 ± 0.2	-6.2 ± 0.4	-5.8 ± 0.2	-6.4 ± 0.2
(-)-Spathulenol	-5.1 ± 0.0	-6.1 ± 0.0	-5.3 ± 0.1	-6.7 ± 0.0	-6.4 ± 0.1	-6.4 ± 0.0
(+)-Spathulenol	-4.9 ± 0.0	-6.1 ± 0.0	-5.6 ± 0.1	-7.1 ± 0.0	-6.4 ± 0.0	-6.2 ± 0.0
S- $\alpha$ -Zingiberene	-5.9 ± 0.1	-4.8 ± 0.0	-5.1 ± 0.4	-6.0 ± 0.1	-5.7 ± 0.0	-5.8 ± 0.3
(+)-Trans-Carveol	-5.1 ± 0.1	-5.2 ± 0.2	-5.2 ± 0.0	-5.8 ± 0.1	-5.4 ± 0.0	-5.5 ± 0.0
Neral	-4.7 ± 0.2	-4.5 ± 0.1	-4.9 ± 0.2	-5.3 ± 0.2	-5.0 ± 0.2	-5.4 ± 0.1
Nerol	-4.6 ± 0.2	-4.7 ± 0.1	-4.8 ± 0.1	-5.3 ± 0.0	-5.0 ± 0.3	-5.6 ± 0.1
Sabinene	-5.1 ± 0.1	-4.8 ± 0.1	-4.9 ± 0.1	-5.4 ± 0.0	-4.9 ± 0.0	-5.2 ± 0.1
$\rho$ -Cimeno	-5.6 ± 0.0	-4.8 ± 0.0	-4.7 ± 0.1	-5.6 ± 0.1	-5.8 ± 0.1	-5.5 ± 0.0

\*Mean ± SD of affinity (Kcal/mol) for 100 run

#### Identification of interactions between ligands with best in silico affinities and dengue virus proteins

Interactions between DENV protein and essential oils components, as predicted by LigandScout 3.0, are shown in Figure 3. Most protein-ligand interactions were hydrophobic in nature. More frequently interacting residues were VAL, ILE, and THR. In the case of the dengue virus protease (2WHX) bound to the inhibitor alpinetine, interacting residues with the ligand include ILE365A and THR408A, interactions were also identify in the (-)-germacrene D-2WHX protein complex, which may suggest a possible indication of (-)-germacrene D binding to 2WHX. On the other hand, important interactions with protein NS5 methyltransferase are hydrogen bonds at both ends of the elongated SAM molecule, with ASP131 and VAL132 fixing the adenine moiety and GLY86, SER56, and ASP146 fixing the amino acid moiety at the opposite end. The elongated binding pocket is lined with predominantly apolar residues (Podvinec *et al.*, 2010). Other important residues are HIS110, LYS105, ASP131 and ILE147, which play an important role in the N7 and 2-O'-methylation (Lim *et al.*, 2011). The molecule (+)-epibicyclosquiphellandrene possesses a hydrophobic interaction with VAL132 (Figure 3F) and one of the residues (ILE147) important in the binding site of

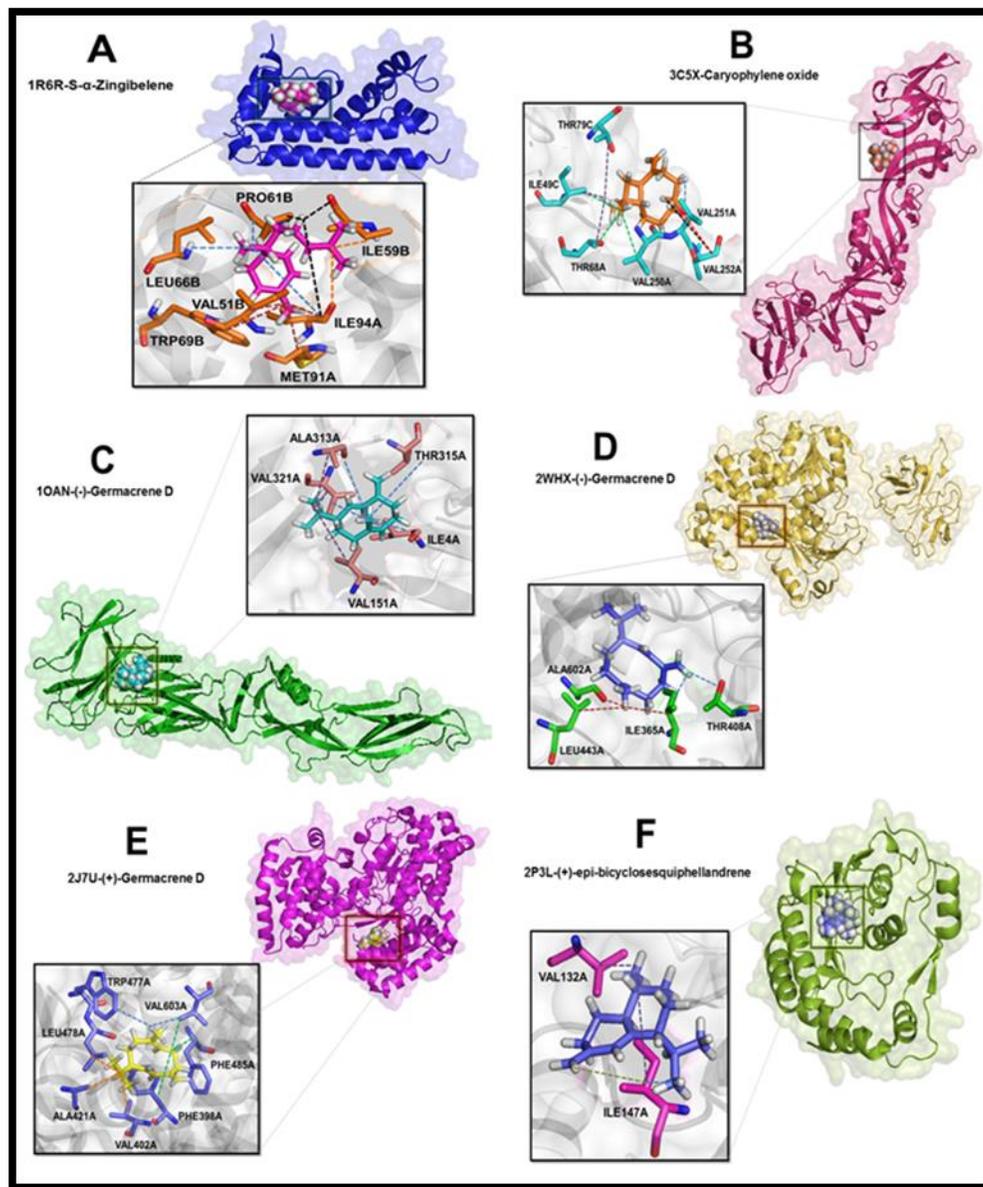
SAM. These results indicate that this compound can block the binding site SAM by interaction with critical residues.

#### Validation of the docking

Docking validation was performed by redocking co-crystallized SAH y RVP into their respective binding sites. We found that the binding conformations of redocked SAH reproduced the binding modes of the co-crystallized ligands with binding affinities  $-7.24 \pm 0.35$  kcal/mol and  $-5.37 \pm 0.35$  kcal/mol for SAH and RVP, respectively (Figure 4). The theoretical binding affinity of SAH y RVP reported using Autodock Vina were -7.3 and -5.6 kcal/mol, respectively (Lim *et al.*, 2011), comparable with the values obtained by us. Moreover, the SAM binding pocket is thought to be more closed than the RNA cap site. SER56, GLY86 and ASP146 interact with the SAH molecule by forming hydrogen bonds. In addition, there were four hydrophobic interactions between the hydrophobic side chains of THR104, LYS105, VAL132 and ILE147 in the SAM binding pocket with the SAH molecule (Lim *et al.*, 2011). We found that LYS105, THR104, GLU111 and GLY86 interact with the SAH molecule by forming hydrogen bonds and SER56 interact with the SAH molecule by bond donor. The RNA cap site of NS5 MTase is an open

and shallow pocket. LEU20, ASN18 and LYS29 forms a hydrogen bond with RVP. A hydrophobic interaction between RVP and LEU17 of the RNA cap site was observed. The aromatic ring of PHE25 forms an aromatic stacking interaction with the pseudobase

ring of RTP (Lim *et al.*, 2011). We found that SER150 and LYS14 interact with the RVP molecule by forming hydrogen bonds and ASN18 interact with the RVP molecule by bond donor.



**Figure 3**

**3D view and interacting residues present in DENV proteins. A. 1R6R-S- $\alpha$ -zingibellene, B. 3C5X-Caryophyllene oxide, C. 10AN-(-)-Germacrene D, D. 2WHX (-)-germacrene D, E. 2J7U-(+)-germacrene D, F. 2P3L-(+)-epi-bicyclosesquiphellandrene complexes**

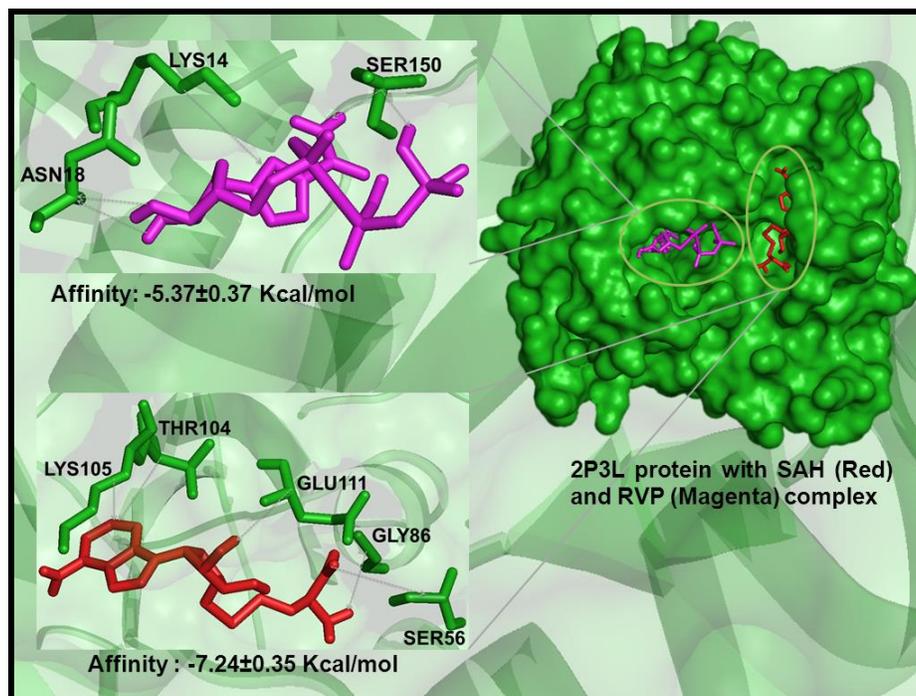
There are slight differences between our result and the result of Lim *et al.*, 2011 in terms of the amino acids that are involved in the interaction between SAH and the SAM binding site, and RVP

and the RNA cap site. We identified one interact between SAH and GLU111, which are not identified in Lim's work. However, our results confirm that THR104 and GLY86 hydrogen bonds with SAH.

Otherwise, we identified two interact between RVP and SER150, and LYS14 which are not identified in Lim's work.

As cited in literature, if the RMSD of the best docked conformation of the native ligand is  $\leq 2.0$  Å from the experimental one, the used scoring function

is successful (Hegazy & Ali, 2012). The RMSD of the docked RVP into NS5 MTase was 0.216 Å, and that of the docked SAH into NS5 MTase was 0.210 Å. The obtained success rates of AutoDock Vina were highly excellent.



**Figure 4**  
Surface representation of NS5 MTase with SAH and RVP in the binding site RNA cap and SAM, respectively.

#### *In vitro* antiviral activity of $\beta$ -Caryophyllene

To examine whether the cell viability is affected by  $\beta$ -caryophyllene, a MTT assay was carried out on monolayers of human cells exposed at various concentrations of compound for 72 h (Figure 5A). For HepG-2, the  $CC_{50}$  was  $293.8 \pm 62.1$   $\mu$ M ( $60 \pm 12.7$   $\mu$ g/mL) and the maximum no-toxic concentration (MNTC) was  $110.5 \pm 20.4$   $\mu$ M ( $22.6 \pm 4.2$   $\mu$ g/mL). For HEK-293, the cell viability was reduced by only 48.3% at the highest concentration used (1600  $\mu$ M [327  $\mu$ g/mL]); therefore, the  $CC_{50}$  value could not be determined and the MNTC value was considered  $\sim 100$   $\mu$ M. It seems that  $\beta$ -caryophyllene would be more toxic to cancerous cells than normal cells. The classification system of Halle and Göres (Halle & Göres, 1987) has defined  $CC_{50}$  values lower than 50  $\mu$ M as toxic. In accordance with this classification and the MTT results,  $\beta$ -caryophyllene would be rated as non-toxic for the

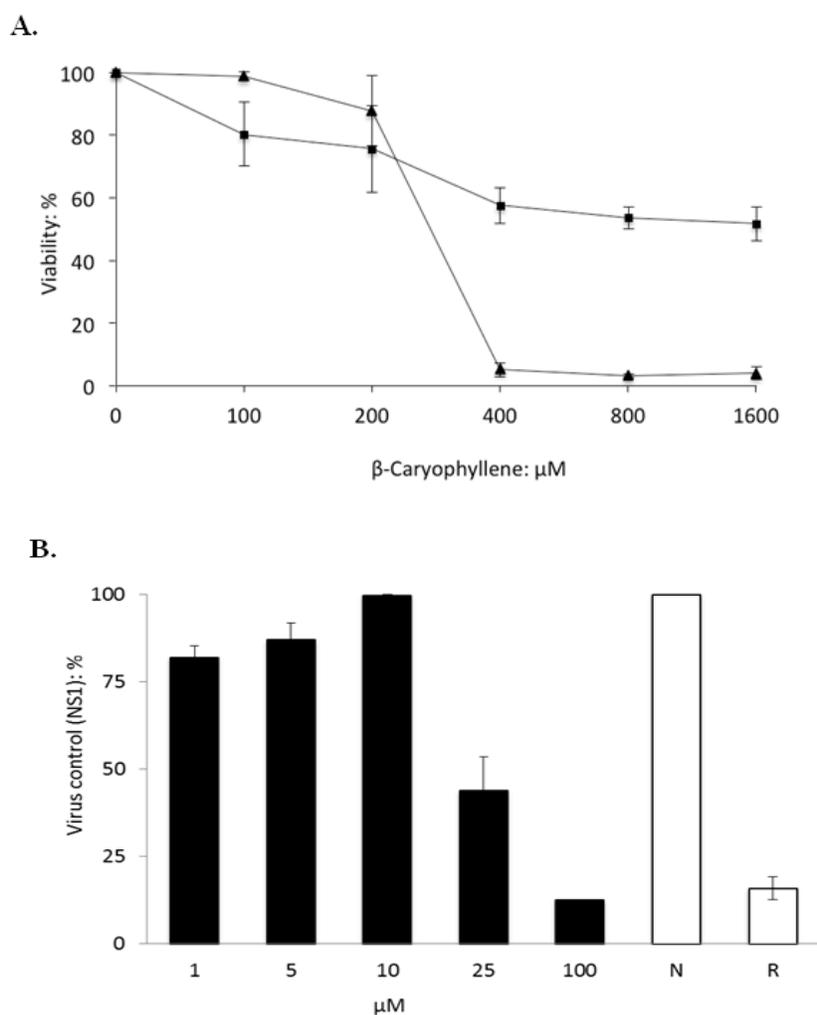
selected human cells. Similar results have been reported by others (Soares *et al.*, 2013).

A virus-NS1 yield reduction assay was next employed to study whether  $\beta$ -caryophyllene at non-toxic concentrations interferes with replication *in vitro* of DENV-2, ribavirin was included as a drug reference. In untreated HepG-2 cells, on average 33.5  $\pm 2.2$  PBU. of NS1 were detected. Addition of  $\beta$ -caryophyllene markedly reduced replication of the virus but not in a dose-dependent manner (Figure 5B). At a concentration of 25  $\mu$ M (5.1  $\mu$ g/mL) and 100  $\mu$ M (20.4  $\mu$ g/mL),  $\beta$ -caryophyllene inhibited NS1 by 56.2% and 87.5%, respectively. Relevant antiviral activity was confirmed in accordance with the 50% inhibitory concentration ( $IC_{50}$ ) and selectivity index ( $SI = CC_{50} / IC_{50}$ ):  $IC_{50}$  value of  $22.5 \pm 5.6$   $\mu$ M ( $4.6 \pm 1.1$   $\mu$ g/mL) and SI value of 71.1. It has been suggested  $IC_{50} < 25$   $\mu$ M and  $SI > 2$  as appropriate for future applications of pure compounds (Amoros *et*

*al.*, 1992; Cos *et al.*, 2006), thereby,  $\beta$ -caryophyllene presents promising antiviral activity against DENV-2.

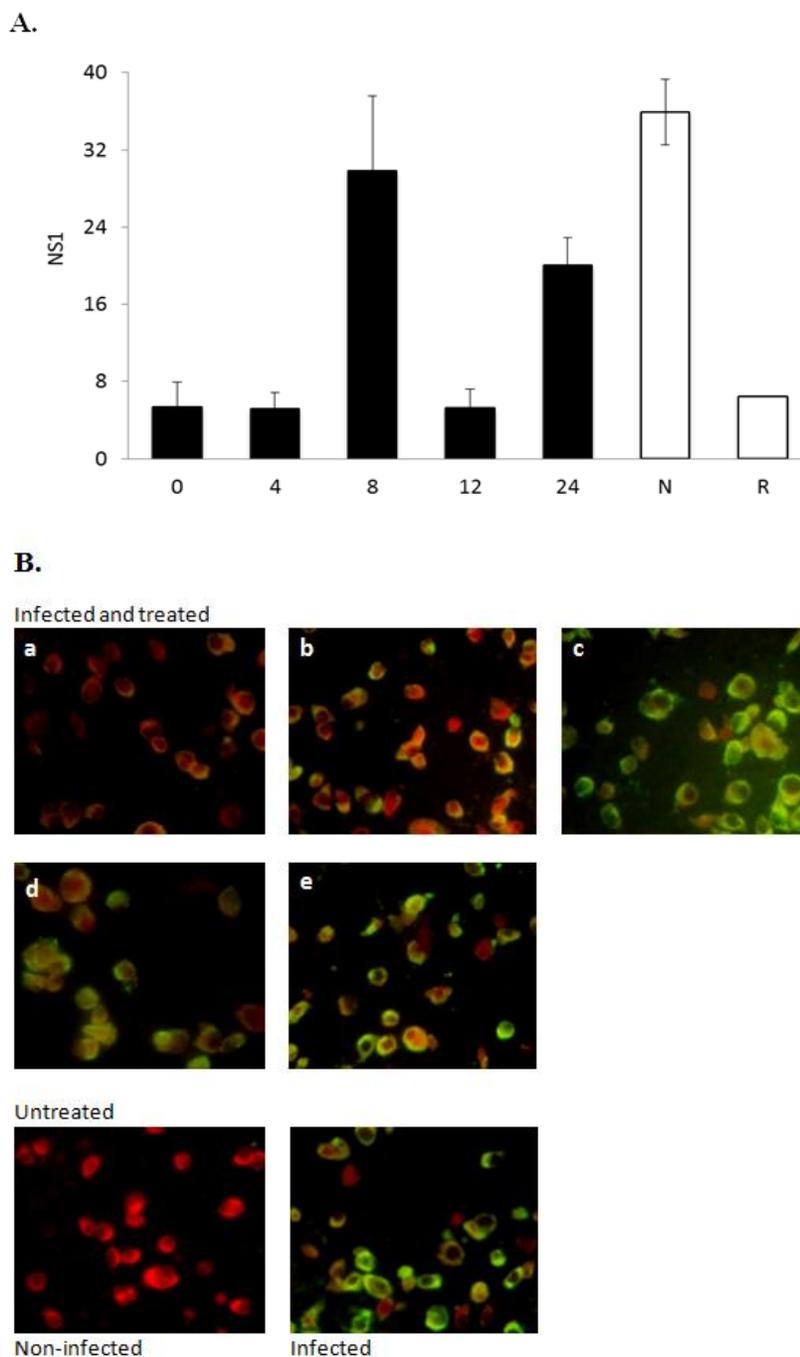
In order to investigate which stage of the virus replication cycle could be inhibited,  $\beta$ -caryophyllene (100  $\mu$ M) was added to virus-infected cells either immediately after virus adsorption or at several time points after that. At 72 h p.i., and supernatants were collected for quantification of NS1 and detection of infectious virus (Figure 6). Addition of compound at earlier stages p.i. resulted in marked reduction of viral replication: at 0 and 4 h, NS1 reduced 85.1% ( $5.3 \pm 2.6$  vs.  $35.9 \pm 3.4$  PBU;  $p <$

0.05.) and 85.6% ( $5.2 \pm 1.7$  vs  $35.9 \pm 3.4$  PBU.;  $p <$  0.05), respectively. Moreover, virus was not detected in C6/36 cells infected with supernatant from 0 h and few cells became infected with supernatant from 4 h.  $\beta$ -caryophyllene failed to inhibit DENV-2 replication when added 8 h after adsorption, concentration of NS1 was not markedly different to control ( $29.8 \pm 7.8$  vs.  $35.9 \pm 3.4$  PBU.;  $p >$  0.05) and high proportion of C6/36 cells were infected. When the compound was added 12 h and 24 h later, there was reduction of NS1 by, respectively, 85.3% ( $5.3 \pm 2.0$  vs.  $35.9 \pm 3.4$  PBU.;  $p <$  0.05) and 44.1% ( $20.1 \pm 2.8$  vs  $35.9 \pm 3.4$  PBU.  $p <$  0.05).



**Figure 5**

**Dose-response curves for  $\beta$ -caryophyllene against DENV-2. Cytotoxicity (A) is expressed as percentage of cell growth (viability) with respect to untreated control on HEK-293 cells (▲) and HepG-2 cells (■). Antiviral activity (B) was determined by means of a viral protein reduction assay and data are expressed as percentage of NS1 PanBio units of untreated virus control. N: untreated. R: treated with ribavirin**



**Figure 6**

**Time-of-drug-addition assay.**  $\beta$ -caryophyllene (100  $\mu$ M) was added to DENV-2-infected cells at various time points p.i., starting at 0 h (immediately after virus adsorption). At 72 h p.i., the culture supernatant was collected to measure NS1 protein (A) or to infect C6/36 cells (B). DENV-2 infection in C6/36 was detected by indirect fluorescent antibody test. N: untreated. R: treated with ribavirin. a,b,c,d,e: 0, 4, 8, 12, 24 h p.i.

The precise mechanism of the antiviral action of essential oil's components is still not fully understood. In this study, inhibition at 0 h and 4 h but

not at 8 h provide evidence that  $\beta$ -caryophyllene acts preventing virus-cell binding but not intracellular events of the DENV cycle. Inhibition at 12 h and 24

h, time when viral particles are present in the supernatant of HepG-2 infected cells (Thepparit *et al.*, 2004), suggests that the compound exerts a virucidal action. This is, inactivate DENV masking proteins on its envelope that are necessary for attachment and/or virus entry into the host cell. In a previous study we demonstrated that citral, a mixture of terpenoids present in some essential oils, inhibits the replication *in vitro* of yellow fever virus (from *flavivirus* genus like DENV) before and after adsorption to cell (Gómez *et al.*, 2013).

## CONCLUSIONS

This computational study has suggested that all DENV proteins are potential targets for simultaneous docking of several essential oils's components. Components identified with high affinity *in silico* for DENV proteins were: (-)- $\alpha$ -copaene, (+)- $\alpha$ -copaene,  $\beta$ -bourbonene, (-)-germacrene D, (+)-germacrene D, (-)-spathulenol, (+)-spathulenol,  $\beta$ -caryophyllene, caryophyllene oxide and (+)-epi-bicyclosquiphellandrene. Most components bind to their respective binding sites by creating hydrophobic interactions with important residues in the binding pockets.  $\beta$ -caryophyllene shows promising antiviral activity since presents a SI of 71.1 and was active at concentration of  $22.5 \pm 5.6 \mu\text{M}$ .

## ACKNOWLEDGEMENTS

This research was carried out thanks to financial support received from the Colombian government - Grants RC-0572-2012 and 11075192058-2010 (Patrimonio Autónomo del Fondo Nacional de Financiamiento para la Ciencia, la Tecnología y la Innovación, Francisco José de Caldas). The authors thank to the University of Cartagena, Cartagena (Colombia), Program to Support Research Groups, sponsored by the Vice-Rector for research (2010–2012).

## REFERENCES

Amoros M, Simoes C, Girre L, Sauvager F, Cormier M. 1992. Synergistic effect of flavones and flavonols against herpes simplex virus type 1 in cell culture. Comparison with the antiviral activity of propolis. **J Nat Prod** 55: 1732 - 1740.

Armaka M, Papanikolaou E, Sivropoulou A, Arsenakis M. 1999. Antiviral properties of isoborneol, a potent inhibitor of herpes simplex virus type 1. **Antivir Res** 43: 79 - 92.

Astani A, Reichling J, Schnitzler P. 2010. Comparative study on the antiviral activity of selected monoterpenes derived from essential oils. **Phytother Res** 24: 673 - 679.

Astani A, Reichling J, Schnitzler P. 2011. Screening for antiviral activities of isolated compounds from essential oils. **Evid Based Complement Alternat Med** 2011: 253643.

Bai F, Town T, Pradhan D, Cox J, Ashish, Ledizet M, Anderson JF, Flavell RA, Krueger JK, Koski RA, Fikrig E. 2007. Antiviral peptides targeting the West Nile virus envelope protein. **J Virol** 81: 2047 - 2055.

Balachandran V, Lalitha S, Rajeswari S. 2012. Density functional theory, comparative vibrational spectroscopic studies, NBO, HOMO-LUMO analyses and thermodynamic functions of N-(bromomethyl)phthalimide and N-(chloromethyl)phthalimide. **Spectrochim Acta A Mol Biomol Spectrosc** 91: 146 - 157.

Bicchi C, Rubiolo P, Ballero M, Sanna C, Matteodo M, Esposito F, Zinzula L, Tramontano E. 2009. HIV-1-inhibiting activity of the essential oil of *Ridolfia segetum* and *Oenanthe crocata*. **Planta Med** 75: 1331 - 1335.

Chu JJ, Yang PL. 2007. c-Src protein kinase inhibitors block assembly and maturation of dengue virus. **Proc Natl Acad Sci USA** 104: 3520 - 3525.

Cos P, Vlietinck A, Berghe D, Maes L. 2006. Anti-infective potential of natural products: How to develop a stronger *in vitro* 'proof-of-concept'. **J Ethnopharmacol** 106: 290 - 302.

DeLano, W.L. The PyMOL **Molecular Graphics System**; DeLano Scientific LLC: San Carlos, CA, USA.

Duschatzky C, Possetto M, Talarico L, García C, Michis F, Almeida N, Lampasona M, Schuff C, Damonte E. 2005. Evaluation of chemical and antiviral properties of essential oils from South American plants. **Antivir Chem Chemother** 16: 247 - 251.

Frisch MJ, Trucks GW, Schlegel HR, Scuseria GE, Robb MA, Cheeseman JR, Montgomery JA, Vreven T, Kudin KN, Burant JC, Millam JM, Iyengar SS, Tomasi J, Barone V, Mennucci B, Cossi M, Scalmani G, Rega N, Petersson GA, Nakatsuji H, Hada M, Ehara M, Toyota K, Fukuda R, Hasegawa J, Ishida M, Nakajima T, Honda Y, Kitao O, Nakai H,

- Klene M, Li X, Knox JE, Hratchian HP, Cross JB, Bakken V, Adamo C, Jaramillo J, Gomperts R, Stratmann RE, Yazyev O, Austin AJ, Cammi R, Pomelli C, Ochterski JW, Ayala PY, Morokuma K, Voth GA, Salvador P, Dannenberg JJ, Zakrzewski VG, Dapprich S, Daniels AD, Strain MC, Farkas O, Malick DK, Rabuck AD, Raghavachari K, Foresman JB, Ortiz JV, Cui Q, Baboul AG, Clifford S, Cioslowski J, Stefanov BB, Liu G, Liashenko A, Piskorz P, Komaromi I, Martin RL, Fox DJ, Keith T, Al-Laham MA, Peng CY, Nanayakkara A, Challacombe M, Gill PMW, Johnson B, Chen W, Wong MW, Gonzalez C, Pople JA. 2003. **Gaussian03**. Gaussian, Inc., Pittsburgh, PA, USA.
- Garozzo A, Timpanaro R, Stivala A, Bisignano G, Castro A. 2011. Activity of *Melaleuca alternifolia* (tea tree) oil on influenza virus A/PR/8: study on the mechanism of action. **Antiviral Res** 89: 83 - 88.
- Gómez LA, Stashenko E, Ocazonez RE. 2013. Comparative study on *in vitro* activities of citral, limonene and essential oils from *Lippia citriodora* and *L. alba* on yellow fever virus. **Nat Prod Commun** 8: 249 - 252.
- Halle W, Göres E. 1987. Is the prediction of LD50 using cell cultures generally valid?. **Pharmazie** 42: 245 - 248.
- Hegazy G, Ali H. 2012. Design, synthesis, biological evaluation, and comparative Cox1 and Cox2 docking of p-substituted benzylidene amino phenyl esters of ibuprofenic and mefenamic acids. **Bioorgan Med Chem** 20: 1259 - 1270.
- Herrero LJ, Zakhary A, Gahan ME, Nelson MA, Herring BL, Hapel AJ, Keller PA, Obeysekera M, Chen W, Sheng KC, Taylor A, Wolf S, Bettadapura J, Broor S, Dar L, Mahalingam S. 2013. Dengue virus therapeutic intervention strategies based on viral, vector and host factors involved in disease pathogenesis. **Pharmacol Ther** 137: 266 - 282.
- Kampmann T, Yennamalli R, Campbell P, Stoermer MJ, Fairlie DP, Kobe B, Young PR. 2009. *In silico* screening of small molecule libraries using the dengue virus envelope E protein has identified compounds with antiviral activity against multiple flaviviruses. **Antiviral Res** 84: 234 - 241.
- Knox JE, Ma NL, Yin Z, Patel SJ, Wang WL, Chan WL, Ranga Rao KR, Wang G, Ngew X, Patel V, Beer D, Lim SP, Vasudevan SG, Keller TH. 2006. Peptide inhibitors of West Nile NS3 protease: SAR study of tetrapeptide aldehyde inhibitors. **J Med Chem** 49: 6585 - 6590.
- Koch C, Reichling J, Schnee J, Schnitzler P. 2008. Inhibitory effect of essential oils against herpes simplex virus type 2. **Phytomedicine** 15: 71 - 78.
- Lauro G, Romano A, Riccio R, Bifulco G. 2011. Inverse virtual screening of antitumor targets: pilot study on a small database of natural bioactive compounds. **J Nat Prod** 74: 1401 - 1407.
- Li Z, Khaliq M, Zhou Z, Post CB, Kuhn RJ, Cushman M. 2008. Design, synthesis, and biological evaluation of antiviral agents targeting flavivirus envelope proteins. **J Med Chem** 51: 4660 - 4671.
- Lim S, Rahman M, Tejo V. 2011. Structure-based and ligand-based virtual screening of novel methyltransferase inhibitors of the dengue virus. **BMC Bioinformatics** 12: S24.
- Lim SP, Wang QY, Noble CG, Chen YL, Dong H, Zou B, Yokokawa F, Nilar S, Smith P, Beer D, Lescar J, Shi PY. 2013. Ten years of dengue drug discovery: progress and prospects. **Antiviral Res** 100: 500 - 519.
- Ma L, Jones CT, Groesch TD, Kuhn RJ, Post CB. 2004. Solution structure of dengue virus capsid protein reveals another fold. **Proc Natl Acad Sci USA** 101: 3414 - 3419.
- Maldonado W, Olivero J. 2011a. Potential interaction of natural dietary bioactive compounds with COX-2. **J Mol Graph Model** 30: 157 - 166.
- Maldonado W, Olivero J, Ortega C. 2011b. Searching of protein targets for Alpha lipoic acid. **J Braz Chem Soc** 22: 2250 - 2259.
- Maldonado W, Olivero J. 2012. Food-Related Compounds That Modulate Expression of Inducible Nitric Oxide Synthase May Act as Its Inhibitors. **Molecules** 17: 8118 - 8135.
- Meneses R, Torres F, Stashenko E, Ocazonez R. 2009a. Aceites esenciales de plantas Colombianas inactivan el virus del dengue y el virus de la fiebre amarilla. **Salud-UIS** 3: 236 - 243.
- Meneses R, Ocazonez R, Torres F, Stashenko E. 2009b. Essential oils from seven aromatic plants grown in Colombia: Chemical composition, cytotoxicity and *in vitro*

- virucidal effect on the dengue virus. **Int J Essent Oil Ther** 3: 1 - 7.
- Meneses R, Ocazonez R, Martínez J. 2009c. Inhibitory effect of essential oils obtained from plants grown in Colombia on yellow fever virus replication *in vitro*. **Ann Clin Microbiol Antimicrob** 8:8.
- Murrell S, Wu SC, Butler M. 2011. Review of dengue virus and the development of a vaccine. **Biotechnol Adv** 29: 239 - 247.
- Noble C, Chen Y, Dong H, Gu F. 2010. Strategies for development of dengue virus inhibitors. **Antiviral Res** 85: 450 - 462.
- O'Boyle NM, Banck M, James CA, Morley C, Vandermeersch T, Hutchison GR. 2011. Open Babel: An open chemical toolbox. **J Cheminform** 3: 33.
- Ocazonez RE, Cortés FM, Villar LA, Gómez SY. 2006. Temporal distribution of dengue virus serotypes in Colombian endemic area and dengue incidence. Re-introduction of dengue-3 associated to mild febrile illness and primary infection. **Mem Inst Oswaldo Cruz** 101: 725 - 731.
- Ocazonez R, Meneses R, Torres F, Stashenko E. 2010. Virucidal activity of Colombian *Lippia* essential oils on dengue virus replication *in vitro*. **Mem Inst Oswaldo Cruz** 105: 304 - 309.
- Perera R, Kuhn R. 2008. Structural proteomics of dengue virus. **Curr Opin Microbiol** 11: 369 - 377.
- Podvinec M, Lim S, Schmidt T, Scarsi M, Wen D, Sonntag LS, Sanschagrin P, Shenkin PS, Schwede T. 2010. Novel Inhibitors of Dengue Virus Methyltransferase: Discovery by in Vitro-Driven Virtual Screening on a Desktop Computer. **Grid J Med Chem** 53: 1483 - 1495.
- Puig-Basagoiti F, Tilgner M, Forshey BM, Philpott SM, Espina NG, Wentworth DE, Goebel SJ, Masters PS, Falgout B, Ren P, Ferguson DM, Shi PY. 2006. Triaryl pyrazoline compound inhibits flavivirus RNA replication. **Antimicrob Agents Chemother** 50: 1320 - 1329.
- Rossi SL, Nasar F, Cardoso J, Mayer SV, Tesh RB, Hanley KA, Weaver SC, Vasilakis N. 2012. Genetic and phenotypic characterization of sylvatic dengue virus type 4 strains. **Virology** 423: 58 - 67.
- Saddi M, Sanna A, Cottiglia F, Chisu L, Casu L, Bonsignore L, De Logu A. 2007. Antiherpevirus activity of *Artemisia arborescens* essential oil and inhibition of lateral diffusion in Vero cells. **Ann Clin Microbiol Antimicrob** 6:10. Available from: <http://www.annclinmicrob.com/content/6/1/10>
- Sampath A, Padmanabhan R. 2009. Molecular targets for flavivirus drug discovery. **Antiviral Res** 81: 6 - 15.
- Schnitzler P, Schuhmacher A, Astani A, Reichling J. 2008. *Melissa officinalis* oil affects infectivity of enveloped herpes viruses. **Phytomedicine** 15: 734 - 740.
- SYBYL 2007. **Molecular modeling software**, Version 8.1. Tripos: St. Louis, MO, USA, 2007.
- Soares DC, Portella NA, Ramos MF, Siani AC, Saraiva EM. 2013. Trans- $\beta$ -Caryophyllene: An effective antileishmanial compound found in commercial copaiba oil (*Copaifera* spp.). **Evid Based Complement Alternat Med** 2013: 761323.
- Stevens AJ, Gahan ME, Mahalingam S, Keller PA. 2009. The medicinal chemistry of dengue fever. **J Med Chem** 52: 7911 - 7926.
- Trott O, Olson AJ. 2009. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization and multithreading. **J Comput Chem** 31: 455 - 461.
- Thepparit C, Phoolcharoen W, Suksanpaisan L, Smith DR. 2004. Internalization and propagation of the dengue virus in human hepatoma (HepG-2) cells. **Intervirology** 47: 78 - 86.
- Wolber G, Langer T. 2005. LigandScout: 3-D pharmacophores derived from protein bound ligands and their use as virtual screening filters. **J Chem Inf Model** 45: 160 - 169.
- Wu QF, Wang W, Dai XY, Wang ZY, Shen ZH, Ying HZ, Yu CH. 2012. Chemical compositions and anti-influenza activities of essential oils from *Mosla dianthera*. **J Ethnopharmacol** 139: 668 - 671.
- Wu S, Patel KB, Booth LJ, Metcalf JP, Lin HK, Wu W. 2010. Protective essential oil attenuates influenza virus infection: an *in vitro* study in MDCK cells. **BMC Complement Altern Med** 10: 69.

Zaheer-ul-Haq, Halim SA, Uddin R, Madura JD. 2010. Benchmarking docking and scoring protocol for the identification of potential acetylcholinesterase inhibitors. **J Mol Graph Model** 28: 870 - 882.