Supplementary Material

_Himatanthus phagedaenicus_ stem extracts present anti-flavivirus activity while an isolated sesquiterpene glucoside present only anti-Zika virus activity _in vitro_

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Abstract
The hexane and ethanol extracts from *Himatanthus phagedaenicus* (Apocynaceae) stems were evaluated for antiviral activity against Zika virus, yellow fever virus and dengue virus 2 and for cytotoxicity in Vero cells by MTT assay. The ethanol extract showed good antiviral activity against the three viruses with selective indexes (SI) > 10 and its fractionation led to the isolation of the known plumieride that was active only against Zika virus (SI of 15.97).

Keywords: *Himatanthus phagedaenicus*; Zika virus; Yellow Fever virus; Dengue virus; Plumieride; Spectroscopic analyses.

Experimental Section

*Plant material*

Plant samples were collected in the city of Nova Lima, state of Minas Gerais, Brazil. Voucher specimens were deposited in the Herbário BHCB, Universidade Federal de Minas Gerais, under the code BHCB 112116.

*Preparation of extracts*

After drying in a circulating air oven at 37ºC, for 48 h, dried stems were milled and the powder was kept in dark flasks. Stems powder (143 g) was submitted to successive extractions with hexane (HEX) and then with ethanol (EtOH), by percolation at room temperature. The extracts were concentrated in a rotary evaporator and kept in a glass desiccator under vacuum.

*Fractionation of Himatanthus phagedaenicus stems ethanol extract (HPE EtOH)*

An aliquot of HPE EtOH (30.0 g) was dissolved in a mixture of methanol/water (6/4), and was extracted with chloroform (3X), that resulted in the fractions HPF CHCl₃ (7.4 g, yield 24.5%), and HPF H₂O (20.8 g, yield 69.2%). Subsequently, from the HPF CHCl₃ fraction (6.0 g), after chromatography on a silica gel column (200.0 g), eluted with solvents of increasing polarities, gave 14 different fractions of which F2 (3.0 g) was rechromatographed in silica gel column, obtaining 9 fractions that were
combined according to their TLC profiles, from group 4, *Himatanthus phagedaenicus* isolated (HPI, 0.5 g) crystallized out.

**Phytochemical analyses**

**Thin layer chromatography (TLC)**

Different secondary metabolites classes in the extract and chromatographic fractions were detected by TLC (silica gel F$_{254}$). Mobile phases and visualization spray reagents were used according to Wagner et al. (1984), in comparison with reference compounds.

**UPLC-DAD, UPLC-DAD-ESI-MS and UPLC-ESI-MS/MS**

Analyses were performed in an ACQUITY UPLC H-Class Core System (Waters) with an ACQUITY UPLC™ PDA DETECTOR in the Laboratório de Fitoquímica, Faculdade de Farmácia, UFMG. The mobile phase consisted of water 0.1% formic acid (solvent A) and acetonitrile 0.1% formic acid (solvent B). The elution protocol was 0-13 min, linear gradient from 5% to 95% B. The flow rate was 0.3 mL min$^{-1}$, and the sample injection volume was 4.0 μL. The UV spectra were registered from 200 to 400 nm. Mass spectrometry analyses by electron spray ionization (UPLC-PDA-ESI-MS) were performed in an ion trap mass spectrometer equipped with an atmospheric pressure ionization (API) interface operated in the following conditions: positive and negative ion mode; capillary voltage, 3500 V; capillary temperature, 320ºC; source voltage, 5 kV; vaporizer temperature, 320ºC; corona needle current, 5 mA; and sheath gas, nitrogen, 27 psi. Analyses were run in the full scan mode (100–2000 Da). The ESI-MS/MS analyses were performed in an UPLC Acquity (Waters) equipped with a Tandem Quadrupole Detector (TQD), fitted with an electrospray source in the positive and negative mode; ion spray voltage: −4 kV; orifice voltage: −60 V, with helium as the collision gas; collision energy was set at 30 eV. An ACQUITY UPLC BEH C-18 column (1.7 μm, 50 × 2 mm i.d.) (Waters) was used.

**Infrared (IR) Spectroscopy and Melting point**

Infrared spectra were recorded on a Spectra One PerkinElmer spectrophotometer, ted with a Paragon ATR accessory and melting point of compound was determined on Microquímica MOAs 301 apparatus.
Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectra were registered on a Bruker AvanceIII 200 MHz using TopSpin™ 3.2 pl 6.0 software for acquisition and processing, in the LAREMAR at Universidade Federal de Minas Gerais – UFMG. The sample was dissolved in CD$_3$OD and the chemical shifts (δ, in ppm) are reported relative to tetramethylsilane (TMS). Coupling constants (J) are given in Hz.

Physical and spectroscopic data on plumieride. Colourless crystals (MeOH); mp 227.9 – 230.0 °C, Lit. mp 227 – 229 °C (Singh et al., 2014). IR ν$_{max}$ 3367, 1754, 1693 and 1633 cm$^{-1}$; ESI-MS m/z: 469.00 [M – H]$^-$ and 471.36 [M + H]$^+$ (26.3%), 291.24 [(M – C$_{15}$H$_{15}$O$_6$)]$^+$ (100.0%), 309.27 [(M – C$_{15}$H$_{17}$O$_7$)]$^+$ (95.0%); $^1$H NMR (200 MHz, CD$_3$OD): 1.42 (d, J 6.0 Hz, CH$_3$-14), 2.96 (m, H-2'), 3.20 (dd, J 8.0 and 4.0 Hz, H-9), 3.40 (m, H-3'), 3.50 (m, H-3'), 3.67 (s, CH$_3$-16), 3.80 (m, H-5'), 3.89 (d, J 12.0 Hz, H-5), 4.44 (m, H-13), 4.50 (dd, H-4'), 4.70 (d, H-1'), 5.27 (d, J 6.0 Hz, H-1), 5.50 (dd, J 6.0 and 4.0 Hz, H-7), 6.40 (dd, J 2.0 and 4.0 Hz, H-6), 7.37 (s, H-3), 7.51 (s, H-10). $^{13}$C NMR (50 MHz, CD$_3$OD): 22.50 (C-14), 40.46 (C-5), 50.60 (C-9), 52.10 (C-16), 61.00 (C-6'), 63.60 (C-13), 71.30 (C-4'), 74.70 (C-2'), 77.80 (C-3'), 78.90 (C-5'), 94.20 (C-1), 97.90 (C-8), 100.10 (C-1'), 111.10 (C-4), 130.00 (C-7), 138.60 (C-11), 141.50 (C-6), 150.30 (C-10), 152.60 (C-3), 168.50 (C-15), 172.80 (C-12).

Biological assays

Cell lines

Vero cells (ATCC number CCL-81) were maintained in minimal essential medium (MEM, Cultilab, Brazil). Culture media were supplemented with 5% fetal bovine serum (FBS, Cultilab, Brazil), 200 U/mL of penicillin (ThermoFischer Scientific, USA), 100 µg/mL of streptomycin (ThermoFischer Scientific, USA) and 2.5 µg/mL of amphotericin B (Merck, Germany).

Viruses

Viruses were obtained from the virus collection of the Laboratório de Vírus from Universidade Federal de Minas Gerais (UFMG). Zika virus (PE243) was isolated in Recife, Brazil, from a male patient in 2015, at the Fundação
Oswaldo Cruz (FIOCRUZ) as described before (Donald et al., 2016). Dengue virus 2 (PI59), lineage II, was identified during an outbreak in Piauí, Brazil, as described before (Figueiredo et al., 2014). Vaccine of yellow fever virus (17DD) was kindly provided by Bio-Manguinhos (FIOCRUZ N. 980FB066Z). All viruses were replicated and titrated in Vero cells.

Viral replication

Culture cell flasks of 75 cm² containing Vero cells were incubated with MEM 5% FBS at 37 °C and 5% CO₂ atmosphere. After 24h, with approximately 60 to 80% confluent monolayer the medium was removed and the cells were washed three times with phosphate-buffered saline (PBS) to remove cellular debris and serum residue. Cells of a control flask were counted and cells were infected at a Multiplicity Of Infection (MOI) of 0.01 of each virus in 2 mL MEM, and flasks were gently homogenized every 10 minutes during an 1h incubation at 37 °C and with 5% CO₂. Following adsorption period of 1 hour, 12 mL of fresh MEM with 2% FBS were added to the flasks and incubated in the same conditions, checked daily to evaluate the cytopathic effects. In monolayers with 80% cytopathic effect, the supernatant was removed, centrifuged at 4 °C to remove debris and stored at -70 °C.

Viral titration

Vero cells were seeded in 6-well plates (2.10⁵ cells per well) and incubated at 37 °C and 5% CO₂ atmosphere. After 24h, plates were infected with 300 μL of a serial dilution (1:10) of each virus (10⁻¹ a 10⁻⁷). After 1h adsorption at the same conditions, 2.0 mL of fresh M199 (Gibco, USA) medium with antibiotics, 2% FBS and 1% of carboxymethylcellulose (CMC) (Synth, Brazil) were added to each well. After five (ZIKV) or seven days (DENV-2 and YFV) of incubation at 37 °C and 5% CO₂ atmosphere, plates were fixed with 10% formalin solution overnight, followed by coloration with 1% violet crystal solution for 15 minutes. Plates were washed and set to dry, counting in each well 30 to 100 plate forming units per millilitres (PFU/mL) to determinate the virus titer (Coelho et al. 2017).

Citotoxicity assay
Cytotoxicity was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay (Mosmann, 1983). Vero cells were seeded in 96-well plates (4.10^4 cells per well) and incubated at 37 °C and 5% CO₂ atmosphere. After 24 hours of incubation, 200 μL of fresh MEM medium (1% FBS) containing serial dilution of extracts (from 500 to 1.95 μg/mL) were added to the plates. A serial dilution of DMSO was used as viability control, as well as an inhibition control (10% DMSO solution) and a blank (medium only). After 72 hours of incubation at 37 °C and 5% CO₂ atmosphere, medium was removed and 100 μL of MTT tetrazolium (ThermoFischer Scientific, USA) solution in MEM (0.5 mg/mL) were added to each well. After 3 hours of incubation at 37 °C and 5% CO₂ atmosphere, the medium was removed and 100 μL of DMSO was added to each well to solubilize formazan crystals. After shaking for 15 minutes, absorbance at 570 nm of each well was read using a spectrophotometer (VersaMax, Molecular Devices, USA). Cytotoxic concentration for 50% of the cells (CC<sub>50</sub>) of each extract was calculated as a percent of cell viability as follow:

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\frac{(ODe) - (ODB)}{(ODc) - (ODB)} \times 100(\%)
\]

OD<sub>e</sub>, OD<sub>c</sub> and OD<sub>b</sub> indicate absorbance of cells with extracts, viability control and blank, respectively. Linear regression was used for analysis, considering R² values superior to 0.9. All conditions were tested in triplicates.

Antiviral activity assay

Vero cells were seeded in 96-well plates (4.10^4 cells per well) and incubated at 37 °C and 5% CO₂ atmosphere. After 24 hours of incubation, medium was removed and 100 μL of fresh medium containing a serial dilution of extracts (below the CC<sub>50</sub> of each extract) were added to the plates. Subsequently, viral suspensions of ZIKV, DENV-2 and YFV were prepared in MEM (1% FBS) to a MOI of 0.1. That is, 100 μL of fresh medium containing 4.10<sup>3</sup> viral particles were added to each well. After 72h of incubation at 37°C and 5% CO₂ atmosphere, medium was removed and 100 μL of MTT (ThermoFischer Scientific, USA) solution in MEM (0.5 mg/mL) were added to
each well. After 3 hours of incubation at 37 °C and 5% CO₂ atmosphere, the medium was removed and 100 μL of DMSO was added to each well solubilize formazan crystals (Takeuchi et al., 1991). After shaking for 15 minutes, absorbance at 570 nm of each well was read using a spectrophotometer (VersaMax, Molecular Devices, USA). Ribavirin and mycophenolic acid (MPA) were used as inhibition controls, and a serial dilution of DMSO was used as viability control. The effective concentration for 50% of the cells (EC₅₀) of each extract was calculated as protection of virus-infected cells, as follow:

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\frac{(ODve) - (ODv)}{(ODc) - (ODv)} \times 100(\%)
\]

ODve, ODv and ODc indicate absorbance of virus-infected cells with extracts, virus-infected cells only and viability control, respectively. Linear regression was used for analysis, considering R² values superior to 0.9. Selectivity index (SI) to each extract was determined, as the ratio between the cytotoxic concentration (CC₅₀) and the effective concentration of 50% (EC₅₀). All conditions were tested in triplicates.

**Statistical analysis**

All data were expressed as means ± standard deviations of triplicate measurements. Antiviral activity and cytotoxicity of extracts, fractions and isolated compound were submitted to ANOVA and the Tukey’s test was used to compare the means (α = 0.05). All statistical analysis were performed using the software SigmaPlot 12.5 (Systat, 2013).

References


Systat, 2013. Software, San Jose, CA, USA.


Figure Legend

Figure S1. Full scan of Total Positive Ion Chromatogram (TIC) of the HPE EtOH (red), HPF H$_2$O (green), and HPF CHCl$_3$ (purple). Conditions: See Experimental Section.

Figure S2. Chemical structure of the sesquiterpene glucoside plumieride.