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Essential oil of *Cordia curassavica* (Jacq) Roem. & Schult: *in vitro* and *in silico* evaluation of effects on dengue virus replication and cytokines production

[Aceite esencial de *Cordia curassavica* (Jacq) Roem. & Schult: evaluación *in vitro* e *in silico* del efecto sobre la replicación del virus dengue y la producción de citoquinas]

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Abstract: The lack of effective conventional therapies against dengue has created an interest in herbal preparations as alternative therapies. In the present study, *in vitro* effects of *Cordia curassavica* essential oil (EO) on both dengue virus replication and cytokine production were examined. Predictions of molecular interactions between EO compounds and virus and cell proteins were performed with AutoDock Vina. The EO inhibited replication of dengue virus serotypes at IC_{50} < 30 µg/mL, and it reduced 87% TNF-α, 67% IL-8 and 46% IFN-α in LPS-stimulated PBMCs. The main EO compounds were trans-β-caryophyllene (21.4%), germacrene D (17.8%), α-copaene (16.5%), trans-β-guaiene (8.2%), and α-pinene (6.0%). The first two compounds, δ-cadinene, α-muurolene, α-cubebene and β-burbonene were coupled to proteins involved in the TLR-4 cytokine effector pathway. 3,7-Guaiadiene was coupled to the viral E and C proteins. This study demonstrates the potential of *C. curassavica* EO as a starting point for discovering novel therapeutic for dengue.

Keywords: *Cordia curassavica*; Essential oil; Dengue virus; Cytokines; Phytotherapeutic

Resumen: La falta de terapias eficaces para el dengue ha suscitado interés por preparados herbales como terapias alternativas. En el presente estudio se examinaron efectos *in vitro* del aceite esencial (AE) de *Cordia curassavica* sobre la replicación del virus dengue y producción de citoquinas. Se realizaron predicciones de interacciones moleculares entre los compuestos del AE y proteínas virales y celulares con AutoDock Vina. El AE inhibió la replicación de serotipos del virus a CI_{50} < 30 µg/mL y redujo 87% TNF-α, 67% IL-8 y 46% IFN-α en MNCP. Los principales compuestos del AE fueron trans-β-cariofileno, germacreno D, α-copaeno, trans-β-guaieno y α-pineno. Los dos primeros compuestos, el δ-cadineno, el α-muuroleno, el α-cubebeno y el β-burboneno se acoplaron a proteínas implicadas en la vía efectora de citoquinas TLR-4. El 3,7-guaiadiene se acopló a las proteínas virales E y C. Este estudio demuestra el potencial del AE de *C. curassavica* como punto de partida para descubrir nuevas terapias para el dengue.

Palabras clave: *Cordia curassavica*; Aceite esencial; Virus dengue; Citoquinas; Fitoterapéutico

INTRODUCTION

Aromatic plant essential oils (EOs) contain a complex mixture of phytochemicals. Their main components include monoterpenes, sesquiterpenes, phenylpropanoids, alcohols, aliphatic aldehydes and esters (Manion & Widder, 2017). EOs are known for their multiple biological activities, they are present in many herbal formulations used to prevent and treat many life-threatening diseases in humans (Raut & Karuppayil, 2014; Tariq *et al*., 2019). There is sufficient evidence of antiviral properties of EOs *in vitro* and *in vivo* studies (Tariq *et al*., 2019; Ma & Yao, 2020). EOs have been found to stimulate the immune system, suppress the response involved in inflammation, and decrease cytokine production. In general, certain components of EOs determine their biological activities (Manion & Widder, 2017; Sandner *et al*., 2020).

Cordia L is a genus of trees or shrubs belonging to the family Boraginaceae with more than 409 species distributed widely in Central and South America, India, Asia, and Africa (Oza & Kulkarni, 2017; Andrade *et al*., 2022). The most cited species is *C. curassavica* (Jacq) Roemer & Schultes (official name), it has *C. verbenacea* A. DC., *Varronia curassavica* Jacq. and *Cordia chacoensis* as synonyms. *C. curassavica* is a medicinal plant traditionally used to treat inflammation, ulcers, arthritis, and pain (Oza & Kulkarni, 2017; Martim *et al*., 2020). *C. curassavica* EO is recognized for its efficacy and safety as an analgesic and anti-inflammatory agent, it is used to treat myofascial pain and tendonitis (Martim *et al*., 2020). The EO extracted from its leaves is used in the formulation of the topic anti-inflammatory phytomedicine Acheflan®, which is widely used in Brazil and other countries (Dutra *et al.*, 2016). Antimicrobial properties of *C. curassavica* EO on bacteria and yeast are reported (Rodrigues *et al*., 2012). Studies on antiviral activities have been not yet documented.

Dengue virus (DENV) has a single-stranded, ~11 kb, positive-sense RNA genome, which encodes for a single polyprotein that is cleaved into 10 individual proteins (Nasar *et al*., 2020). Three proteins are present in the viral particle (virion), that is, E (envelope), prM/M (membrane), and C (capsid). Seven non-structural (NS) proteins, that is, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. are mainly involved in virus entry, attachment, genome replication, and virion assembly. DENV causes a wide spectrum of clinical manifestations ranging from mild febrile illness to severe life-threatening dengue (Bhatt *et al*., 2021). Dengue remains a major public health burden in Latin American and Caribbean countries, with a significant number of hospitalizations and deaths each year (Perez *et al*., 2019). Currently, there are no antivirals to treat dengue despite extensive research on synthetic DENV inhibitors (Low *et al*., 2017; Lim, 2019). There is a need for alternative therapies that can shorten the duration of the disease and prevent the development of severe dengue. Traditional herbal preparations are being validated as supportive tools to minimize the risk of severe dengue (Sood *et al*., 2015; Singh & Rawat, 2017; Sathyapalan *et al.*, 2020; Lim *et al*., 2021).

Fourteen species of *Cordia* are recognized as medicinal plants in Colombia (Bernal *et al*., 2011). The aim of the present work was to study the chemical composition of the EO from *C. curassavica* grown in Colombia and evaluate its potential as a primary source of herbal preparations for the dengue treatment. *In vitro* effects on both DENV serotypes replication and cytokine production by human peripheral blood mononuclear cells (PBMCs) were investigated. Predictions of molecular interactions between EO compounds and virus and cell proteins were also investigated.

MATERIALS AND METHODS

Plant

C. curassavica was collected from the experimental pilot of CENIVAM (Centro Nacional de Investigaciones para la Agroindustrialización de Especies Vegetales Aromáticas y Medicinales Tropicales at Universidad Industrial de Santander, Bucaramanga, Colombia). The plant was botanically identified by Prof. Dr. José Luis Fernandez, a voucher specimen (COL559446) was deposited at the Colombian National Herbarium (Bogota). Fresh leaves and flowers from plants were employed for EO extraction.

EO extraction and chemical analysis

Microwave-assisted hydro-distillation method was used for extracting EO as described (Stashenko *et al.*, 2013). Plant leaves were subjected to hydro-distillation 2 h in a Clevenger-type apparatus, which was placed inside a domestic microwave oven (Samsung MS-1242zk, 1600 W, 2.4 GHz) with a side

orifice, through which an external glass condenser joined the 2-L round flask with the plant material (210 g) and water (300 mL). The oven was operated for 45 min (3 x 15 min) at full power, and the EO obtained was dried using anhydrous sodium sulfate. Stock solutions of EO were prepared in dimethyl sulfoxide (DMSO, 0.5% final concentration). The EO chemical composition was investigated by gas chromatography-mass spectrometry (GC/MS) as described (Stashenko *et al.*, 2013). Analysis were performed using an Agilent Technologies 6890 N Series Network System chromatograph coupled to a 5975 Inert XL mass spectrometer (Palo Alto, CA, USA), and equipped with a DB-5MS fused-silica capillary column (5% phenylmethylsiloxane; 60 m, 0.25 µm, film thickness 0.25 lm; J & W Scientific, Folsom, CA, USA). The mass selective detector was operated in electron ionization mode (70 eV) in a mass scan range from m/z 40 to 350. Helium was the carrier gas at a flow rate of 1 mL/min. Compounds were identified by matching their linear GC retention indices, and in the NIST (2017) mass spectral data base, using MS ChemStation AT G1701-DA software.

Virus and cells

DENV reference strains (C.D.C., Dengue Branch, USA) were used: DENV-1/Hawaii, DENV-2/ NGC, DENV-3/H-87 and DENV-4/H-241. Virus stocks were prepared and titrated on Vero cells using the plaque method as described (Meneses *et al.*, 2009). Viruses were stored at -80ºC until needed. HepG-2 cells were cultured in Dulbecco's medium (DMEM-F12, Gibco), and Vero cells in minimal essential medium (MEM, Gibco). PBMCs were isolated from peripheral blood of healthy donors. Briefly, blood was diluted with RPMI 1640 medium (Gibco) and layered on Ficoll Hypaque 1077 (Sigma Aldrich), followed by centrifugation at 2800 rpm for 30 min at room temperature; next, the upper layer was discarded and the fluffy layer at the interphase was harvested, rinsed, and re-suspended in RPMI-1640 culture medium.

MTT assay

The MTT assay was used for evaluation of EO cytotoxicity as described (Meneses *et al.*, 2019). Cells in 96-well plate were treated with EO over a six-point concentration, and then the microplates were incubated at 37°C in a 5% CO_2 humidified atmosphere. Not-treated cells were run in parallel. MTT (Sigma Aldrich, Co) solution (5 mg/mL) was added to each plate well and the plate was kept at 37ºC for 4 h. Next, DMSO solution (100 µL) was added to each well and the absorbance values were measured at 580 nm using a plate reader. Cell viability was calculated using the formula: % viability=[O.D. EO sample / O.D. cell control) × 100].

In-situ ELISA assay

The assay evaluated the inhibitory effect of the test EO on DENV replication in Vero cells. A standardized routine procedure was used. Virus (MOI of 0.5) was adsorbed for 1 h onto cells grown in 96-well plates; the virus inoculum was aspirated and the monolayer was rinsed; and next fresh culture medium containing EO (five-point concentration) was added. Not-treated virus-infected cells and not-infected not-treated cells were run in parallel. The virus was allowed to replicate for 5 days, and the presence of DENV-E protein on cells was determined by ELISA with antibody anti-DENV MAB 4G2 antibody (C.D.C., Dengue Branch, USA). Percent reduction of DENV-E protein was calculated using the formula: %DENV-E = [(O.D. treated-virus - O.D. not-treated virus) / (O.D. cell control – O.D. not-treated virus) × 100].

Cytokine assay

The assay evaluated the effect of the test EO on cytokines secretion in LPS-stimulated PBMCs. Cells were seeded in 24-well plates (10^5 cells/well) in culture medium containing LPS (100 ng/mL; Sigma Cat. # L2654). Next, EO (50 µg/mL) was added in a final volume of 500 µL, and the plates were incubated 24 h at 37°C in a 5% CO_2 humidified atmosphere. Positive (LPS-stimulated cells treated with dexamethasone at 50 µg/mL) and negative (LPS-stimulated non-treated cells) controls were run in parallel. The plates were centrifuged (3000 rpm, 10 minutes) to obtain clarified culture medium for quantification of TNF-α (tumor necrosis factor alpha), IL-8 (interleukin 8), IFN-γ (interferon gamma), and RANTES (regulated upon activation, normal T cell expressed and secreted) by using ELISA kits (Invitrogen). Percent inhibition of cytokine was calculated using the formula: % inhibition = [(cytokine level in the presence of EO - cytokine level in the absence of EO/cytokine level

in the presence of EO)] x 100.

Docking analysis

Target DENV-2 proteins

3D crystal structures for proteins were download from the Protein Data Bank (PDB). Viral proteins with important roles in the virus replication cycle (Nassar *et al*. 2020) were screened: E (PDB1OAN), prM (PDB3C5X), and C (PDB1R6R), which form the viral particle; NS1 (PDB4O6B), which is a pathogenic factor; the complex NS2B-NS3 protease (PDB2FOM), which is essential for viral replication; and the NS5 protein, which is a large oligomer with a methyltransferase (MTase) domain at the N-terminal and an RNA-dependent RNA polymerase (RdRp) domain at its C-terminal that play key enzymatic roles such as catalyzing 5'-RNA methylation and RNA synthesis, respectively.

Target cell proteins

3D crystal structures for proteins were download from the Protein Data Bank (PDB). Proteins involved in the Toll-like receptor 4 (TLR-4) cytokine effector pathway (Park *et al*., 2009) were screened: TLR4-MD-2 (myeloid differentiation 2 protein) complex (PDB3XFI); the multiprotein complex Myddosome (MyD88, myeloid differentiation primary response gene 88 protein / IL-1 receptor-associated kinases 2 and 4, IRAK2/IRAK4) (PDB 3MOP); and NF-κB (nuclear factor kappa B) protein (PDB 1NFI).

Protein structures were optimized by removing water and solvent molecules and co-crystallized ligands through the PyMOL 2.3.0 software followed by energy minimization (1000 kJ/mol) using the OPLS force field-AA/L implemented in the GROMACS 5.0 package; structures were solvated using a cubic box and the SPC-216 water model under 1 ns long, 310.15 K, 1 bar, and 0.09 M Na^+Cl^- conditions; kollman charges were added to each atom, and the non-polar hydrogen atoms were merged to the protein structure employing Autodock Tools (Morris *et al.*, 2009).The structures were then saved in PDBQT file format for docking analysis.

Ligands

Structures of EO compounds and reference compounds (epigallocatechin gallate, baicalin, CID_54692801, imperatorin and quinolone) were retrieved from ZINC v.1.0 (**www.zinc.docking.org**), PubChem (**https://pubchem.ncbi.nlm.nih.gov**) and chEBI (**https://www.ebi.ac.uk/chebi**) databases. Compounds were formatted in the MOL2 file. Structures were optimized by adding gasteiger charges to each atom, and merging non-polar hydrogen atoms using AutoDock Tools The structures were saved in PDBQT file format.

Molecular docking was performed using the Autodock Vina 1.5.6 software (Trott & Olson, 2010). Default parameters were used, and the search exhaustiveness parameter was set to 100. For each ligand, 27 docked conformations were generated using global docking simulations, i.e., the grid box was defined to cover all protein structure to search for the best binding site in the protein. Three simulations were performed for each ligand-protein pair by using seeds 6, 12, and 18. The binding free energy was approximated by the average of docking scores for each protein. Discovery Studio Visualizer v21.1.0.20298 was used to view ligand-protein interaction.

Data analysis

R software for Windows v.5 (**http://www.R-project.org**) was used. Dose-response curve was plotted and the half maximal cytotoxic concentration (CC_{50}) and maximal inhibitory concentration (IC_{50}) were determined from the plot. Level of significance in cytokine assays were calculated by One-Way ANOVA followed by Turkey post- hoc test. Results were represented as means ± standard deviation.

RESULTS

EO chemical composition

The GC/MS analysis identified a total of twenty compounds that represented 96.2% of the EO, from which 70% corresponded to sesquiterpenes and 30% to oxygenated monoterpenes (Table No. 1). The most abundant compounds found were: *trans*-β-caryophyllene (21.4%), germacrene D (17.8%), α-copaene (16.5%), *trans*-β-guaiene (8.2%) and α-pinene (6.0%), followed by bornyl acetate (3.5%), δ-cadinene (3.2%) and α-humulene (2.7%). All EO compounds identified in this study, except 3,7-guaiadiene, are reported as chemical constituents of *C. curassavica* EO (Facanali *et al*., 2020; Andrade *et al*., 2022).

Table No. 1
Cordia curassavica essential oil chemical composition

Compound	Linear retention indices		Relative GC area, %
	Experimental	Literature a	
α-Pinene [b]	938	936	6.0
Sabinene	972	973	0.4
β-Pinene [b]	980	978	2.9
β-Myrcene [b]	990	989	0.5
1,8-Cineole [b]	1034	1032	0.7
Bornyl acetate	1290	1284	3.5
α-Ylangene	1372	1370	0.8
α-Copaene	1374	1376	16.5
β-Bourbonene	1386	1384	2.7
α-Cubebene	1390	1387	1.7
trans-β-Caryophyllene [b]	1430	1420	21.4
β-Copaene	1434	1433	0.8
3,7-Guaiadiene	1448	1444	1.4
α-Humulene [b]	1460	1453	2.7
γ-Muurolene	1474	1476	0.8
Germacrene D	1482	1481	17.8
α-Muurolene	1500	1498	2.9
trans-β-Guaiene	1502	1499	8.2
δ-Cadinene	1520	1523	3.2
Caryophyllene oxide [b]	1588	1581	1.3

[a]Babushok *et al*., 2011. [b]Standard compounds (Sigma-Aldrich, St. Louis, MO, USA) were used

Antiviral action of C. curassavica EO

***In vitro* analysis**

Prior to the assessment of antiviral activity, cytotoxicity of the EO in Vero cells was evaluated. The MTT assay revealed that 80% of cells were viable after 5 days of treatment with EO at concentration of 30 µg/mL, the CC_{50} value was 41 µg/mL (Figure No. 1A). The antiviral potency of the EO was examined at range of non-toxic concentrations, levels of viral E protein on cell membrane was used to monitor virus replication. The treatment with EO inhibited replication of three DENV serotypes after adsorption on Vero cells at IC_{50} values lower than 30 µg/mL (Figure No. 1B): DENV-1, 26 ± 6.1 µg/mL; DENV-2, 24 ± 4.3; DENV-3, 18 ± 3.4 µg/mL; and DENV-4, 26 ± 8.3 µg/mL.

In-silico analysis

All twenty compounds identified in the GC/MS analysis were docked with DENV-2 proteins, which have roles in virus internalization into the cell (E, pM and C), polyprotein cleavage (protease complex NS2B/NS3), and viral RNA replication (NS1 and NS5). AutoDock Vina binding energies are presented in Supplementary Table No. 1. Five sesquiterpenes including 3,7-guaiadiene, α-muurolene, tran-β-guaiene, γ-muurolene, α-cubebene exhibited the highest docking affinity (energies from -8.03 kcal/mol to -7.01 kcal/mol) with E protein at the βOG (β-octylglucoside) pocket. 3,7-Guaiadiene (-7.08 kcal/mol) also bound to C protein at the α2-α4 dimer interface. Hydrophobic interactions are the main driving force in compound-protein interactions. The best docking results per DENV protein and the contact amino acid residues of the complex are presented in Table No. 2 and Figure No. 2. The docking analysis did not predict interactions between sesquiterpenes and the other DENV target proteins including prM, NS1, the protease NS2B/NS3 complex, and NS5-MTase domain (binding energies ranged from -6.88 kcal/mol to -5.02 kcal/mol). In

addition, the analysis did not predict molecular interactions between monoterpenes present in the EO and the selected DENV proteins (energies from -6.45 kcal/mol to -4.01 kcal/mol).

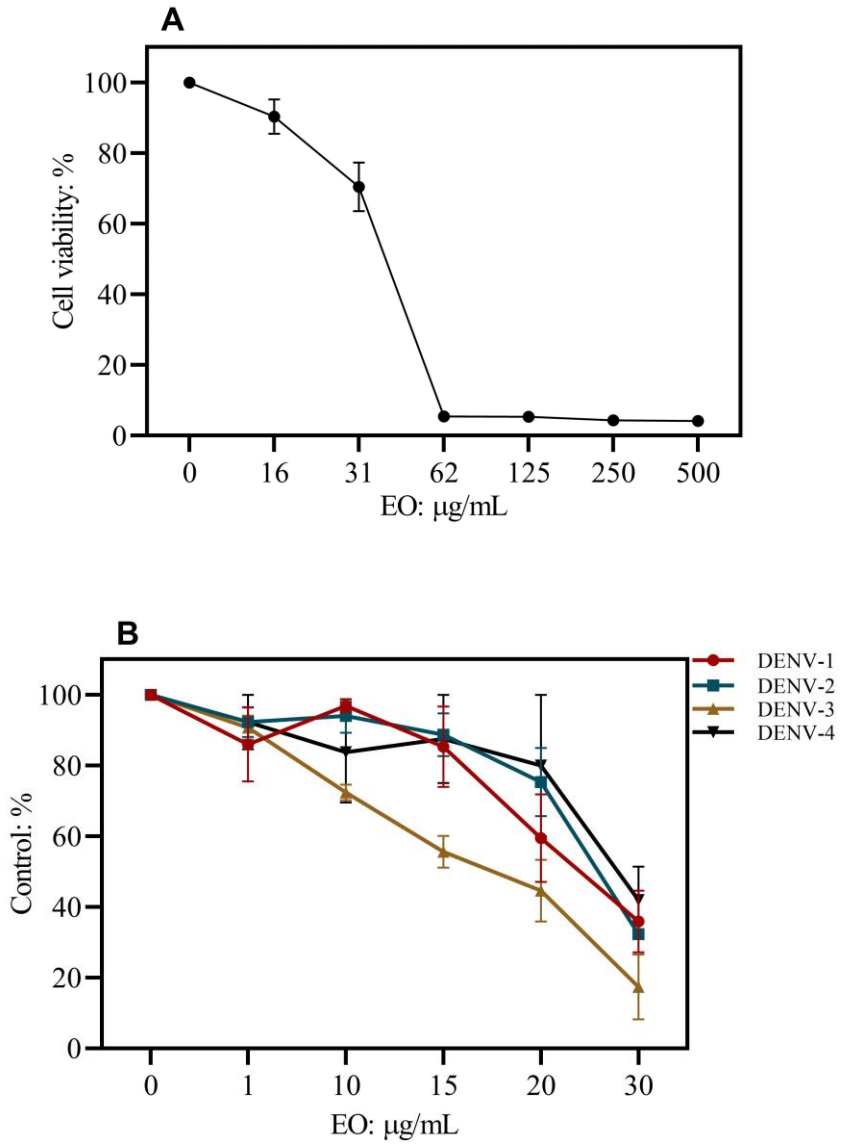


Figure No. 1
***In vitro* antiviral effect of *Cordia curassavica* essential oil (EO) on Dengue Virus (DENV) replication. A. Cytotoxicity, non-infected cells were treated with EO at the indicated concentrations and viability was measured in the MTT assay. B. Antiviral effect, virus (DENV-1, -2, -3 and -4) was adsorbed on Vero cells and allowed to replicate for 5 days in the presence of EO, viral E protein was measured by *in-situ* cell ELISA. Data are the mean ± SD of three independent experiments in triplicate**

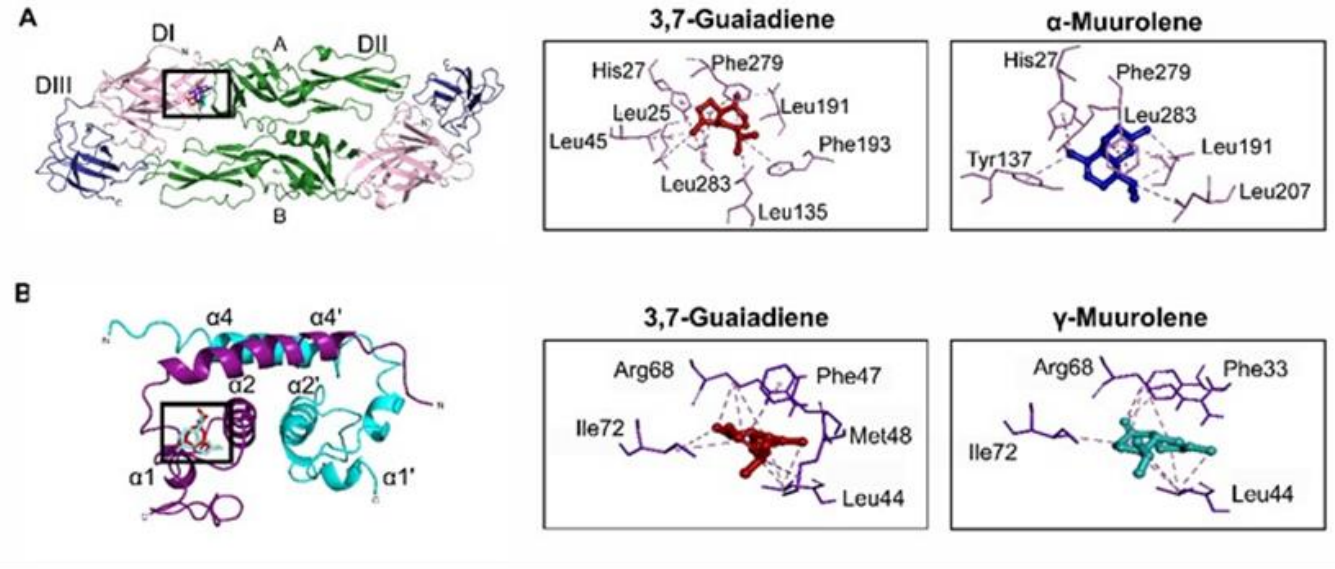
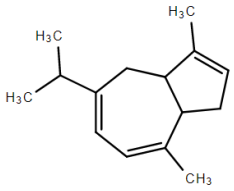
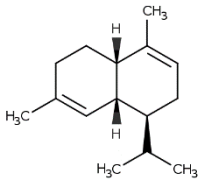
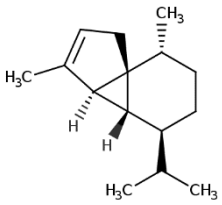
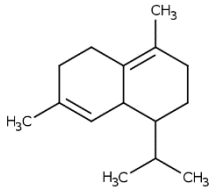
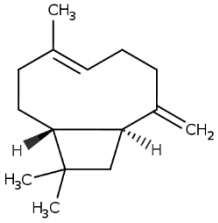
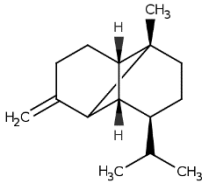
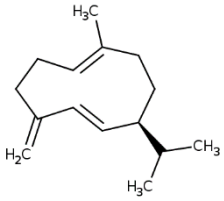
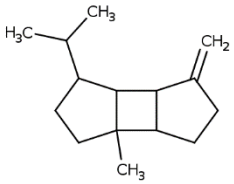


Figure No. 2
Predicted binding sites of sesquiterpenes present in Cordia curassavica essential oil to DENV-2 proteins to. A. Envelope (E) protein with 3,7-guaiadiene (-8.03 kcal/mol) and α-muurolene (-7.31 kcal/mol). B. Capside (C) protein with 3,7-guaiadiene (-7.08 kcal/mol) and γ-muurolene (-6.79 kcal/mol)

Table No. 2
Sesquiterpenes present in *Cordia curassavica* essential oil with the lowest negative binding energy with target proteins

Name	Structural formula	Target protein	Amino acids*	Kcal/mol
3,7-Guaiadiene		DENV-E	His27, Leu25, Leu45, Leu135, Leu191, Phe193, Phe279, Leu283.	-8.03
		DENV-C	Leu44, Phe47, Met48, Arg68, Ile72.	-7.08
		MD-2	Ile44, Ile46, Ile63, Tyr65, Phe76, Val113, Phe147.	-7.57
α-Muurolene		DENV-E	His27, Tyr137, Leu191, Leu207, Phe279, Leu283.	-7.31
α-Cubebene		MD-2	Ile44, Ile46, Ile63, Tyr65, Phe76, Val113, Phe147, Leu149.	-7.69
		IRAK2/4	Lys34, Lys44, Ile45, Trp63, Met66, Phe93	-7.40

δ-Cadinene		MD-2	Ile44, Ile63, Tyr65, Phe76, Phe147, Leu149.	-7.69
trans-β-Caryophyllene		MD-2	Ile44, Ile63, Ile46, Phe76, Phe104, Ile117, Val135, Leu149, Phe147, Phe151.	-7.66
β-Copaene		MD-2	Ile44, Ile46, Ile63, Leu74, Ile117, Leu146, Phe147.	-7.58
Germacrene D		MD-2	Ile44, Ile63, Tyr65, Phe76, Leu74, Phe147.	-7.56
β-Bourbonene		IRAK/2/4	Lys34, Lys44, Trp62, Trp63, Met66, Phe93.	-7.43

*** Hydrophobic interactions with ligand**

Effect on cytokine production

In vitro analysis

Prior to the assessment of cytokine production, cytotoxicity of the EO in PBMCs was evaluated. The MTT assay revealed that 75% of the cell were viable after three days of treatment with EO at concentration of 50 µg/mL, the CC_{50} value was 96 ± 3.0 µg/mL (Figure No. 3A). LPS-stimulated PBMCs treated with

EO at concentration of 50 µg/mL reduced TNF-α (86.9%; $p<0.01$) and IL-8 (67.2%; $p<0.01$) to a greater extent than IFN-γ (46%; $p>0.05$), respect to not-treated LPS-stimulated PBMCs (Figure No. 3B and Supplementary Table No. 2). A significant reduction of RANTES was not observed respect to not-treated cells (2,388 ± 1,08.1 *vs* 1,923 ± 77.5; $p>0.05$).

In-silico analysis

A docking analysis was carried out to examine molecular interactions between all compounds identified in the test EO and proteins involved in the activation of the TLR4 signaling pathway. AutoDock Vina binding energies are presented in Supplementary Table No. 3. Twelve sesquiterpenes exhibited docking affinity with MD-2 protein (binding energies from -7.69 kcal/mol to -7.12 kcal/mol) at LPS pocket, α-cubebene, δ-cadinene, *trans*-β-caryophyllene, β-copaene and 3,7-guaiadiene showed the highest binding energies. Nine of these twelve sesquiterpenes docked the IRAK2-IRAK4 complex at the interaction region (binding energies from -743 kcal/mol to -7.01 kcal/mol), β-burbonene, α-cubebene, *trans*-β-caryophyllene and 3,7-guaiadiene showed the highest docking affinity. The docking analysis did not predict interactions between EO-sesquiterpenes and NF-kB, binding energies ranged from -6.03 kcal/mol to -5.54 kcal/mol. In addition, the analysis did not predict molecular interactions between EO-monoterpenes and the selected TLR4 pathway proteins (energies from -6.45 kcal/mol to -4.38 kcal/mol).

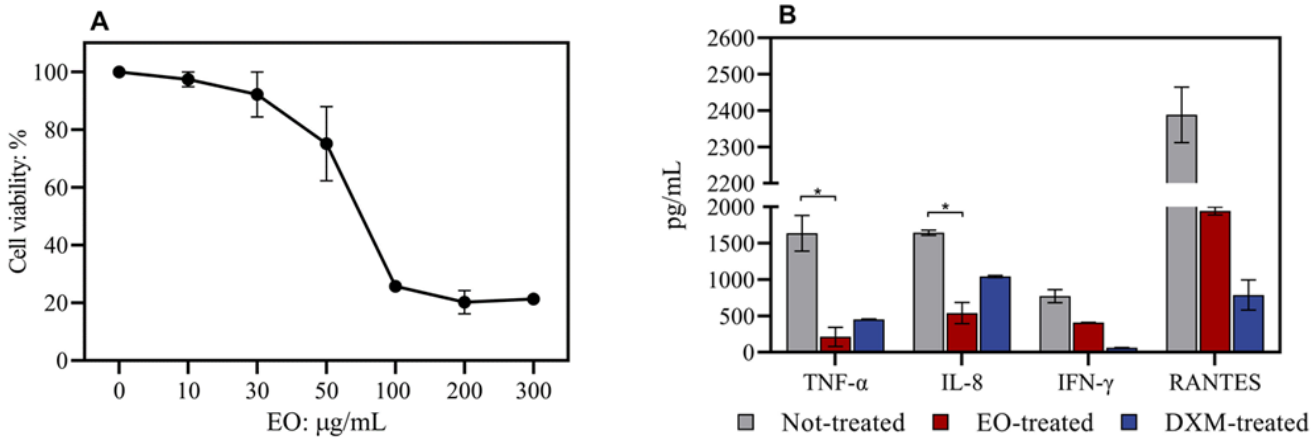


Figure No. 3

Effect of *Cordia curassavica* essential oil (EO) on cytokine production. A. Cytotoxicity, human peripheral mononuclear cells (PBMCs) were treated with EO at the indicated concentrations and viability was measured in the MTT assay. B. Effect on cytokines, LPS-stimulated PBMCs were treated with EO (50 µg/mL) and cytokines in the supernatants were measured 72 h later by ELISA. Dexamethasone (DXMT: 100 µM) is a reference anti-inflammatory drug. Data are means ± SD of three independent experiments in duplicate. *$p<0.01$

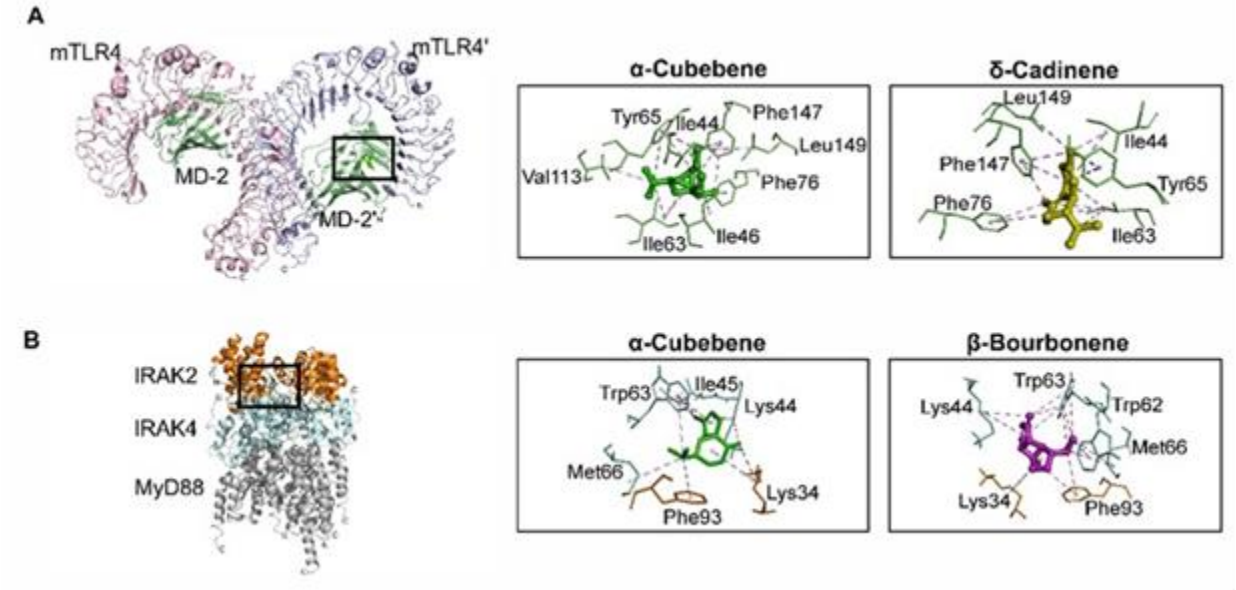


Figure No. 4
Predicted binding sites of sesquiterpenes present in Cordia curassavica essential oil to TLR4 signaling pathway proteins to. A. A. TLR4-MD-2 protein complex with α-cubebene (-7.69 kcal/mol) and δ-cadinene (-7.69 kcal/mol). B. IRAK4/IRAK2 protein complex with α-cubebene (-7.40 kcal/mol) and β-bourbonene (-7.43 kcal/mol)

DISCUSSION

This work reports for the first time the potential of *C. curassavica* EO as a starting point for research and the discovery of herbal preparations for treatment of dengue. The antiviral assay revealed that the EO reduced replication of more than one DENV serotype at IC_{50} values lower than 30 µg/mL. A standardized reference is not available to assess antiviral *in vitro* effect of plant material, a threshold of $IC_{50} \leq 50$ µg/mL has been suggested as a strict endpoint criterion for the antiviral activity of plant samples (Butterweck & Nahrstedt, 2012; Grienke *et al*., 2018). Thus, *C. curassavica* EO examined in this study could have good antiviral action against DENV viruses. Studies suggest that EOs and their isolated chemical constituents majorly act on free viruses directly and therefore blocking early steps of the viral cycle (Ma & Yao, 2020; Reichling, 2021). We (Ocazionez *et al*., 2010) and other authors (Garcia *et al*., 2010) reported the antiviral action of EOs by direct treatment of DENV before and during adsorption on cells.

In silico modelling analyses in this study revealed molecular interactions between sesquiterpenes constituents of the EO and DENV-2 proteins. We used docking scores higher than -6.99 kcal/mol as interaction energy, although docking scores higher than -6.50 kcal/mol are frequently used in molecular docking studies. Five sesquiterpenes displayed docking scores higher than -6.99 kcal/mol with the E protein at the βOG pocket. This protein is a key factor implicated in the binding host receptor and mediating membrane fusion processes for virus entry into the host cells (Zhang *et al*., 2017; Nasar *et al*., 2020), and the βOG pocket has been established as a target for developing antivirals against DENV (De Wispelaere *et al.*, 2018; Nasar *et al.*, 2020). Five sesquiterpenes displayed docking scores higher than -6.75 kcal/mol with the C protein at the dimer (α2/α4) interface. The C protein plays important role in the protection of the viral (RNA) genome and virus particle assembly (Byk & Gamarnik, 2016), mutations in the dimer interfaces affect structural stability of the protein and impair RNA-capsid interaction (Figueira-Mansur *et al*., 2019). Three sesquiterpenes also displayed docking scores higher than -6.70 kcal/mol with the RdRp domain of NS5 protein, which play key enzymatic role catalyzing RNA synthesis (Nassar *et al.*, 2020).

It is plausible that sesquiterpenes present in *C. curassavica* EO prove efficacious as DENV entry inhibitors due to their ability to fit into the βOG

pocket of the E protein. In addition, they could affect viral RNA replication and virus assembly by interacting with NS5 and C protein, respectively. *In-silico* investigations of phytochemicals as antiviral agents against DENV have predicted molecular interactions between sesquiterpenes and viral proteins (Pajaro-Castro *et al*., 2015; Powers & Setzer, 2016). In a previous study we reported *in vitro* inhibitory effect of *trans*-β-caryophyllene on DENV replication (Flechas *et al*. 2018). Antiviral activities of sesquiterpenes are documented (Ma & Yao, 2020).

Anti-inflammatory and anti-allergic properties of *C. curassavica* EO and its chemical compounds are well documented (Martim *et al*., 2020; Andrade *et al*., 2022). The LPS-stimulated PBMCs assay in this work shows effects on cytokine production after treatment of cells with the EO: inhibition of TNF-α and IL-8 to a greater extent than IFN-γ, and absence of RANTES inhibition. It is important to note that increased levels of pro-inflammatory cytokines including TNF-α and IL-8 are associated to progressing of severe dengue, and RANTES deficient condition favors the virus pathogenesis (Islam *et al.*, 2019; Malavige *et al.*, 2020). In contrast, low IFN-γ response during early dengue is likely to result in an altered antiviral response (Bhatt *et al.*, 2021).

TLR4 signal pathway plays an important role in the cytokine production process in LPS-stimulated PBMCs, LPS triggers activation of the TLR4/MD-2 complex receptor binding to hydrophobic residues located on the pocket of MD-2 (Park *et al*., 2009). Upon activation, the TLR4:MD-2 complex initiates oligomerization of a multiprotein complex (MyD88/IRAK2/IRAK4) named Myddosome, which initiates signal transduction pathway leading to the activation of the transcription factor NF-kB and the production of cytokines. Sesquiterpenes can down-regulate TNF-α and IL-8 gene expression by interfering with TLR4 signaling (Wang *et al*., 2017; Kim *et al*., 2020).

The *in-silico* modeling analyses in this work suggest that sesquiterpenes might interfere with the TLR4 signaling pathway, and thus play a role in the effect on cytokine production in LPS-stimulated PBMCs treated with *C. curassavica* EO. Twelve sesquiterpenes docked MD-2 close to residues located on the LPS binding pocket, they could fit into the pocket because of their small size and hydrophobic properties. Molecules that can fit into the LPS-pocket are recognized inhibitors of the TLR4 pathway (Ain *et al*., 2020). The *in-silico* modeling also revealed that sesquiterpenes docked the Myddsome at a region located in the IRAK2/IRAK4 interaction, which could interfere with the complex formation and therefore the signal transduction. Molecules that disrupt the Myddosome formation are candidate drugs as inhibitors of the TLR4 pathway (Turney *et al*., 2014; Wang *et al*., 2019). Sesquiterpenes can interact with key mediators of signaling pathways involved in cytokine production, which result in antiinflammatory effect (Sandner *et al*., 2020; Gandhi *et al*., 2020). Reduction of proinflammatory cytokines has been reported following treatment with *trans*-β-caryophyllene, α-humulene and germacrene D of LPS-stimulated human macrophages (Liu *et al*., 2014). Although the docking analysis did not predict molecular interactions between EO-monoterpenes and the selected signaling proteins of the TLR4 pathway, there is sufficient evidence that these terpenes have immunomodulatory and antiinflammatory properties (Sandner *et al*., 2020; Silveira e Sá *et al*., 2013).

Finally, important limitations of this work should be recognized. An antiviral assay to measure viral progeny was not used, although it is likely that reduction of E protein indicated that virus released into the culture medium was reduced. Also, a time-of-addition experiment to elucidate the antiviral mode of action of the EO was not carried out. Thus, antiviral effect of the EO needs to be confirmed using more robust cell-based assays including all four DENV serotypes. Further analysis is needed to elucidate the potential of *C. curassavica* EO as an immunomodulator of the DENV-induced cytokine response. Despite these limitations, the present study had contributed with knowledge about the potential of *C. curassavica* EO as a primary source for discovery and development of alternative therapies for dengue treatment.

CONCLUSIONS

The results of this work suggest that *C. curassavica* EO could be a good candidate for further analysis to fully characterize its anti-dengue potential. The EO could serve as a primary source for developing herbal preparations with antiviral and immunomodulatory properties to prevent progressing to severe dengue. Sesquiterpenes present in the EO such us 3,7-guaiadiene, γ- muurolene, *tran*-β-guaiene, α-cubebene, δ-cadinene, *trans*-β-caryophyllene and β-copaene might serve as a starting point for discovery of antivirals and immunomodulators drugs. Research on the pharmacological potential of plants used in

traditional medicine is especially needed for low- and middle-income countries where dengue is endemic and traditional medicine is widely used. It can help to find alternative options for the treatment and prevention of severe dengue.

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SUPPLEMENTARY MATERIAL

Table No. 1
Docking energies (kcal/mol) of *Cordia curassavica* essential oil compounds with DENV-2 proteins

Compound	C	prM	E	NS1	NS2B/NS3	NS5MTase	NS5RdRp
3,7-Guaiadiene	-7.08±0.49	-5.56±0.18	-8.03±0.82	-5.95±0.26	-5.15±0.10	-5.93±0.24	-6.78±0.37
α-Muurolene	-6.56±0.38	-5.19±0.23	-7.31±1.05	-5.64±0.33	-4.87±0.22	-5.81±0.41	-6.35±0.15
tran-β-Guaiene	-6.71±0.25	-5.32±0.33	-7.20±1.10	-5.82±0.14	-5.16±0.14	-5.82±0.08	-6.37±0.28
γ-Muurolene	-6.79±0.32	-5.14±0.19	-7.18±0.89	-5.75±0.31	-5.14±0.26	-5.76±0.22	-6.76±0.47
α-Cubebene	-6.76±0.25	-5.31±0.18	-7.01±1.28	-6.08±0.36	-5.42±0.26	-5.74±0.18	-6.52±0.61
Germacrene D	-6.54±0.17	-5.33±0.10	-6.75±1.17	-6.21±0.53	-5.12±0.34	-5.81±0.27	-6.88±0.41
β-Bourbonene	-6.78±0.37	-5.02±0.15	-6.62±1.08	-6.25±0.34	-5.09±0.22	-5.74±0.19	-6.23±0.53
δ-Cadinene	-6.46±0.17	-5.18±0.24	-6.30±0.81	-5.99±0.44	-4.93±0.30	-5.50±0.15	-6.41±0.64
α-Humulene	-6.20±0.28	-5.09±0.13	-6.21±0.95	-5.62±0.20	-5.04±0.26	-5.84±0.36	-6.37±0.48
Sabinene	-5.67±0.41	-4.23±0.25	-6.20±0.62	-5.41±0.17	-4.30±0.17	-4.52±0.13	-5.78±0.30
β-Myrcene	-5.11±0.45	-4.24±0.16	-6.08±0.20	-4.87±0.26	-4.03±0.07	-4.42±0.10	-5.21±0.14
trans-β-Caryophyllene	-6.61±0.41	-5.36±0.19	-6.02±0.38	-5.89±0.21	-5.09±0.18	-5.87±0.27	-6.48±0.42
β-Copaene	-6.27±0.37	-5.20±0.22	-5.90±0.26	-6.45±0.61	-4.19±0.17	-5.37±0.17	-6.47±0.71
Caryophyllene oxide	-6.10±0.41	-5.18±0.17	-5.65±0.19	-6.10±0.69	-5.11±0.19	-5.71±0.33	-6.21±0.30
α-Copaene	-6.04±0.38	-4.95±0.13	-5.51±0.54	-6.28±0.54	-4.82±0.15	-5.47±0.29	-5.96±0.26
α-Ylangene	-6.16±0.47	-4.98±0.13	-5.41±0.44	-6.28±0.44	-4.83±0.82	-5.52±0.37	-6.13±0.49
β-Pinene	-5.37±0.27	-4.23±0.09	-5.07±0.37	-5.42±0.11	-4.05±0.13	-4.63±0.14	-5.65±0.26
Bornyl acetate	-5.23±0.29	-4.48±0.12	-4.97±0.21	-6.03±0.29	-4.55±0.19	-4.76±0.10	-6.03±0.30
α-Pinene	-5.45±0.30	-4.25±0.14	-4.98±0.27	-5.41±0.09	-4.09±0.18	-4.49±0.14	-5.65±0.31
1,8-Cineole	-5.13±0.26	-4.26±0.15	-4.80±0.14	-5.46±0.09	-4.14±0.06	-4.73±0.28	-5.51±0.35
Reference compounds: Epigallocatechin gallate[¶]	-7.67±0.4	-7.37±0.12	-7.85±0.3	-7.31±0.19	-	-	-
CID_54692801[Φ]					-8.52±0.31		
Baicalin*	-	-	-	-	-	-8.38±0.11	-10.0±0.29

[¶] Kaihatsu *et. al*., 2018; Ismail & Jusoh, 2017; Kaihatsu *et al*., 2018; Qamar *et al*., 2014
[Φ] Cabarcas-Montalvo *et al*., 2016
*** Hassandarvish *et al*., 2016**

Table No. 2
Levels of cytokines in LPS-stimulated PBMCs treated with *Cordia curassavica* essential oil

Treatment	TNF-α	INF-γ	IL-8	RANTES
None	1640±346.1	773±127.6	1644 ± 50.6	2388±108.1
Essential oil: 30 µg/mL	214±186.6 (86.9)*	408±4.6 (47.2)	539±206.2 (67.2)*	1923±77.5 (19.4)
Dexamethosone: 100 µM	453±10.8	63±5.0	1042±21.5	789±293.3

Data (pg/mL) are mean ± SD from independent experiments done in triplicate. Percentage of reduction respect to untreated cells in parenthesis. *: $P < 0.01$, ANOVA followed by Tukey *post-hoc* test

Table No. 3
Docking energies (kcal/mol) of Cordia curassavica essential oil compounds with TLR4 pathway proteins

Compound	MD-2	IRAK2/IRAK4	NF-kB
α-Cubebene	-7.69±0.48	-7.40±0.45	-5.88±0.22
δ-Cadinene	-7.69±0.56	-7.01±0.22	-5.63±0.15
trans-β-Caryophyllene	-7.66±0.43	-7.35±0.31	-5.84±0.11
β-Copaene	-7.58±0.44	-7.13±0.37	-5.81±0.22
3,7-Guaiadiene	-7.57±0.28	-7.35±0.13	-6.03±0.19
tran-β-Guaiene	-7.55±0.31	-7.23±0.13	-5.84±0.14
Germacrene D	-7.56±0.38	-7.14±0.22	-5.85±0.14
β-Bourbonene	-7.45±0.38	-7.43±0.42	-5.74±0.18
α-Humulene	-7.41±0.67	-7.14±0.26	-5.73±0.07
α-Muurolene	-7.40±0.22	-6.93±0.10	-5.93±0.20
γ-Muurolene	-7.23±0.30	-6.82±0.25	-5.88±0.19
α-Ylangene	-7.12±0.46	-6.84±0.24	-5.54±0.11
α-Copaene	-6.95±0.26	-7.14±0.46	-5.58±0.16
Caryophyllene oxide	-6.71±0.47	-7.06±0.17	-5.77±0.12
Bornyl acetate	-6.35±0.35	-6.30±0.13	-5.13±0.12
α-Pinene	-6.19±0.16	-5.68±0.13	-4.83±0.11
β-Pinene	-6.18±0.17	-5.7±0.12	-4.77±0.10
β-Myrcene	-6.10±0.15	-5.14±0.08	-4.38±0.22
1,8-Cineole	-6.00±0.21	-5.82±0.20	-4.87±0.13
Sabinene	-5.96±0.20	-5.76±0.13	-4.68±0.15
Reference compounds:			
Imperatorin*	-7.50±0.30	-	-
Quinoline[£]	-	-8.15±0.17	-
Epigallocatechin gallate[¶]	-	-	-8.38±0.20

[*] Huang *et al.*, 2021; [£] Zhong *et al.*, 2016; [¶] Suhail *et al.*, 2019