



Original Article

Cissampelos sympodialis has anti-viral effect inhibiting dengue non-structural viral protein-1 and pro-inflammatory mediators



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ARTICLE INFO

Article history:

Received 2 November 2015

Accepted 6 March 2016

Available online 19 May 2016

Keywords:

Cissampelos sympodialis

Dengue

Huh-7 cells

Cytokines

NS1

Warifteine

ABSTRACT

Dengue is the most important viral infection transmitted among humans by arthropod-borne. There are currently no vaccines or specific therapeutical treatment. Therefore, immunomodulatory compounds from plants have been widely examined for their antiviral effects. *Cissampelos sympodialis* Eichler, Menispermaceae, has scientifically proven to present immunomodulatory activities. Here we assessed the antiviral activity of leaf hydroalcoholic extract, warifteine or methylwarifteine from *C. sympodialis* in an *in vitro* dengue virus infection model. The results demonstrated that leaf hydroalcoholic extract or warifteine/methylwarifteine treatment did not reduce dengue virus-Ag+ hepatocyte (Huh-7 cell) rates in present experimental conditions. However, we assessed the potential antiviral effect of leaf hydroalcoholic extract or warifteine/methylwarifteine on dengue virus-infection by the production of inflammatory molecules, TNF- α , MIF, IL-8 and PGE₂. Dengue virus infection enhanced TNF- α , MIF, IL-8 and PGE₂ production in infected Huh-7 cells and leaf hydroalcoholic extract but not warifteine/methylwarifteine treatments, significantly reduced these molecules in infected cells. In dengue virus-infected Huh-7 cells, non-structural protein-1 is produced and leaf hydroalcoholic extract significantly inhibited it independently of alkaloids. Our findings imply that leaf hydroalcoholic extract may attenuate dengue virus infection in Huh-7 cells by inhibiting the enhanced of pro-inflammatory mediators and non-structural protein-1 production induce by dengue virus independently of warifteine/methylwarifteine its major compound.

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Introduction

Dengue virus (DENV) is an important human infectious pathogen in the tropics and subtropics; it remains an important public health burden requiring continuing attention. The clinical manifestations of dengue disease range from asymptomatic infection, undifferentiated fever and dengue fever, to dengue hemorrhagic fever with plasma leakage and potentially life-threatening dengue shock syndrome (Amorim et al., 2014).

Ethnobotanical studies have been the primary source for selection of molecules in scientific investigations and they represent a rich trial for immunomodulation products. *Cissampelos sympodialis* Eichler, Menispermaceae, popularly known as milona occurs in several Brazilian states such as Paraíba. The aqueous infusion of the leaves has been used in folk medicine to treat inflammatory diseases (Piuvezam et al., 2012). The leaf hydroalcoholic extract (AFL) and its bisbenzylisoquinoline alkaloids presented immunomodulatory effect in several experimental models of inflammations (Piuvezam et al., 2012).

In the present study, we hypothesized that AFL, warifteine or methylwarifteine display antiviral effect *via* its immunomodulatory properties. To prove it we used the *in vitro* model of DENV infection in Huh-7 cells (human hepatocyte cell lineage).

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Materials and methods

Plant material, obtaining and preparation of leaf hydroalcoholic extract (AFL) of *Cissampelos sympodialis*

The leaves of *Cissampelos sympodialis* Eichler, Menispermaceae, were obtained from the Botanical Garden of the Federal University of Paraíba (voucher specimen AGRA 1456) and the extract of the leaves (AFL) as well as its alkaloids warifteine (WAR) and methylwarifteine (MWAR) were gently provided by Dr. Jose Maria Barbosa Filho (De Freitas et al., 1996; Vieira et al., 2013). In brief, 3 kg of fresh leaves was collected, dried and pulverized. After, three successive alcohol extractions were performed in a percolator at room temperature (25–30 °C). The AFL was obtained with a mixture of water and ethanol (30/70, v/v). Then solvent was removed and the dry weight of the extract was 79.9% based on the present solid waste. The AFL was prepared in sterile saline (Piuvezam et al., 1999) to posterior standardization using the alkaloid warifteine as a pattern (Cerqueira-Lima et al., 2010). AFL was dissolved in dimethyl sulfoxide (DMSO) and stock solutions (1 mg/ml) stored at –20 °C. AFL was diluted to the indicated concentrations (0.1–100 µg/ml) with culture medium before use in experiments. DMSO concentration did not exceed 0.01%.

Bisbenzylisoquinoline alkaloids extraction and purification

To obtain warifteine and methylwarifteine, the *C. sympodialis* extract was dissolved in 3% HCl and extracted with CHCl₃. Aqueous fraction was basified with NH₄OH at pH 9 and again extracted with CHCl₃. The CHCl₃ extract was washed with H₂O and dried with MgSO₄ to get the tertiary alkaloid fraction. After, the tertiary alkaloid fraction was subjected to chromatography column over alumina, eluting with hexane containing CHCl₃/MeOH. Fractions eluted with CHCl₃-MeOH (49:1) were further purified by thin layer column (1 mm layer) for isolation of WAR and MWAR (Cerqueira-Lima et al., 2010; Melo et al., 2003; Vieira et al., 2013). WAR or MWAR powder (purity 90%) was dissolved in 0.1 N HCl. For each experiment, the stock solution was further diluted with 0.1 N HCl to desired concentrations (1, 2.5, 5, 10 µM).

Culture medium and preparation of Huh-7 cells

Huh-7 cells (hepatocarcinoma cell line), obtained from American Type Culture Collection (ATCC, cell line-615), were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Kibbutz Beit Haemek, Israel), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen Life Technologies).

MTT assay for cell viability cytotoxicity of AFL, warifteine and methylwarifteine

Huh-7 cells were incubated in 96-well plates at 1×10^5 cells per well containing 100 µl of DMEM medium and different concentrations of AFL (0 at 200 µg/ml), warifteine (WAR, 0–200 µM) or methylwarifteine (MWAR, 0–200 µM) for 72 h. Cells were washed once before adding 50 µl FBS-free medium containing MTT (5 mg/ml). After 4 h of incubation at 37 °C, the medium was discarded and the formazan blue that formed in the cells was dissolved in DMSO (SIGMA). The optical density was measured at 540 nm (Mosmann, 1983).

Cell cultures, virus stock preparation and virus titration

The DENV serotype 2 strain 16681 has provided by Dr. SB Halstead (Naval Medical Research Center, USA) and propagated in

Aedes albopictus C6/36 cell clone to obtain the virus stock, as described before (Reis et al., 2008). In brief, *A. albopictus* C6/36 cell clone was grown as monolayers at 28 °C on Leibovitz medium (L-15) supplemented with 200 mM glutamine, 1% non-essential amino acid solution, 0.5% tryptose phosphate broth, 100 U/penicillin, 10 µg/streptomycin and 5% fetal bovine serum (FBS) and infected with DENV-2 for 8 days. After, the supernatant containing the virus particles was ultracentrifuged ($100,000 \times g$) for 1 h at 4 °C. The pellet was stored at –70 °C and Virus titer was calculated as 50 percent tissue culture infectious dose (TCID₅₀). The virus stock used was at a concentration of 1.6×10^9 TCID₅₀/ml (Reis et al., 2008; Lima-Junior et al., 2013).

Huh-7 cells infection and treatment with AFL, warifteine or methylwarifteine

Huh-7 cells were resuspended in supplemented RPMI 1640 medium, plus 10% FCS and seeded at 2×10^6 cells/ml on 96- or 24-well plates. After an overnight incubation, infection was effected with a diluted inoculum (30 or 300 µl) in cell culture medium containing 1.6×10^9 TCID₅₀/ml. After a 2 h-incubation period for adsorption, the cell culture supernatant was replaced with a 2% FBS medium and incubated with leaf hydroalcoholic extract (AFL) of *C. sympodialis* (0.1, 1 or 10 µg/ml), WAR (0.1, 1 or 10 µM) or MWAR (0.1, 1 or 10 µM) and subsequently incubated at 37 °C with 5% CO₂. After 24, 48 or 72 h, supernatants were collected and stocked at –20 °C for cytokine measurement and cells recovered for viral antigen determination, cell viability determined in culture by MTT assay. Well content with cell control, inactivated and infectious DENV was assayed.

Viral antigen determination in Huh-7 cells by flow cytometry

Huh-7 cells were recovered in a cold cell culture medium, set at 1×10^6 cells/microtube, then centrifuged ($350 \times g$, 10 min) and washed once with phosphate buffered saline pH 7.4 containing 1% bovine serum albumin and 0.1% NaN₃ (PBS/BSA). Afterwards, cells were fixed with paraformaldehyde 2% in PBS/BSA at 4 °C for 20 min and permeabilized with saponin 0.15% in PBS/BSA. Permeabilized cells were then blocked with 5% inactivated plasma in PBS/BSA at 4 °C for 30 min and incubated with mouse anti-Dengue Complex monoclonal antibody (MAB8705, Millipore) at 4 °C for 60 min. Cells were then washed and incubated for 30 min at 4 °C with anti-mouse IgG Alexa Fluor 488 (A20181, Life Technologies). After incubation, cells were washed with PBS/BSA, resuspended in paraformaldehyde 2%, and kept at 4 °C until cell acquisition (5000 events for gated monocytes) by FACS[®] Calibur flow cytometer (Beckon & Dickinson) and analyzed with FlowJo Software (TreeStar Inc.). An isotype-matched antibody was adopted as a staining negative control (Lima-Junior et al., 2013).

Cytokine quantification

ELISA cytokine kits was used to measure TNF-α, IL-8, and MIF in the cells supernatant, and the assay was performed according to the manufacturer's instructions (R&D Systems, CA, USA) as described previously (Assuncao-Miranda et al., 2010). Data analyses of all assays were performed with Bio-Plex Manager software (Bio-Rad). Prostaglandin (PG) E₂ concentrations in the cell culture supernatants from Huh-7 cells were determined by an enzyme immunoassay (ELISA) kit according to the procedures supplied by the manufacturer (Cayman Chemical, Ann Arbor, MI, USA).

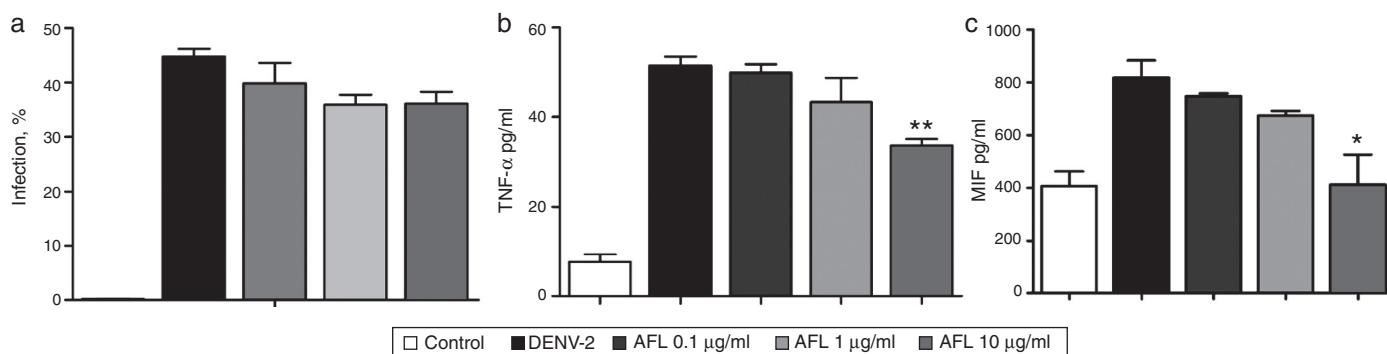


Fig. 1. Effect of AFL on Dengue virus type 2 (DENV-2 strain 16681) D infection, TNF- α and MIF production in infected Huh-7 cells. (A) DENV antigen positive cells detected by flow cytometry analysis. (B) TNF- α and (C) MIF- α levels detected by ELISA. Results were presented as mean \pm SEM where * $p < 0.05$ was considered significant by one-way ANOVA and a Dunnett test, as a post-test when the test substance were compared with DENV-2. Data are representative of three independent experiments performed in triplicates.

NS1 quantification

NS1 was measured in culture medium from Huh-7 cells infected with dengue virus and pre-treated using a commercial sandwich ELISA kit (Platelia NS1; BioRad Laboratories). The tests were conducted with according to the manufacturer's protocols.

Results and discussion

Immunomodulatory compounds of plants have been widely examined for their antiviral effects (Abd Kadir et al., 2013; Reis et al., 2008). In this study, anti-DENV activity of leaf hydroalcoholic extract (AFL) of *C. sympodialis*, warifteine (WAR) and its methylated form methylwarifteine (MWAR) the major alkaloids isolated from the plant were evaluated. The concentration of AFL or WAR/MWAR used was tested by MTT assay (see section *Culture medium and preparation of Huh-7 cells*) and they do not show toxic effects on Huh-7 cells (data not shown).

To determine whether AFL has anti-DENV effect, we firstly measured DENV-antigen expression, TNF- α and MIF production in Huh-7 cells infected by DENV-2. We pre-infected Huh-7 cells for 2 h and treated with AFL (0.1, 1 and 10 $\mu\text{g/ml}$) and cells were collected at 72 h post-infection. As shown on Fig. 1A, the plant extract (AFL) did not interfere with the expression of DENV. However, AFL (10 $\mu\text{g/ml}$) reduced the production of TNF- α at 72 h post-infection (Fig. 1B) and reduced MIF production by Huh-7 infected cells (Fig. 1C).

TNF- α is the main cytokine that induces vascular leakage and its serum levels have been implied in DENV-infected patients with serious forms of disease (Braga et al., 2001; Sellahewa, 2013). In

mice, vascular hemorrhage can be induced by injection of DENV and is less severe in TNF- α deficient mice or blocking by anti-TNF- α antibodies (Chen et al., 2007; Shresta et al., 2006). Furthermore (Perez et al., 2010) demonstrated an association between high levels of expression of TNF- α allele and susceptibility of dengue hemorrhagic fever (DHF).

Another important cytokine in DENV infection is macrophage migration inhibitory factor (MIF). Assuncao-Miranda et al. (2010) showed enhanced of MIF on plasma of patients with severe forms of dengue. Therefore, endogenous MIF contributes to the pathogenesis of dengue infection. In the present study, *in vitro* infection of Huh-7 cells with DENV-2 increased MIF levels that were successfully suppressed by AFL treatment (Fig. 1C)

To confirm the immunomodulatory activity of AFL we measured on supernatant, IL-8 and PGE2 levels. AFL (10 $\mu\text{g/ml}$) inhibited both IL-8 and PGE2 (Fig. 2A and B). Interleukin-8 and PGE2 induce alterations in endothelial functions (Purwati et al., 2011; Lima-Junior et al., 2013). Elevated levels of these molecules were detected in serum and pleural fluid of patients with DHF (Raghupathy et al., 1998; Purwati et al., 2011) suggesting that the fluid effusion into the pleura and other serous cavities in DHF patients may be at least in part attributed to IL-8 and/or PGE2. The production of IL-8 appears to be dependent on IL-1 and TNF- α (Raghupathy et al., 1998) and as it was related above AFL decreased the amount of TNF- α on DENV-2 infected Huh-7 cells suggesting a cytokine down regulatory effect of AFL.

In DENV infection, hepatocytes produce and activate cyclooxygenase-2 (COX-2) with production of PGE2 (Liou et al., 2008). The so-called break-bone pain in dengue disease is probably related to the overproduction of PGE2 and this molecule

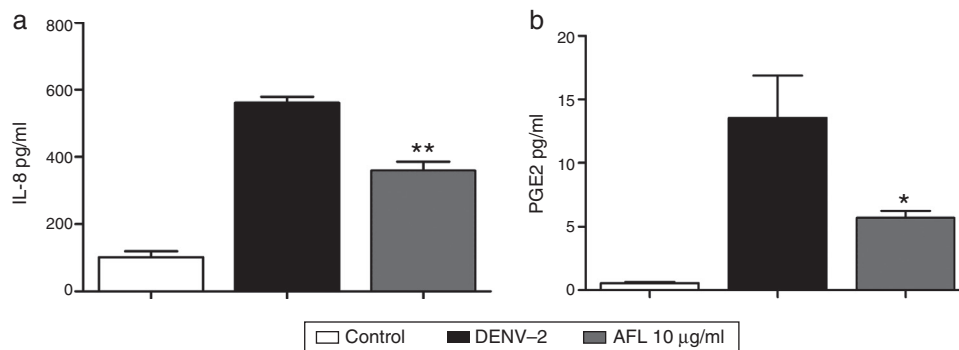


Fig. 2. Effect of AFL on DENV-2 induced IL-8, PGE₂ or NS1 production in infected Huh-7 cells treated with AFL (10 $\mu\text{g/ml}$) for 2 h pre-infection. The supernatant of cells were collected at 48 h post-infection with Dengue virus type 2 (DENV-2 strain 16681). (A) IL-8 level in DENV infected Huh-7 cells treated with AFL (B) PGE₂ level in DENV infected Huh-7 cells treated with AFL. IL-8 or PGE-2. where * $p < 0.05$, ** $p < 0.01$ was considered significant by one-way ANOVA and Dunnett test, as a post-test when the test substance were compared with DENV-2. Data are representative of 3 independent experiments performed in three replicates.

has important effect on replication and infectivity of the virus (Steer and Corbett, 2003; Tsatsanis et al., 2006). Therefore COX-2 modulation by the plant extract may be a promising target for controlling not only inflammation and pain but also viral infection indicating an important role of the plant in this disease (Rocca and FitzGerald, 2002; Steer and Corbett, 2003).

DENV is an enveloped virus, and its genome is composed of a positive-sense, single-stranded RNA coding for three structural proteins present in the virion and infected cells and seven non-structural (NS) proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) not present in the virion (Amorim et al., 2014). NS1 is a glycoprotein expressed in infected mammalian cells as soluble monomers that is subsequently transported to the cell surface where the protein remains and/or it is released into the extracellular milieu been essential for viral replication and viability. In addition, this protein appears into the blood stream and several laboratorial tests use NS1 to diagnose DENV infections (Amorim et al., 2014). In this study, we showed that AFL significantly reduced NS1 production in DENV-2 infected Huh-7 cells (Fig. 3).

To determine the mechanism of action of AFL on *in vitro* dengue infection we tested two major alkaloids from the plant: warifteine (WAR) and its methylate form methylwarifteine (MWAR). As it is shown on Fig. 4A, WAR/MWAR treatment did not reduce DENV-2 antigen in infected Huh-7 cells and did not inhibit TNF- α or MIF production (Fig. 4B and C). In addition, neither WAR nor MWAR reduced IL-8, PGE2 nor NS1 production by DENV-2 infected Huh-7 cells (Fig. 5A–C) indicating that AFL effect is independently of

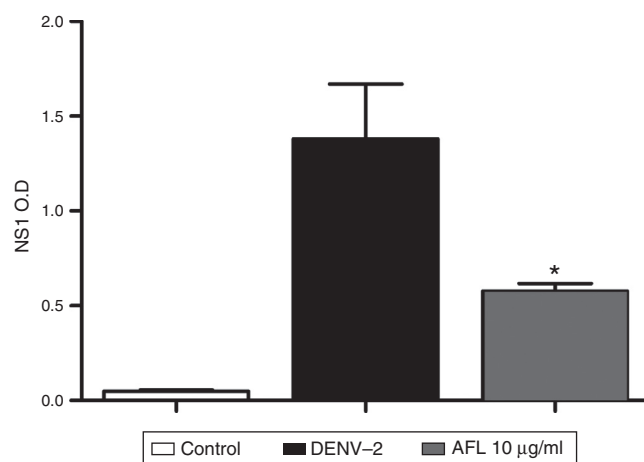


Fig. 3. Effect of AFL on DENV-2 induced NS1 production in infected Huh-7 cells treated with AFL (0.1, 1 or 10 µg/ml) for 2 h pre-infection. The supernatant of cells were collected at 24, 48 or 72 h post-infection with dengue virus type 2 (DENV-2 strain 16681). NS1 level in DENV-infected Huh-7 cells treated with AFL were determined by ELISA in the supernatants of Huh-7 cells 72 h post infection, where * $p < 0.05$, ** $p < 0.01$ was considered significant by one-way ANOVA and a Dunnett test, as a post-test when the test substance were compared with DENV. Data are representative of three independent experiments performed in triplicates.

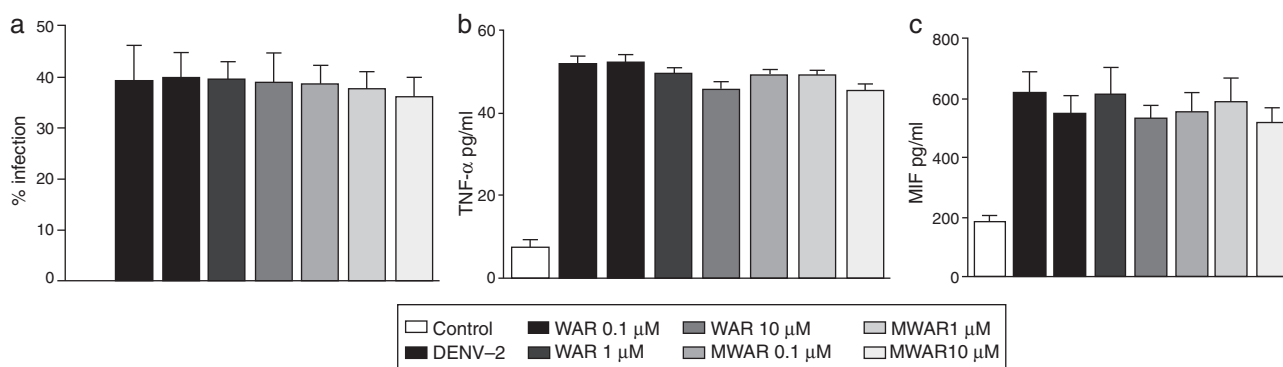


Fig. 4. Effect of WAR or MWAR on Dengue virus type 2 (DENV-2 strain 16681) D infection, TNF- α and MIF production in infected Huh-7 cells. (A) DENV antigen positive cells detected by flow cytometry analysis. (B) TNF- α and (C) MIF- α levels detected by ELISA. Results were presented as mean \pm SEM where * $p < 0.05$ was considered significant by one-way ANOVA and a Dunnett test, as a post-test when the test substance were compared with DENV-2. Data are representative of three independent experiments performed in triplicates.

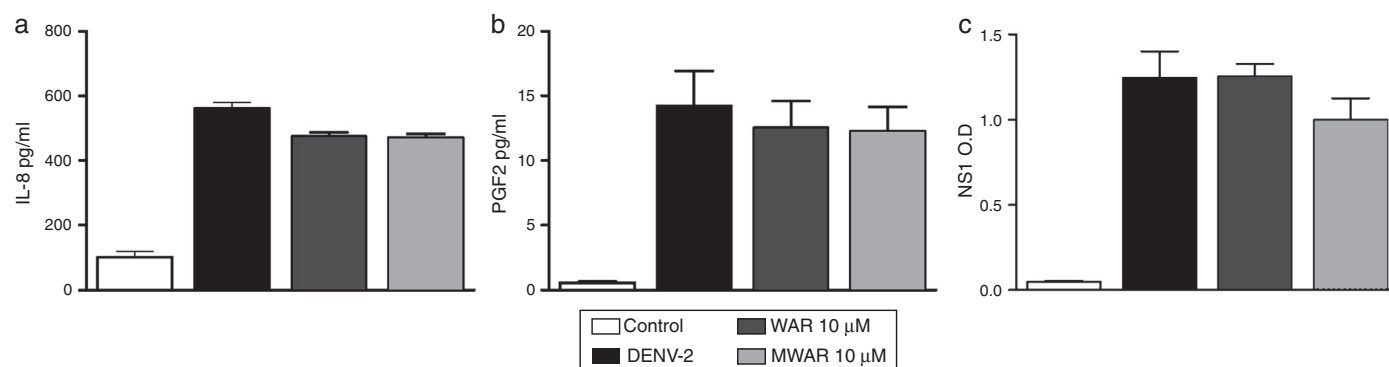


Fig. 5. Effect of WAR or MWAR on DENV-2 induced IL-8, PGE2 or NS1 production in infected Huh-7 cells treated with AFL (10 µg/ml) for 2 h pre-infection. The supernatant of cells were collected at 48 h post-infection with Dengue virus type 2 (DENV-2 strain 16681). (A) IL-8 level in DENV infected Huh-7 cells treated with AFL (B) PGE2 level in DENV infected Huh-7 cells treated with AFL. IL-8 or PGE-2 and (C) NS1 level in DENV-infected Huh-7 cells treated with WAR were determined by ELISA in the supernatants of Huh-7 cells 72 h post infection, where * $p < 0.05$, ** $p < 0.01$ was considered significant by one-way ANOVA and a Dunnett test, as a post-test when the test substance were compared with DENV-2.

WAR/MWAR and that the plant extract presents other compounds responsible for the antiviral effect.

Conclusion

In conclusion, DENV2 infected Huh-7 cells produce TNF, MIF, IL-8 and PGE2 that are correlated with NS1 production and AFL suppressed the viral-induced inflammatory response by decreasing the cytokine production as well as NS1 release, but failed to affect virus production. Conversely, warifteine/methylwarifteine did not affect the inflammatory mediators nor virus production in DENV-2 infected Huh-7 cells. These data shed light on dengue virus infection and *C. sympodialis* extract capable to reduce NS1 and pro-inflammatory mediators demonstrating the potential anti-inflammatory and antiviral therapy of this herbal medicine.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors' contributions

FCL, CFM and LGF (PhD students) contributed to the development of the biological protocols. CSM and ALAL (MS students) contributed to the ELISA assay. JMBF contributed in plant identification and alkaloid isolation. CFK and MRP designed the study, supervised the laboratory work and contributed to critical reading of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

This study was financially supported by CNPq-Universal14/2012-472853/2012-0; CAPES/Brazil; PROEP, IOC and Plataforma Tecnológica (RPT11D)/FIOCRUZCSM, LGF and CM are graduated students at Posgbp IOC, Fiocruz.

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