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Original Article

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

Fagner Carvalho Leite[a,b], Cíntia da Silva Mello[c], Luciana Gomez Fialho[c], Cintia Ferreira Marinho[c], Ana Luisa de Araujo Lima[a], José Maria Barbosa Filho[d], Claire Fernandes Kubelka[c], Marcia Regina Piuvezam[a,*]

[a] *Laboratório de Imunofarmacologia, Departmento de Fisiologia e Patologia, Universidade Federal da Paraíba, João Pessoa, PB, Brazil*
[b] *Faculdade São Francisco da Paraíba, Cajazeiras, PB, Brazil*
[c] *Laboratório de Imunologia Viral, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil*
[d] *Laboratório de Fitoquímica, Departmento de Ciências Farmacêuticas, Universidade Federal da Paraíba, João Pessoa, PB, Brazil*

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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 PGE_2. Dengue virus infection enhanced TNF-α, MIF, IL-8 and PGE_2 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/methywarifteine its major compound.

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).

* Corresponding author.
E-mail: mrpiuvezam@ltf.ufpb.br (M.R. Piuvezam).

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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 Paraiba (vouche specimen AGRA 1456) and the extract of the leaves (AFL) as well as it 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 $CHCl_3$. Aqueous fraction was basified with NH_4OH at pH 9 and again extracted with $CHCl_3$. The $CHCl_3$ extract was washed with H_2O and dried with $MgSO_4$ to get the tertiary alkaloid fraction. After, the tertiary alkaloid fraction was subjected to chromatography column over alumina, eluting with hexane containing $CHCl_3$/MeOH. Fractions eluted with $CHCl_3$–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 Techonologies).

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 obtained 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 × *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 × *g*, 10 min) and washed once with phosphate buffered saline pH 7.4 containing 1% bovine serum albumin and 0.1% NaN_3 (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).