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Comparative Anti-dengue Activities of Ethanolic and Supercritical Extracts of *Lippia origanoides* Kunth: *in-vitro* and *in-silico* Analyses

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Abstract: Despite intense research efforts, no approved effective therapies are available for dengue treatment. Standardized plant extracts could serve as potential candidates for developing plant-based treatments for dengue. We have compared the anti-dengue potential of ethanolic (EtOH) and supercritical (SCO_2) extracts of *Lippia origanoides* Kunth (Verbenacea). The mode of antiviral action and the effect on the release of IL-β from DENV-1-stimulated macrophages (PMA/THP-1) were evaluated. *In silico* analyses were performed to predict molecular interactions between flavonoids identified in the UHPLC–ESI+–Orbitrap–MS analysis and target proteins. The EtOH extract showed a strong antiviral effect (IC_{50}, 22 and 17 µg/mL; SI, 20 and 23) before and during DENV-1 adsorption to liver (HepG-2) cells. SCO_2 showed a weak antiviral effect. EtOH, but not SCO_2, reduced the level of IL-1β released from PMA/THP-1 cells by 45-55%. Flavonoid glycosides were identified in EtOH but not in SCO_2. Seventeen flavonoids were predicted to bind to DENV E, GRP78, CLEC5A, and NLRP3 proteins involved in DENV replication and IL-1β production. The data provide a first step towards defining the potential of *L. origanoides* extract as a candidate for developing phytotherapeutics for dengue.

Keywords: Plant extracts; *Lippia origanoides*; dengue; dengue virus; macrophages; cytokine. © 2023 ACG Publications. All rights reserved.

1. Introduction

Medicinal plants have been used in folk medicine for thousands of years in virtually all countries. Crude extracts from many plant species exhibit *in vitro* antiviral effects against pathogenic viruses [1,2], and they are recognized as effective mediators of immune responses to improve the defense system and reduce harmful inflammatory responses [3,4]. Plant extracts are rich sources of flavonoids, which exert antiviral activities and can inactive pathways that produce inflammatory cytokines [5,6]. Traditional and

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alternative medicine systems use different phytotherapeutics based on plant extracts, which constitute an important part of therapies for various human diseases [7].

The dengue virus (DENV) causes a spectrum of diseases ranging from asymptomatic to dengue fever and severe dengue shock syndrome [8]. DENV productively infects macrophages and liver cells during the natural infection, resulting in viremia and an increase of cytokines levels in the blood [9,10]. Many studies have demonstrated an association between exacerbated DENV-stimulation of macrophage and dengue severity [9]. IL-1β is a potent inflammatory cytokine released by macrophages, and high blood levels are associated with the severity of dengue [11]. Despite intensive research efforts, no approved effective therapies are currently available to treat dengue patients [12].

Phytotherapy has emerged as a promising alternative to treat and prevent dengue [13,14]. Formulations based on extracts of *Carica papaya* L. and *Euphorbia hirta* L. are used in traditional and complementary medicine for treating dengue in India and the Philippines, which have been validated in clinical studies [15,16]. In *vitro* and *in vivo* studies have demonstrated the potential of plant extracts as primary sources for discovering phytotherapeutics to treat dengue [17,18]. Extracts of at least twenty plant species (e.g., *Cymbopogan citratus, Andrographis paniculata*, *Momordica charantia*, *Ocimum sanctum*, *Pelargonium citrosum,* and *Citrus limon*) showed potential as inhibitors of the DENV replication. Extracts of *Azadirachta indic*a [19]*, Curcuma longa* L. [20], and *Cissampelos pareira* Linn [21] remarkably reduced viral loads in DENV-infected mice.

DENV transmission in Colombia causes thousands of dengue cases annually [22]. Several plant preparations are traditionally used in different parts of the country to treat common symptoms of infectious diseases, including dengue [23]. Despite the richness of the Colombian flora, between 29,000 and 31,000 species [23,24], a few plant species have been studied to search for their pharmacology potential. As a traditional medicine in Latin American countries, *Lippia origanoides* Kunth (Verbenacea) is used to treat various diseases and pains [25]. *L. origanoides* is one of the most studied plants in Colombia; its chemical composition and anticancer, antimicrobial, and antioxidant activities are documented [26-29]. We have documented the *in vitro* antiviral efficacy of *L. origanoides* essential oil against DENV [30] and yellow fever virus [31].

Plant extracts chemical composition is affected by the extraction method, which consequently can impact their antiviral activity [32,33]. Plant extracts tested for anti-dengue activity are most commonly obtained by conventional techniques, such as ethanolic extraction and aqueous extraction [15-21]. Studies showed supercritical fluid extraction as an alternative process to obtain antiviral agents from plant extracts because it avoids degradation of the active compounds [32-34]. Variations in the biological activities of ethanolic and supercritical extracts of the same plant are documented [32-34]. In previous studies [26,27], we obtained supercritical extracts of *L. origanoides* with high pinocembrin content, a pharmacologically active flavonoid with antiviral and anti-inflammatory activities [35]. To our knowledge, studies comparing the anti-dengue potential of ethanolic (EtOH) and supercritical (SCO_2) extracts of *L. origanoides* have yet to be reported. In this study, we have compared *L. origanoides* EtOH and SCO_2 extracts for *in vitro* efficacy in reducing DENV replication in human liver cells (HepG-2) and IL-β released from DENV-stimulated human macrophages (PMA/THP-1). We also conducted an *in silico* analysis to hypothesize how the flavonoids identified in the plant extract might act on both DENV replication in HepG-2 cells and IL-β production by macrophages.

2. Materials and Methods

2.1. L. origanoides Phellandrene Chemotype

The plant was grown in experimental plots at the Agroindustrial Pilot Complex of the National Center for Agroindustrialization of Aromatic and Medicinal Tropical Vegetal (CENIVAM. N 07°08,422' W 073°06,960'), Universidad Industrial de Santander, Bucaramanga, Colombia. The taxonomic identification was performed at the Colombian National Herbarium with voucher number 22035.

2.2. Preparation of Plant Extract

Dried leaves and stems were triturated and steam-distilled to remove essential oils. The ethanolic extract (EtOH) was obtained as described in a previous study [26-28]. Briefly, the powder was mixed with acidified ethanol solution (20 mL, 0.5% HCl, 1:1 v/v) and put for 5 min in an S15H ultrasound bath (Elmasonic, Singen, (Germany). The mixture was filtered through cellulose filter paper, the residue was extracted twice, and the extract was evaporated to dryness using a rotary evaporator (VirTis AdVantage Plus tray lyophilizer). Supercritical extract (SCO_2) was prepared in a previous study [26] using an ethanol-modified supercritical CO_2 method and a Thar SFE-2000-2-FMC50 one-step pilot scale equipment (Thar Instruments, Pittsburgh, PA, USA). Extraction was carried out using 99.8 % pure CO_2 and industrial-quality ethanol; the process conditions and the schematic diagram of the experimental apparatus were described [26]. EtOH and SCO_2 solutions [$1x10^5$ µg/mL; 1% dimethyl sulfoxide (DMSO) final concentration] for analysis of biological activities were prepared in culture medium (MEM, Gibco) and filtered through cellulose filter paper (0.45 µM) to eliminate contaminant microorganisms.

2.3. UHPLC–ESI+–Orbitrap–MS Analysis

The plant extracts were analyzed by a UHPLC Dionex™ UltiMate™ 3000 (Thermo Fisher Scientific, Bremen, Germany) coupled to an Orbitrap™ mass detector (Exactive Plus, TFS, Bremen, Germany), using a heated-electrospray interface (HESI-II) operated in positive-ion acquisition mode (350 °C). The process conditions and data analysis were described in previous studies [27-29]. The SCO_2 extract was analyzed in the previous study [27], and data were used in the present study. Briefly, separation of the metabolites was performed using a Hypersil GOLD aQ (Thermo Scientific, Sunnyvale, CA; 100×2.1mm i.d., 1.9 µm) column with a mobile phase of LCMS –grade acetonitrile with 0.5 % formic acid as phase B and water with 0.5% formic acid as phase A. The gradient setting of the mobile phase was divided as follows: 100 % A changed linearly to 100 % B in 8 min, maintained constant for 4 min, returned to 100 % A in 1 min, and remained unchanged for the last 3 min. The mass spectra were obtained in positive mode (injection volume, 1 µL; gas temperature, 350 °C; drying gas, 7 L/min, N_2; nebulizer gas, 40 psi; capillary voltage, 3.5 kV; mass range 100-1100 m/z. All analyses were performed and monitored using Thermo XCalibur™ Roadmap software, version 3.1.66.10. The external calibration methodology was employed for quantification using the calibration curves of available standard substances [36]. The quantification of the substances for which no standards were available was obtained with the internal standard method [37] using kaempferol as a surrogate.

2.4. Virus and Cells

The DENV serotype 1 (DENV-1) Hawaii reference strain (C.D.C.; Dengue Branch, U.S.A.) was used. The virus was titrated by the plaque formation assay. Cells were cultured in a medium supplemented with 10% fetal bovine serum: HepG-2 and HepaRG cells in Dulbecco´s Modified Eagle Medium (DMEN) high glucose; HEK293 cells in DMEM-F12; Vero cells in Minimum Essential Media (MEM); and THP-1 cells in RPMI-1640 medium.

2.5. Cytotoxicity Assays

Cells in 96-well cell culture microplates were treated with extract at six points concentrations (8 to 512 µg/mL) for 72 h (37 °C; 5% CO_2). Cell viability was measured using a crystal violet uptake assay and the MTT assay. DMSO at range concentrations was included as a positive control. Each extract was analyzed in triplicate in three independent experiments. Experimental conditions and data analysis were described in previous studies [30,31].

2.6. Mode of Antiviral Action

To determine the mode of antiviral action, the extracts were tested at six points (3.12 to 100 µg/mL) non-cytotoxic concentrations against DENV-1 using three different experimental approaches. The virus

was incubated (2 h; 37 ºC, 5% CO_2) with extract before adsorption to cells, the extract was added during virus adsorption (1.5 h; 37 ºC, 5% CO_2), or the extract was added immediately after viral adsorption. SDS (sodium dodecyl sulfate) was used as a reference antiviral (positive control). The non-adsorbed virus was removed by washing with PBS, and the virus in the cells was allowed to replicate 72 h at 37º C. Next, culture supernatants were used for measuring the DENV non-structural protein 1 (NS1) using an ELISA kit (Panbio™ Dengue Early). The concentration of extract that inhibited DENV NS1 by 50% (IC_{50}) was obtained by performing nonlinear regression followed by the construction of a concentration-response curve (GraphPad Prism software version 8.0, San Diego, CA, U.S.A.). Selectivity index (SI) values were calculated by dividing the non-cytotoxic concentration for uninfected cells by the IC_{50} value. A favorable SI > 10 indicated antiviral efficacy.

2.7. Assessment of the Effect of Extracts on the Release of IL-1β from Human Macrophages

THP-1 cells were cultured in 24-well plates in RPMI-1640 medium with 15 ng/mL of PMA (phorbol 12-myristate 13-acetate) for 48 h. Cells were then washed with serum-free medium before each experiment to remove undifferentiated cells. Macrophage-like cells were exposed to DENV-1 (MOI of 5.0) for 2 h, and the virus was removed by washing; then, cells were incubated in medium with or without extract (50 and 100 µg/mL) for 12 h and 24 h. Dexamethasone, an anti-inflammatory drug, was included as a positive control. The cytokine IL-1β was measured in culture supernatants using an ELISA kit (Invitrogen).

2.8. Molecular Docking

3D structures of target proteins were downloaded from the Protein Data Bank (PDB ID). The target proteins selected for analysis were the following: DENV-2 E protein (1OAN) and its GRP78 (6EOC) receptor in HepG-2 cells; CLEC5A (2YHF), and NLRP3 (6NPY), which are macrophage proteins involved in signaling leading to the release of IL-1β. All EtOH constituents identified in the UHPLC–ESI+–Orbitrap–MS analysis were selected for the analysis, and structures were retrieved from the PubChem database (supplementary material). The molecular docking analysis was performed using AutoDock Vina (version 1.5.6, La Jolla, CA, USA), as described in a previous study [30]. 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. Three simulations were performed for each ligand-protein pair using seeds 6, 12, and 18. The average docking scores for each protein approximated the binding free energy. Discovery Studio Visualizer v21.1.0.20298 was used to view ligand-protein interaction.

2.9. Statistical Analysis

Data were analyzed using GraphPad Prism software (version 8.0, San Diego, CA, U.S.A.). Analysis of variance (ANOVA) followed by Tukey's post hoc test was used for data comparisons.

3. Results and Discussion

3.1. Chemical Composition Analysis

Table 1 shows a comparison of the chemical profiles of EtOH and SCO_2 extracts according to the UHPLC–ESI+–Orbitrap–MS analysis. The exact mass characteristic positive ions of compounds and mass spectra are presented in the supplementary material (Table S1 and Figure S1). The content of EtOH was represented by twenty flavonoids of the type flavanone, flavone, flavonols, and chalcone, from which thirteen were glycosides. The predominant flavonoids were quercetin glycoside (9.3 µg/mg), luteolin glycoside (6.7 µg/mg), eriodictyol glycoside (2.5 µg/mg), and eriodictyol (2.2 µg/mg). The UHPLC-ESI+-Orbitrap-MS analysis in the previous study [26] identified the seven flavonoid aglycones of EtOH but not flavonoids glycosides as constituents of the SCO_2 extract, with pinocembrin (48.3 µg/mg) being the predominant compound followed by galangin (9.3 µg/mg).

We have optimized extraction conditions for *L. origanoides* chemotypes to enhance yield and extract antioxidant activity [26,27]. The pinocembrin content was more than ten times the amount found in

alcoholic extracts from the same plant and other species. The pressure employed during the supercritical extraction was high (50 MPa) to favor the extraction of polar substances without using a co-solvent. However, the number of polyphenols and their concentrations were lower compared to alcoholic extracts. The high concentration of pinocembrin identified in the SCO_2 extract indicated a higher selectivity for the extraction of this flavonoid. A recent publication from our group examined the obtainment of pinocembrin-enriched *L. origanoides* extracts under various conditions [38]. The pinocembrin concentration changed from 5.5 mg/g to 48 mg/g and 145 mg/g when the extraction media changed from ethanol to ethanol-modified $scCO_2$, and to a coexisting mixture of two fluid phases of $scCO_2$ with two proportions of aqueous ethanol, respectively.

3.2. Cytotoxicity

Cytotoxicity is a key indicator for biological assessment using *in vitro* models. Cytotoxicity can vary depending on the cell type and assay. Using two viability assays, the EtOH and SCO_2 extracts were tested against two human cells (liver and kidney) and Vero cells (monkey). Both extracts can be classified as noncytotoxic to all four cells according to results from the crystal violet and MTT assays (Figure 1). A 50% reduction in cellular viability was not observed at the highest concentrations (128 to 512 µg/mL) of EtOH and SCO_2 extracts. Other *L. origanoides* extracts from Colombia showed low cytotoxicity for normal human fibroblast (MRC-5) cells [29].

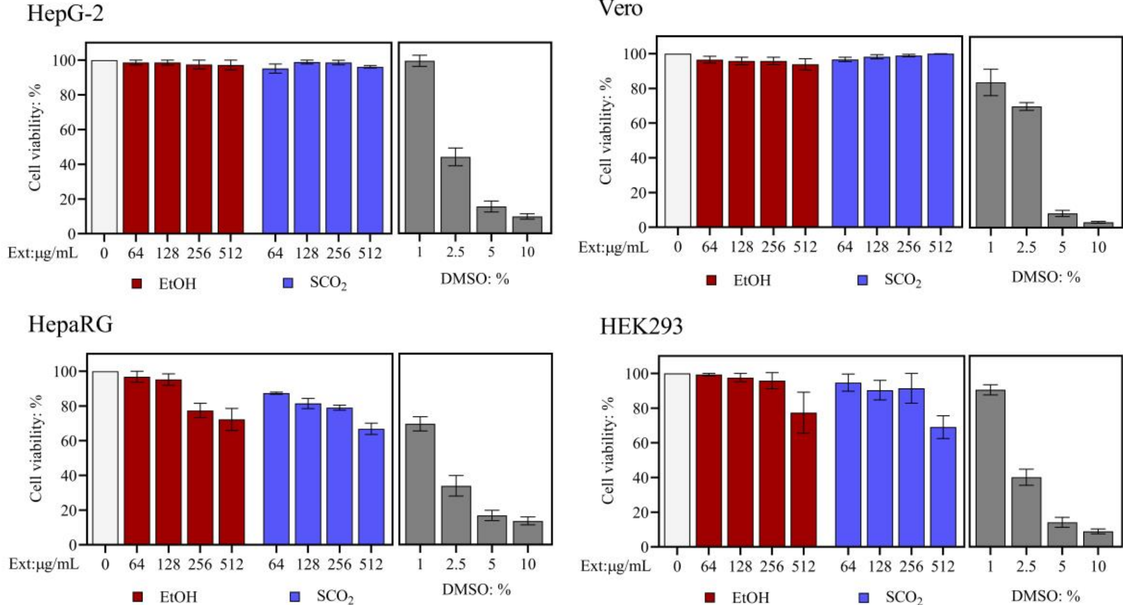

Figure 1. Representative results of the cytotoxicity assays. Cells were cultured in medium containing ethanolic (EtOH) and supercritical (SCO_2) extract, and viability was measured 72 h later. Human (HepG-2) and monkey (Vero) liver cells were analyzed in the crystal violet assay. Human liver (HepaRG) and kidney (HEK293) cells were analyzed in the MTT assay. Dimethyl sulfoxide (DMSO) was included as a positive control. All values represent the mean ± SD of independent experiments (n=3) in triplicate.

Table 1. Composition of *L. origanoides* extracts according to UHPLCESI (+)-Orbitrap-MS analysis

No	Compound	Formula	$[M+H]^+$		Δ ppm	μg/mg	
			Experimental	Calculated		EtOH	SCO_2
1	Taxifolin glucoside	$C_{21}H_{22}O_{12}$	467.1182	467.1184	0.24	0.12 ± 0.01	-
2	Quercetin glucoside	$C_{21}H_{20}O_{12}$	465.1025	465.1027	0.35	9.3 ± 1.3	-
3	Eriodictyol rhamnoside	$C_{21}H_{22}O_{10}$	435.1284	435.1285	0.28	0.61 ± 0.06	-
4	Luteolin rutinoside	$C_{27}H_{30}O_{15}$	595.1652	595.1657	0.86	0.12 ± 0.01	-
5	Eriodictyol glucoside	$C_{21}H_{22}O_{11}$	451.1232	451.1234	0.48	3.5 ± 0.5	-
6	Luteolin glucoside	$C_{21}H_{20}O_{11}$	449.1079	449.1083	1.07	6.7 ± 0.9	-
7	Taxifolin	$C_{15}H_{12}O_7$	305.0654	305.0655	0.30	0.73 ± 0.01	-
8	Galangin glucoside	$C_{21}H_{20}O_{10}$	433.1129	433.1129	0.02	0.60 ± 0.05	-
9	Phloridzin	$C_{21}H_{24}O_{10}$	437.1438	437.1442	0.82	0.26 ± 0.04	-
10	Eriodictyol	$C_{15}H_{12}O_6$	289.0710	289.0712	0.57	2.2 ± 0.1	0.44 ± 0.1
11	Quercetin	$C_{15}H_{10}O_7$	303.0504	303.0504	0.12	1.08 ± 0.03	-
12	Luteolin	$C_{15}H_{10}O_6$	287.0552	287.0555	0.92	0.93 ± 0.04	-
13	Naringenin	$C_{15}H_{12}O_5$	273.0762	273.0762	0.28	0.11 ± 0.01	1.06 ± 0.05
14	Chrysoeriol	$C_{16}H_{12}O_6$	301.0703	301.0706	0.99	0.07 ± 0.01	0.04 ± 0.01
15	Cirsimaritin	$C_{17}H_{14}O_6$	315.0860	315.0863	0.83	0.07 ± 0.01	0.05 ± 0.01
16	Sakuranetin	$C_{16}H_{14}O_5$	287.0913	287.0914	0.28	0.01 ± 0.01	1.42 ± 0.08
17	Pinocembrin	$C_{15}H_{12}O_4$	257.0813	257.0813	0.20	0.47 ± 0.04	48.3 ± 1.0
18	Tricetin trimethyl	$C_{18}H_{16}O_7$	345.0965	345.0968	0.80	0.02 ± 0.02	-
19	Galangin	$C_{15}H_{10}O_5$	271.0605	271.0606	0.49	0.40 ± 0.04	7.2 ± 0.2
20	Methylated galangin	$C_{16}H_{12}O_5$	285.0756	285.0757	0.42	0.10 ± 0.01	-

The ethanolic extract (EtOH) was analyzed in the present study, data are mean ± standard deviation (n = 3). The supercritical extract (SCO_2) extract was analyzed in a previous study, and data were reported [26].

3.3. Mode of Antiviral Action

Three different experimental approaches were used to characterize the mode of antiviral effect of the extracts against DENV-1 by measuring the reduction of the DENV NS1 protein in HepG-2 cells (Figure 2A). The DENV NS1 protein is a surrogate for viral progeny in human liver cells [39]. Untreated infected cells showed a DENV NS1 protein content in supernatants of 2.72 ± 0.27 and 2.26 ± 0.43 absorbance units. The EtOH extract reduced DENV NS1 by more than 50% in a concentration-dependent manner by treatment before and during DENV-1 adsorption to cells (Figures 2B and C). EtOH showed similar ($p < 0.05$, ANOVA) antiviral potency before (IC_{50} of 22 ± 1.2 µg/mL; SI = 23) and during (IC_{50} of 17 ± 2.0 µg/mL; SI = 20) viral adsorption. In contrast, the SCO_2 extract at the maximum concentration (100 µg/mL) reduced DENV NS1 protein by 25% ($p < 0.05$) and 35% - 45% ($p < 0.05$) by treatment before and during viral adsorption, respectively, in a concentration-independent manner. As for treatment after DENV-1 adsorption, EtOH at maximum concentration reduced DENV NS1 by 25%; ($p = 0.05$), while SCO_2 did not cause a reduction. Many studies document *in vitro* anti-DENV activity of plant extracts before and after virus adsorption to cells [15-21].

Currently, a standardized reference is not available to define the *in vitro* antiviral effect of a plant extract. Inhibitory concentration 50 (IC_{50}) values lower than 100 µg/mL and a selectivity index (SI) value higher than 4.0 were used to classify a plant extract as having antiviral potency [40]. The results of the antiviral assays indicated that the EtOH extract had a strong antiviral effect against DENV-1. Since the SCO_2 extract did not reduce DENV NS1 by more than 50%, IC_{50} values were not estimated, and therefore the extract can be classified as having weak antiviral activity.

Glycoside and glycoside-free (aglycone) forms of flavonoids present in plant extracts are responsible for their biological activities [41,42]. Flavonoid glycosides are composed of a flavonoid aglycone linked to a sugar moiety such as glucose, galactose, rhamnose, arabinose, and rutinose [41]. Flavonoids are poorly soluble in aqueous solutions, and their solubility can be increased by glycosylation, which improves their pharmacological properties [42,43]. In this study, we analyzed extracts of *L. origanoides* that differed in the content of flavonoids. The EtOH extract was characterized by a higher content of flavonoids, including glucosides, than SCO_2. We can hypothesize that flavonoid glycosides in EtOH played a crucial role in its strong antiviral effect against DENV-1. Glycosides flavanone and flavonols, rather than their aglycones, show enhanced *in vitro* antiviral efficacy against pathogenic human viruses [5,42,44]. It has been suggest that glycosylated moiety might enhance the antiviral activity of flavonoids probably by favoring its internalization into the cell. α-Glucosidase is a cell enzyme required for the N-linked oligosaccharide processing event for the secretion of DENV infectious particles, and flavonoid glycosides exhibit strong inhibitory effects on α-glucosidase [45].

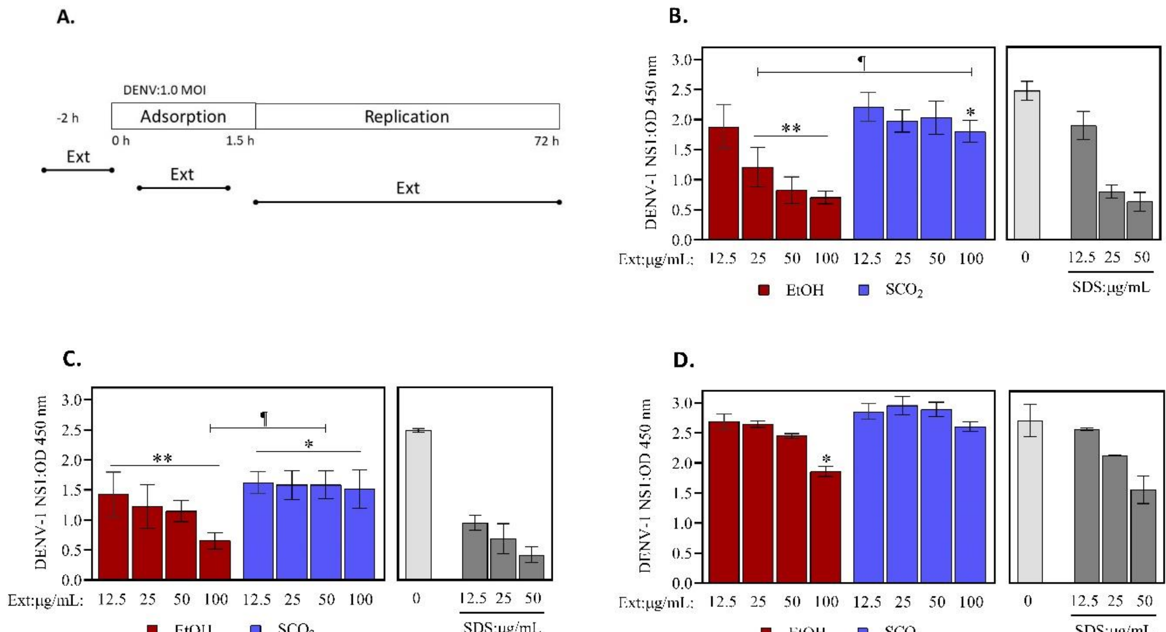

Figure 2. Mode of antiviral action of *L. origanoides* extracts. Representative results of antiviral assays. (A) Experimental design scheme of treatments: DENV-1 was incubated for 2 h with extract (Ext) before adsorption on HepG-2 cells. EtOH or SCO_2 extract was added during and immediately after virus adsorption to cells. The DENV NS1 protein in supernatants was measured 72 h postinfection using ELISA. Treatment before (B), during (C), and after (D) viral adsorption. All values represent mean ± SD, N=3. ***p* < 0.001, **p* < 0.05 versus untreated control. ¶*p* < 0.001, EtOH versus SCO_2.

3.4. Effect on the Release of IL-1β

Studies document the inhibitory effect of plant extracts on the release of cytokines from DENV-activated macrophages [46,47]. IL-1β is a potent inflammatory cytokine released by DENV-stimulated macrophages, which play an important role in the progression of dengue to severe disease [11]. We have evaluated the effect of EtOH and SCO_2 extracts on activated human macrophages using the release of IL-1β from DENV-1-stimulated PMA-THP-1 cells as an activation marker. The MTT assay revealed that differentiated macrophages treated with 100 μg/mL of both extracts were 84.5% and 79.5% viable (Figure 3A). Untreated DENV-1-stimulated macrophages showed IL-1β content in culture supernatants between 442 ± 93.4 pg/mL and 412 ± 94.2 pg/mL at 12 h and 24 h, respectively (Figure 3B). DENV-1-stimulated cells treated with 50 μg/mL and 100 μg/mL of EtOH extract for 12 h and 24 h reduced IL-1β by 55-45% ($p < 0.01$) and 60-42% ($p < 0.01$), respectively. In contrast, macrophages treated with SCO_2 extract at the same concentrations showed a nonsignificant reduction in IL-1β after 12 h (23-11%) and 24 h (32-17%) of treatment.

It is known that glycosides and aglycones flavonoids exhibit different biological activities, and their therapeutic effect can differ markedly [41,42]. Studies show that the O-glycosidation of flavonoid aglycones significantly reduced the inhibition of inflammation mediators in murine immune cells [42]. A fraction of extract from green tea rich in flavonol aglycones showed a higher inhibitory effect on IL-1β released by murine macrophages than the fraction rich in flavonol glycosides [41]. As the EtOH extract had a higher diversity of flavonoid aglycones (n = 13) than the SCO_2 extract (n = 7), this difference could partly explain the higher anti-IL-1β effect of EtOH. In addition, flavonoids in the extracts could block signaling pathways of IL-1β secretion in DENV-1-stimulated PMA-THP-1 cells.

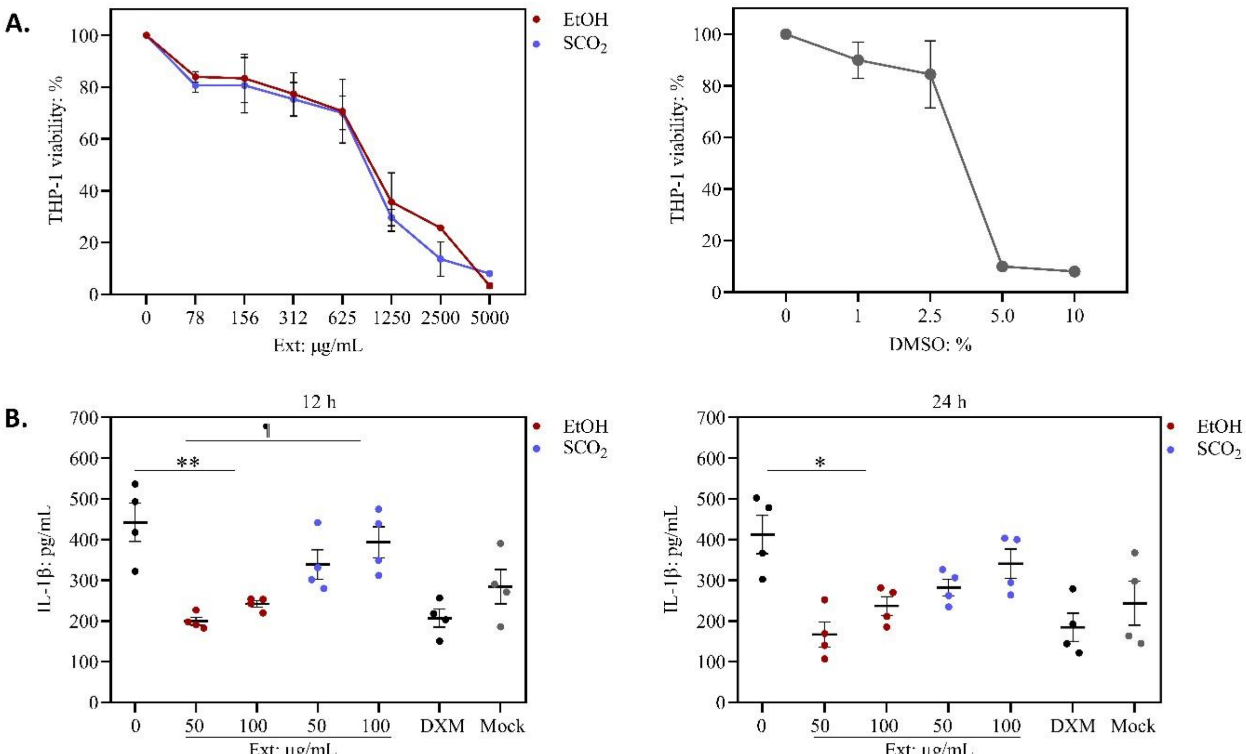

Figure 3. Effect of *L. origanoides* extracts on the release of IL-1β from human macrophages (PMA-THP-1). (A) Viability of cells following treatment with the EtOH and SCO_2 extracts for 72 h, DMSO is a positive control. (B) The extracts were added to DENV-1-stimulated PMA-THP-1 cells for 12 h and 48 h, and IL-1β levels in supernatants were measured using ELISA, dexamethasone (DXM) is a positive control. All values represent the mean ± SD, N=4. $^{**}p < 0.001$, $^{*}p < 0.05$ versus untreated control· ¶$p < 0.001$, EtOH versus SCO_2.

3.5. Molecular Docking Analysis

Molecular docking analysis was performed to evaluate binding affinities between the twenty flavonoids identified in the EtOH extract and target proteins involved in the DENV replication in HepG-2 cells and the release of IL-1β from PMA-THP-1 cells. Binding energy below the upper threshold of -7.00 kcal/mol predicted a good binding affinity between ligand and target. The twenty flavonoids showed variable docking scores depending on the protein target (Figure 4). The top flavonoids with the lowest predicted binding affinities are presented in Tables 2 and 3.

The DENV particle is surrounded by a lipid envelope containing the E protein, which consists of three β-barrel domains [48]. DENV enters the host cell when the E protein binds to a receptor and undergoes conformational rearrangement, which induces fusion of the virus envelope and host-cell membrane [48,49]. The DII domain of the E protein contains a highly conserved site responsible for fusion between the viral envelope and cell membrane, and the DI-DII hinge region “kl loop” structure is crucial for initiating the pH-mediated conformational change of the E protein during fusion [48]. A hydrophobic pocket in the hinge region, occupied by the detergent n-octyl-β-D-glucoside (βOG), plays an important role in the major conformational change during fusion [50]. DENV invades different cells, including liver cells [9,10]. The stress-inducible molecular chaperone protein 78 (GRP78) has been reported to facilitate viral entry and intracellular replication for a wide variety of pathogenic viruses [51], and cell-surface GRP78 was proposed as DENV receptor in HepG-2 cells [52].

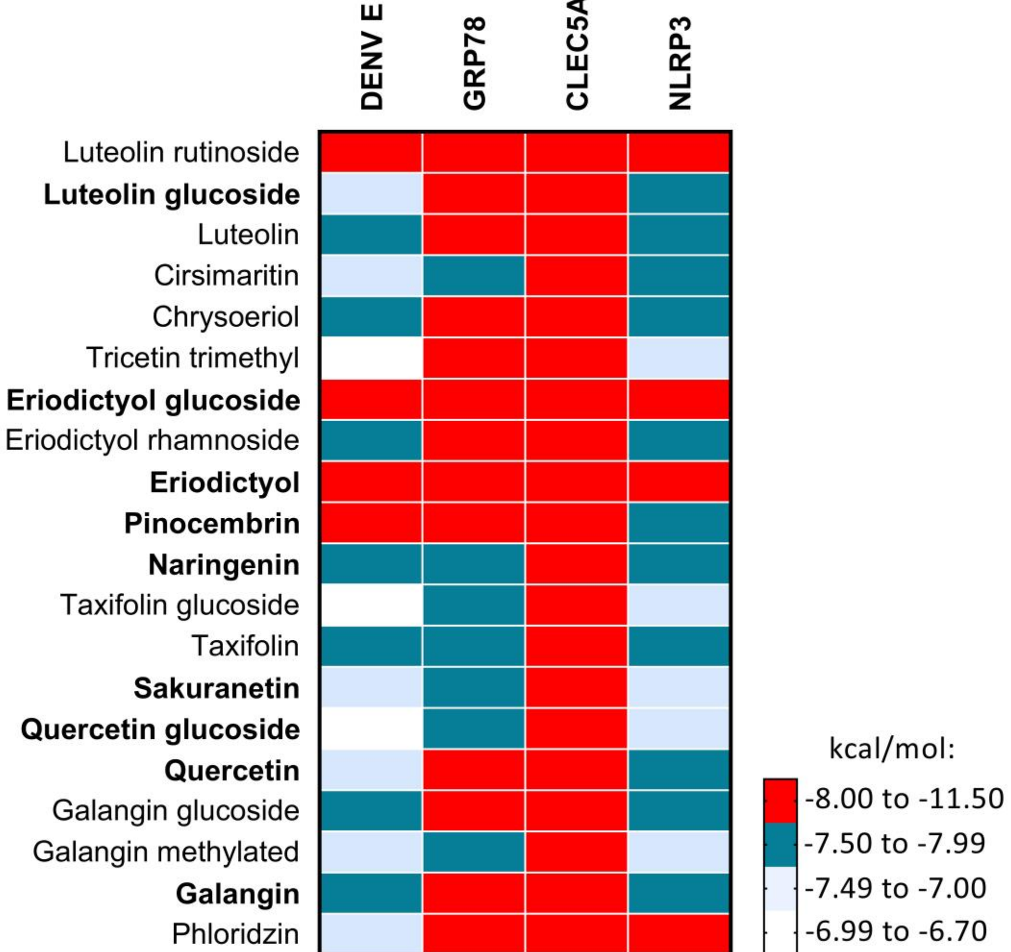

Figure 4. Heat map of AutoDock Vina scores for binding affinities between flavonoid constituents of the EtOH extract and target proteins. Predominant flavonoids in bold.

Docking analysis predicted binding affinities between flavonoids of the EtOH extract and the DENV E and GRP78 proteins, which have formed hydrogen bonds with amino acids of consensus binding sites (Figure 5). As for flavonoid glycosides, five out of seven docked (-7.47 to -9.16 kcal/mol) to E, luteolin rutinoside, and eriodyctiol glucoside showed the lowest binding energies. All flavonoid glycosides except galangin were accommodated into a pocket of the DII domain in the interface formed between chain A and B (DII: A/B) of the E dimer, and galangin into a pocket of the DI-DII hinge region "kl loop" structure. As for flavonoid aglycones, twelve out of thirteen docked (-7.10 to -8.05 kcal/mol) to E; the top were eriodictyol, pinocembrin, naringenin, and chrysoeriol, which were accommodated into the βOG pocket along with four others; and some flavonoids (n = 4) also were accommodated into the DII: A/B pocket. In addition, the analyses predicted binding affinities between all twenty flavonoids of the EtOH extract and GRP78, which docked into two consensus-binding sites. All flavonoid glycosides (-8.55 to -10.07 kcal/mol), chrysoeriol (-8.18 kcal/mol), and trimethylated tricetin (-8.32 kcal/mol) were accommodated into a pocket of the nucleotide binding domain (NBD) and substrate-binding domain) (SBD) interface; and the remaining ten flavonoid aglycones (-7.58 to -8.22 kcal/mol) into a pocket of the NBD domain.

The E protein of DENV plays an important role in the process that allows virus entry into host cells and is the target of antiviral agents [48,53]. The docking analysis results suggest that flavonoids present in the EtOH and SCO_2 extracts may have blocked the interaction between E and its receptor GRP78 and thus interfered with DENV-1 entry into HepG-2 cells. The E protein sites where flavonoids docked were selected as target sites for antiviral agents, as the ligands can block the conformational rearrangement in E that is crucial in the membrane fusion process [50,52,53]. The

antiviral effect of flavonoids on DENV is well documented. More than 30 flavonoids have been evaluated in *in vitro* models, of which twelve showed relevant efficacy [5,54]. In addition, *in silico* activity of about 30 flavonoids was predicted and some were potent ligands for DENV E protein [53]. Some of the flavonoids identified in the present study have been predicted as potent inhibitors of DENV E and non-structural proteins (NS5, NS3/NS2B), including galangin agylcone and its glycosidic form, luteolin and its glycosidic form, naringenin, phloridzin, pinocembrin and quercetin (Table S3). The GRP78 has been proposed as protein target to reduce the infectivity of viruses, and diverse anti-GRP78 agents can interfere with both entry and virions production of different enveloped viruses [51,55]. In current literature, a few flavonoids have been evaluated for *in silico* activity as GRP78 inhibitors and naringin showed higher affinities to the protein [56,57]. We could not find studies reporting anti-DENV and anti-GRP78 activities of the majority of flavonoids identified in the EtOH and SCO2 extracts.

Table 2. Flavonoid glycosides with the lowest binding energies for target proteins

Flavonoid	Target	Interacting amino acids: hydrogen bonds	kcal/mol
Luteolin rutinoside	DENV E[a]	Lys204, Ala205, Trp206, Val250, Thr262, Glu269, Ile270.	-9.16 ± 0.18
	CLEC5A	Lys100, Gln119, Thr122, Asp123, Ala124, His134, Glu136, Asn145, Leu166.	-10.07 ± 0.26
	GRP78	Ile207, Gly210, Lys213, Arg214.	-9.97 ± 0.35
	NLRP3	Thr437, Gln642, Tyr647, Met701.	-8.85 ± 0.18
Luteolin glucoside	CLEC5A	Phe77, Arg96, Lys116, Asp120, Tyr133, Gly150.	-9.13 ± 0.27
	GRP78	Ile206, Assp212, Lys213, Gly403.	-9.04 ± 0.23
Eriodictyol glucoside	DENV E[a]	Asp203, Lys204, Ala205, Trp206, Lys241, His261, Leu264, Thr265, Glu269.	-8.08 ± 0.14
	CLEC5A	Phe77, Lys116, Asp120, Tyr133, Phe148, Gly150, Asn156, Gln157, Phe159.	-9.48 ± 0.18
	GRP78	Tyr209, Gly210, Lys213, Arg214, Asp357, Ile359, Lys382, Glu383, Arg386.	-8.65 ± 0.16
	NLRP3	Arg165, Thr167, Thr231, Arg235, Tyr379, Phe506, Ile519.	-8.92 ± 0.42
Eriodyctiol rhamnoside	CLEC5A	Phe77, As93, Lys116, Asp120, Tyr130, Trp140. Gly150, Gln155, Asn156.	-9.08 ± 0.22
	GRP78	Ala208, Tyr396, Gly403.	-8.71 ± 0.11
Taxifolin glucoside	CLEC5A	Glu76, Lys116, Asp120, Tyr133, Lys138, Trp140, Phe148, Gly150, Gln155.	-8.55 ± 0.19
Quercetin glucoside	CLEC5A	Glu76, Gln79, Asp120, Trp140, Phe148, Gln155.	-8.90 ± 0.23
Galangin glucoside	DENV E[b]	Arg2, Ser72, Asp98, Arg99, Asn103, Asn193, Thr156.	-7.82 ± 0.30
	CLEC5A	Glu76, Phe77, Lys116, Asp120, Trp140, Phe148, Gly150.	-9.31 ± 0.27
	GRP78	Gly48, Gly210, Lys213, Gly403.	-8.64 ± 0.16

DENV E[a], DII domain into the interface formed between each chain (A and B) of the dimer. Domain DENV E[b], II chains interface. CLECSA: extracellular domain. GPR78: the NBD/SBD interface.

Macrophages are the primary target cells for DENV replication and the major source of inflammatory cytokines [9-11]. Flavonoids (e.g., apigenin, quercetin, and rutin) can down-regulate the production of proinflammatory cytokines in DENV-stimulated macrophages [58], which could be attributed to an inhibitory effect on signaling pathways of cytokine secretion [6]. The CLEC5A (C-

type lectin domain containing 5a) protein is a signaling receptor to sense the DENV infection in macrophages [59]. The E protein of DENV interacts with the glycans of the CLEC5A extracellular domain, facilitating the entry of the virus into macrophages and stimulating the release of cytokines, especially IL-1β [59]. The NLRP3 inflammasome is a cytosolic multi-protein complex of macrophages, and its activation is required to release IL-1β triggered by the CLEC5A-DENV interaction [11,59]. Inflammasome activation plays an important role in dengue pathogenesis [11]. We have carried out docking analysis to identify flavonoids as ligands of CLEC5A and the NLRP3 complex. All twenty flavonoids in the EtOH and SCO_2 extracts docked (-8.03 to -10.07 kcal/mol) to CLEC5A, which have formed hydrogen bonds with amino acids of a pocket of the extracellular domain (Figure 5A). In addition, the analysis predicted binding affinities between flavonoids and NLRP3, which have formed hydrogen bonds with amino acids of two consensus sites (Figure 5B). All flavonoid alycones, eriodyctiol rhamnoside and eriodyctiol glucoside (-7.55 to -8.92 kcal/mol) were accommodated in a consensus site corresponding to a pocket of the nucleotide-binding site (NBS) domain; and luteolin rutinoside, tricetin trimethyl, taxifolin glucoside, quercetin glucoside and galangin glucoside (-7.30 to -8.85 kcal /mol) into a pocket of the oligomerization domain (NACHT).

Table 3. Flavonoid aglycones with the lowest binding energies for target proteins

Flavonoid	**Target**	**Interacting amino acids: hydrogen bonds**	**kcal/mol**
Crysoeriol	CLEC5A	Glu76, Phe76, Lys116, Asp120, Lys138, Trp140, Phe148.	-8.54 ± 0.19
	GRP78	Thr37, Thr38, Tyr39, Lys96, Arg297, Gly364.	-8.18 ± 0.45
Trimethylated tricetin	CLEC5A	Met70, Asp74, Asp97.	-8.18 ± 0.25
	GRP78	Lys296, Arg297, Ser365.	-8.32 ± 0.28
Eriodictyol	DENV E	Leu25, His27, Asp192, Gly281, His282.	-8.05 ± 0.43
	CLEC5A	Arg96, Leu106, Asp123, Leu166.	-8.53 ± 0.15
Pinocembrin	DENV E	Leu25, His27, Gly281, His282.	-8.00 ± 0.77
	CLEC5A	Glu76, Phe77, Trp140, Gly150, Gln155.	-8.31 ± 0.13
	GRP78	Thr37, Tyr175, Thr229.	-8.12 ± 0.54
Naringenin	DENV E	His27, Thr48, Gly281, His282.	-7.94 ± 0.66
	CLEC5A	Cys71, Pro72, Glu76, Ser90, Arg181.	-8.34 ± 0.12
Taxifolin	CLEC5A	Leu106, Asp123, Leu166.	-8.46 ± 0.21
Sakuranetin	CLEC5A	Cys71, Glu76, Phe77, Ser90, Arg181.	-8.26 ± 0.15
Quercetin	CLEC5A	Leu106, Asp123, Leu166.	-8.53 ± 0.22
Methylated galangin	GRP78	Thr37, Thr38, Tyr39, Lys96, Glu201, Gly227, Arg297, Gly364.	-8.08 ± 0.52
	CLEC5A	Cys71, Lys116.	-8.17 ± 0.28
Galangin	CLEC5A	Glu76, Phe77, Trp140, Phe148, Gly150.	-8.09 ± 0.30
	GRP78	Thr37, Thr38, Tyr39, Lys96, Tyr175, Gly227, Thr229.	-8.20 ± 0.77
Phloridzin	CLEC5A	Phe77, Phe117, Gln155, Phe159.	-8.82 ± 0.23
	GRP78	Lys213, Tyr396, Ala399, Val400, Gly403.	-8.03 ± 0.25
	NLRP3	Arg165, Thr167, Tyr379.	-8.00 ± 0.30

CLEC5A, extracellular domain. DENV E, βOG pocket. GRP78, NBD domain. NLPR, NACHT domain.

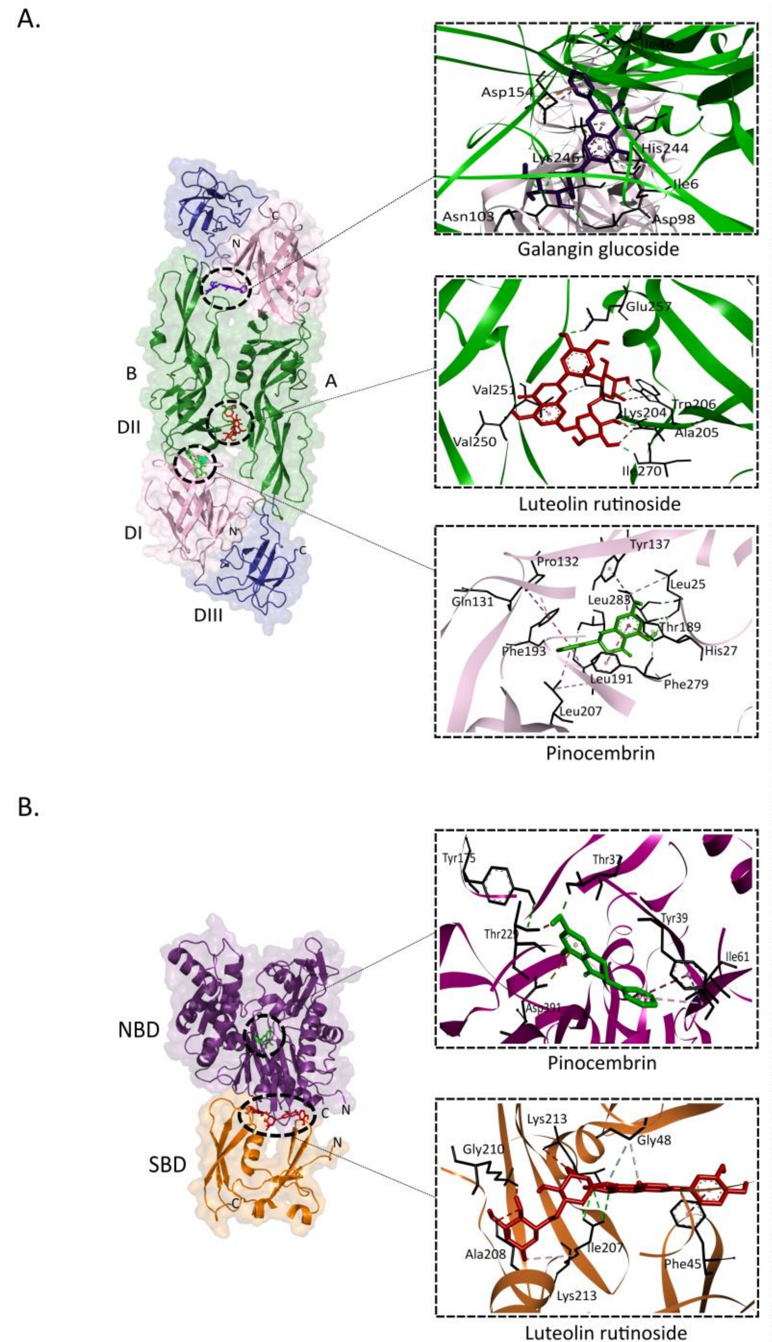

Figure 5. Docked poses of *L. origanoides* flavonoids with target proteins. (**A**) Docked flavonoids in complex with the dengue virus (DENV) type 2 envelope (E) protein. Pinocembrin representing flavonoid aglycones (n = 8), which were accommodated into the βOG pocket; luteolin rutinoside representing flavonoid aglycones (n = 4) and gylcosides (n = 1), which accommodated into a pocket of the chain A and B interface; and galangin glucoside, which accommodated into a pocket of the DI-DII hinge region "kl loop" structure. (**B**) Flavonoids in complex with the stress-inducible molecular chaperone protein 78 (GRP78). Pinocembrin representing flavonoid aglycones (n = 10), which accommodated into a pocket of the nucleotide binding domain (NBD); and luteolina rutinoside representing flavonoid glycosides (n = 7) and aglycones (n = 2), which accommodated into a pocket of the NBD and substrate binding domain (SBD) interface.

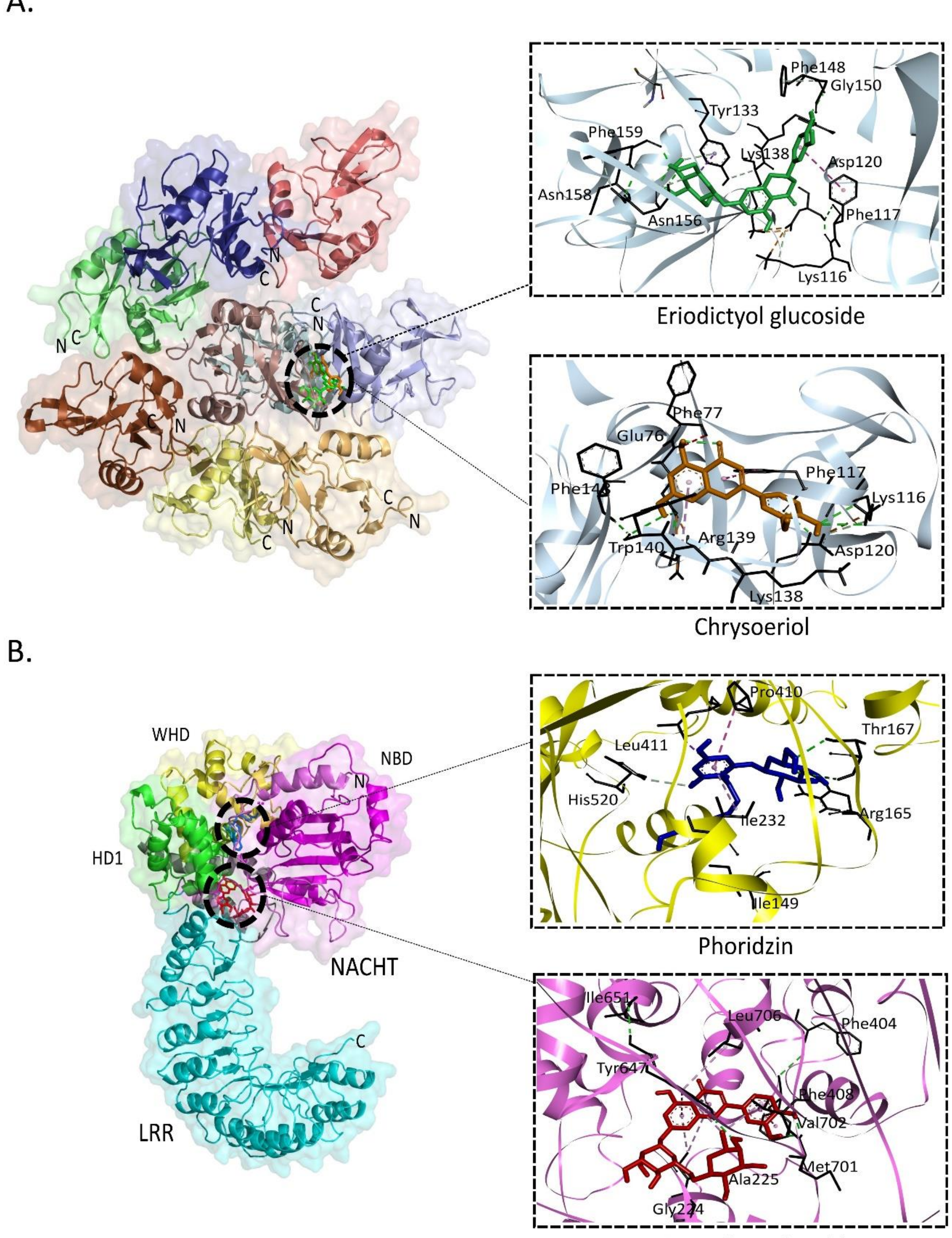

Figure 6. Docked poses of *L. origanoides* flavonoids with macrophage proteins involved in the IL-1β production. (**A**) Eriodictyol glucoside and chrysoeriol representing flavonoid glycosides (n = 2) and aglycones (n=12), respectively, in complex with the CLEC5A protein, which accommodated in a pocket of the extracellular domain. (**B**) Luteolin rutinoside representing flavonoids glycosides (n = 5) in complex with the NLRP3 inflammasome, which accommodated in a pocket of the nucleotide-binding and oligomerization domain (NACHT).

4. Conclusions

This *in vitro* study compared the anti-dengue potential of ethanolic (EtOH) and supercritical (SCO_2) extracts of *L. origanoides,* a plant used in folk medicine in Colombia. The EtOH extract showed a strong antiviral effect against DENV-1 and reduced the release of IL-1 β from DENV-stimulated macrophages. In contrast, the SCO_2 extract showed a weak antiviral effect and had no inhibitory effect on IL-1β. According to the docking analyses, the EtOH extract might have exerted its effects on DENV-1 and IL-1β through flavonoids, which can bind to the DENV E protein and its corresponding receptors GRP78 and CLEC5A in HepG-2 and macrophage-THP-1 cells, respectively. Flavonoids could interfere with both the virus entry into the cells and the stimulation of macrophages by DENV to produce IL-1 β. It appears that the flavonoid glycoside content was responsible for the better anti-dengue potential of the EtOH extract. The data provide a first step towards defining the potential utility of *L. origanoides* extract as a starting point for research and discovery of phytotherapeutics for dengue.

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Supporting Information

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