Virucidal and Antiviral Activity of Extract of *Punica Granatum* Cultivated in Brazil

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Abstract

Background: The arthropod-borne Mayaro virus (MAYV) causes 'Mayaro fever', a disease of medical significance, primarily affecting individuals in permanent contact with forested areas in tropical South America. Recently, MAYV has attracted attention due to its likely urbanization. Currently, there are no licensed drugs against most mosquito-transmitted viruses. *Punica granatum* (pomegranate) fruits cultivated in Brazil were submitted to a phytochemical investigation for the identification and isolation of antiviral compounds. In the present study we studied the antiviral activity of pomegranate extracts in Vero cells infected with Mayaro virus.

Methods: The Ethanol extract and Punicalagin of Pomegranate were extracted solely of the shell and purified by chromatographic fractionation and chemically identified using spectroscopic techniques. Cytotoxicity of purified compounds was measured by the dye-uptake assay while their antiviral activity was evaluated by a virus yield inhibition assay.

Results: Pomegranate ethanol extract ($CC_{50} = 588.9$, $IC_{50} = 12.3$) and a fraction containing punicalagin as major compound ($CC_{50} = 441.5$, $IC_{50} = 28.2$) were shown to have antiviral activity (SI 49 and 16, respectively) against Mayaro virus, an alphavirus. Immunofluorescence analysis showed the virucidal effect of Pomegranate extract and Transmission Electron Microscopy (TEM) revealed damage in viral particles treated with this extract.

Conclusions: The *P. granatum* extract is a promising source of antiviral compounds against the alphavirus MAYV and are excellent candidates for future studies with other enveloped RNA viruses.

Background

Mayaro fever is a dengue-like but usually non-fatal illness, occurring in tropical South America and, endemic to the Amazon region[1], where outbreaks have been registered. The virus was first isolated in Trinidad and Tobago [2] and imported cases of human infection occur, but not frequently, outside the Amazon region [3], although some cases have been reported in other regions [4, 5].

Mayaro virus (MAYV) belongs to the *Togaviridae* family, alphavirus genus, is closely related to Chikungunya and other human alphaviruses [6], has two known genotypes (D and L) and is transmitted mainly by *Haemagogus janthinomys* mosquitoes [1]. Although MAYV has not been associated with fatal human disease, primary infections are often debilitating, with loss of productivity for weeks or even months due to severe arthralgia [7]. MAYV is an enveloped virus, and its genome is a single strand, positive polarity RNA; the virus particle has an icosahedral symmetry and 70 nm in size. Its genome has approximately 11 kbp coding for two polyproteins that are cleaved into non-structural proteins (nsP1, nsP2, nsP3 and nsP4) and structural proteins (C, E2, E3, 6k, E1)[2].

The Mayaro disease consists of a mild or moderate fever of abrupt onset and short duration, chills, and pain of the muscles, joints, and headache. Mosquitoes of *Haemagogus* sp. and *Aedes* sp. act as vectors

of MAYV in rural and urban areas, respectively [8]. A recent finding shows the potential for MAYV transmission by the urban vectors *Aedes aegypti* and *Aedes albopictus* thus contributing to the classification of the MAYV as an emerging virus, having a potential for inclusion in the urban cycle, as occurred more recently with the Chikungunya virus [9–11].

The plant *Punica granatum* L. (*Punicaceae*) has been used popularly as a medicinal plant since ancient times, for several purposes. As antiviral, pomegranate extracts were effective against herpes and influenza viruses. Furthermore, a topic micro biocide could potentially be made for HIV prevention [12]. Punicalagin, the main ellagitannin from pomegranate fruits, targeted and inactivated HSV-1 viral particles and could prevent binding, penetration, and cell to cell spread, as well as secondary infections [13]. A pomegranate polyphenol extract with punicalagin as a major compound inhibited the influenza virus and had a synergistic effect with oseltamivir [14]. In the search for new biological properties associated with fruits cultivated in Brazil, we studied *Punica granatum* for anti-Mayaro virus activity.

Materials And Methods

Extract of *Punica granatum*

Fruits from a local supermarket (1.2 kg) were washed, halved, washed again to extract all the juice and seeds, which were discarded. The shells were ground and extracted with EtOH at room temperature for 15 days. Part (10 g) of the dried ethanol extract (42.7 g) was submitted to chromatography on cellulose acetate with water/MeOH gradient (100% H₂O, 9:1 → 1:9, 100% MeOH) as mobile phase to produce 34 fractions, which were analyzed by thin layer chromatography (TLC) on cellulose (H₂O:AcOH/9:1). Cellulose acetate, XAD-16 and Sephadex LH-20 were used as stationary phases for column chromatography. Those (23–25) with a large purple spot on the TLC plate sprayed with a sodium nitrite/acetic acid 10% solution were pooled and analyzed by HPLC/DAD, ESI/MS, and NMR.

Structure analysis

Optical rotations were measured on a digital polarimeter. Circular dichroism spectra of biflavonoids were obtained with a Chirascan TM CD spectrometer (Applied Photophysics, UK). Low-resolution ESI-MS were obtained on a mass spectrometer and high-resolution ESI-MS on a mass spectrometer. An irregular C18 reversed-phase silica gel (RP-18) was used for analytical HPLC. ¹H-NMR, APT, HSQC and HMBC NMR spectra in MeOD or DMSO as solvents and TMS as internal standard were recorded on Bruker DRX 400 and 500 MHz spectrometers.

Cells and viruses

Vero cells from African green monkey kidney were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and grown in DMEM medium supplemented with 2.5% fetal bovine serum, 0.23% NaHCO₃ and antibiotics anfotericin B (25 µg/mL), 5 mL solution of penicillin (100 U/mL) and streptomycin (100 µg/mL). MAYV was obtained from the ATCC (VR-66, lineage TR 4675).

Cytotoxic Assay

The cytotoxicity of compounds was determined using a technique called "dye-uptake" [15], which consists of the incorporation of neutral red dye by living cells, followed by fixation with 20% formaldehyde. Subsequently, the dye was extracted with an extraction solution consisting of 50% methanol and 10% acetic acid in Phosphate Buffer Saline (PBS) and finally quantified with the plate reader (BIO-RAD model 3550) with a wavelength of 490 nm. Assays were performed on confluent monolayers of 96-well microplate-grown Vero cells (TTP), which were treated with triplicate serial dilutions of the substances and kept in a greenhouse under appropriate conditions for 24 hours. After this period, 100 μ L of DMEM culture medium solution and neutral red at 50 μ g/L were added to the cells. The plates were incubated for 3 hours in the 37°C 5% CO₂. Next, cells were fixed for 20 minutes with 100 μ L of 20% formaldehyde solution in PBS. The fixative solution was then removed, and the dye extracted for 20 minutes with 100 μ L of the extraction solution (50% methanol and 1% acetic acid). Then the plates were read in a spectrophotometer.

Viral inhibition by extracts obtained from *P. granatum*

Confluent Vero cells in 24-well microplates were infected at multiplicity of infection (MOI) of 0.01 for 1 h. After adsorption, the culture medium was added with or without the substances at the indicated concentrations. After 24 h, supernatants of cell cultures were collected for determination of viral titer. Vero cells were cultured in 96-well microplates (TPP, USA). Supernatants collected from the infectivity inhibition experiments were added in serial dilutions to the cells. Tissue Culture Infective Dose (TCID₅₀) was calculated according to the Reed-Muench method[16]. The TCID₅₀ experiments were performed in 96 well plates in quadruplicate and three independent experiments.

Virucidal activity

For the virucidal activity, which is the effect of the substances directly on the virus particles, the substances were incubated 1:1 with 100 plaque-forming unit (PFU) of purified MAYV. The virus:substance incubation period was either 1 hour at 37°C before the mixture was added to Vero cell monolayers, or the substance and virus were mixed right before adding to Vero cell monolayers. Confirmation of the virucidal effect was evidenced by the plaque assay [17, 18]. We used confluent monolayers of Vero cells infected with MAYV. After 1 hour adsorption, virus inoculum was removed, monolayers rinsed with PBS and incubated with growth medium with or without the different substances to be tested. Twenty-four hours post infection the cell culture supernatants were recovered and used for titration of extracellular infective virus. The latter was performed by plaque assay in Vero cells that had just reached confluence. The monolayers were overlaid with growth medium, supplemented with 10% FCS and 6% carboxymethylcellulose (CMC)(Sigma Chemical Co), and were further incubated in an atmosphere of 37°C, 5% CO₂ for 3 days. The monolayers were then stained with crystal violet (1%) and the virus plaques were counted [19]

Immunofluorescence

For immunofluorescence analysis, infected Vero cells in 24-well plates were washed with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. Cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min and then blocked for 15 min at 25°C with a blocking solution that contained 3× rinsing with 3% BSA in PBS with fish gelatin (PBSA). Fixed cells were incubated for 2 h at 25°C with the primary antibody (anti-HA) diluted in blocking solution. The secondary antibody (Cy3-conjugated anti-mouse IgG) was diluted in blocking solution and incubated with the cells for 1 h at 25°C, followed by dilution with 546 Phalloidin blocking solution (Invitrogen) for 40 min at 25°C. The cells were washed five times with PBSA after each antibody treatment. Finally, cells were treated with the DAPI Prolonggold (Invitrogen). Fluorescence was analyzed microscopically with an Automated Fluorescence Microscope (Olympus, Tokyo, Japan) and the cells were photographed with a digital camera DP70 (Olympus, Tokyo, Japan).

Transmission electron microscopy (TEM)

MAYV was purified by ultra-centrifugation in a tartrate two-step gradient (15% and 35%) at 24,000 rpm (sw-28 rotor) in a Beckman ultracentrifuge. The resulting virus band was collected and incubated with the test substances, THA, AAF, and the ethyl acetate extract for 1 h at 37°C. Untreated purified MAYV was used as control. The viruses were then placed on 400-mesh carbon grids for 1 min. Then the grids were washed three times and contrasted with 1% uranyl acetate. Finally, the material was visualized under a transmission electron microscope[15].

Statistical Analysis

The selectivity index (SI) was determined by the ratio of CC_{50} to IC_{50} . All experiments were performed in triplicate, and three independent experiments were conducted. Data were presented as mean $\pm$ SD and t-test was used to evaluate the difference between the test and control. One-way ANOVA and Dunnett's multiple comparisons test were performed with Prism 7 (GraphPad Software). All significant values had p-values less than 0.05.

Results

Structure Identification of *P. granatum* isolate

The chromatographic fractionation of the EtOH extract fruits led to isolation of 34 fractions, which were analyzed by TLC. Those (23–25) with a large purple spot on the TLC plate sprayed with a sodium nitrite/acetic acid 10% solution were pooled and analyzed by HPLC/DAD, ESI/MS, and NMR.

Two major peaks at 20.9 min (237, 276 and 390 nm) and 22.1 min (232, 276 and 393 nm) were observed on HPLC/DAD chromatogram. ESI-MS spectrum of this sample furnished a pseudomolecular ion $[M-H]^+$ –1083. The NMR data are in accordance with the two anomers (α and β) of punicalagin, a typical ellagitannin of *P. granatum* pericarp. Furthermore, α -Punicalagin anomeric hydrogen was detected on 1H -NMR as a doublet at 5.12 ppm ($J = 3.74$ Hz) and a doublet at 4.51 ppm ($J = 7.87$ Hz) for β punicalagin. HSQC spectrum showed for the anomeric carbons 92.28 and 96.30 respectively. Ellagic acid $[M-H]^+$ –301,

gallagic acid [M-H]⁺– 601 and punicalin [M-H]⁺– 781 could be identified in the isolate as minor compounds. Interestingly, cellulose acetate was used for the first time as stationary phase for column chromatography of ellagitannins and the sample with α and β punicalagins as major compounds was obtained with a unique chromatographic run.

Cytotoxicity

The cytotoxicity of the compounds was determined by "dye-uptake" [14, 20–29], which consists of the incorporation of neutral red dye by living cells and subsequent extraction and quantification by a spectrophotometer at a wavelength of 490 nm (Fig. 1A), to which it is possible to determine a viable concentration to be used in the antiviral test, and the data were used to determine the CC₅₀ (Table 1). The antiviral activity of the compounds was evaluated as the ability of the substances to inhibit Mayaro virus replication in Vero cells at a nontoxic concentration (Fig. 1B).

Table 1
Cytotoxicity and anti-MAYV activity of Punicalagin and Ethanol extract of Pomegranate

Substance	CC 50 ^a	IC 50 ^b	IS 50 ^c	RP ^d
Pomegranate ethanol extract	590.8 ± 17.7	12.3 ± 0.4	48	6
Punicalagin	425 ± 12.8	29.9 ± 0.9	14	1.75
Ribavirin	523.1 ± 10.5	62.5 ± 4.4	8	-
^a 50% cytotoxic concentration. ^b 50% inhibitory concentration of viral replication. ^c Selectivity Index = CC50/IC50. ^d Relative Potency = IS ₅₀ (substance)/IS ₅₀ (Ribavirine).				

Antiviral Activity

Vero cells infected with MAYV were treated with different concentrations of the compounds. All the compounds exhibited strong antiviral activity (IC₅₀) when compared to ribavirin, a known antiviral used in the treatment of Hepatitis C, Respiratory Syncytial Virus and other viral infections. The antiviral activity was dose-dependent and reached values above 95% inhibition at the highest nontoxic concentrations tested (CC₅₀). The selectivity index (SI) was determined by the ratio of CC₅₀ to IC₅₀. All experiments were performed in experimental triplicate, and three independent experiments were conducted. Data were presented as mean ± SD and t-test was used to evaluate the difference between the test and control. A P-value of < 0.05 was considered statistically significant (Table 1).

Virucidal activity

For the virucidal activity, we used confluent monolayers of Vero cells infected with MAYV that had been exposed to the substances according to methods. The Pomegranate ethanol extract showed 98% virucidal activity on MAYV particles whereas Punicalagin did not show detectable virucidal activity (Fig. 2).

We also confirmed virucidal activity by immunofluorescence microscopy (IFM) and transmission electron microscopy (TEM). Pomegranate ethanol extract at a concentration of 200 ug/mL was mixed with a MAYV suspension (moi = 0.2), or cell media as control and incubated for 1 hour at 37°C. We observed a reduction in cell fluorescence when we infected the cells with virus-treated particles when compared to the viral control (Fig. 3A). A similar experiment was performed for TEM. Purified MAYV was mixed Pomegranate ethanol extract (200 ug/mL), or cell media (control) and then incubated for 1 hour at 37°C. In Fig. 3B we can see damaged virus particles, apparently fused together with other virus particles as indicated by arrows (Fig. 3B).

Discussion

The virus is a unique pathogen that is incapable to replicate without the host cell. Cell metabolism and cellular machinery are important for viral replication. Therefore, a bioactive substance that attacks virus replication often times will have adverse effects on the host cell also. In vitro antiviral studies (screening) are important for the evaluation of the safety, efficacy, and identification of the mechanism of action before they can be further tested in animals and humans [20, 29]. When there is a cell culture system in which the virus undergoes a complete virus replication cycle, it becomes possible to investigate the antiviral activity and generate clear data, such as the magnitude of inhibition, steps of the virus replication cycle which is (are) affected by the prospective drug and evaluation of cytotoxicity to vertebrate cells among other approaches [20]. In the case of natural compounds, there is an increasing number of potentially useful plants and herbs that need to be exploited and their therapeutic applications are important weapons to be used against most virus families, and even for emerging viruses. The in vitro studies of antiviral effect, therefore, provide the relevant information necessary and the concentrations of the substances to be used for further tests, before it can that go to clinical trials, making it possible to access the risk-benefit issue [3, 17, 18, 26, 30–35].

Our results indicate that all tested substances have potential antiviral activity in concentrations that are non-toxic for Vero cells. According to our knowledge, this is the first report of antiviral activity for the substances pomegranate ethanol extract and Punicalagin against an alphavirus [13, 22, 36]. For the *Punica granatum* L. (Punicaceae), the Pomegranate ethanol extract showed a Selective index of 49, and the fraction containing Punicalagin as the main component had a Selective Index of 15 as shown in Table 1, surprisingly, this substance was previously described with antiviral activity against other viruses, such as HSV-1 and HSV-2, Influenza A and B [12, 14, 23, 28, 37–39]

It has been reported antiviral activity for several partitions from this plant, for Herpes simplex virus, Influenza, Respiratory Syncycial Virus and HIV [12, 14, 23, 28, 37–39]. The fact that this plant can inhibit the replication of viruses from different families, we understand that components from this plant may have several mechanisms of action against virus replication, including virucidal effect for some viruses. Our results show that these substances pomegranate ethanol extract and Punicalagin have antiviral and virucidal activity against an alphavirus and is a potent candidate for further studies.

Conclusions

In summary, the *Punica granatum* L. (Punicaceae), the Pomegranate ethanol extract showed a high Selective index of 49, and significant virucidal activity of approximately 98%, while the fraction containing Punicalagin as the main component had a Selective Index of 15, with strong antiviral activity. The fact that this plant has great potential as an antiviral, can inhibit the replication of viruses from different families with different replication strategies is rather important and suggests multiple targets of action. Our results show that these substances have good antiviral activity against the alphavirus MAYV and are excellent candidates for future studies with other enveloped RNA viruses.

Abbreviations

American Type Culture Collection (ATCC); 50% cytotoxic concentration (CC₅₀); Mayaro virus (MAYV); carboxymethylcellulose (CMC); Herpes Simplex Virus 1 (HSV-1); Herpes Simplex Virus 2 (HSV-2); Human Immunodeficiency Virus (HIV); 50% inhibitory concentration (IC₅₀); Immunofluorescence Microscopy (IFM); Multiplicity of Infection (MOI); Phosphate Buffer Saline (PBS); Plaque-Forming Unit (PFU); Relative Potency (RP); Selectivity Index (SI); Thin Layer Chromatography (TLC); 50% Tissue Culture Infective Dose (TCID₅₀); Transmission Electron Microscopy (TEM)

Declarations

Ethics approval and consent to participate

Not applicable.

Availability of data and material

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

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Authors' contributions

The authors T.S.S., M.D.F.M., R.M.K. and D.F.F initiated and designed the study. D.M.O., J.A.V. and R.M.K. conducted experiments on the production, preparation and purification of extracts from Pomegranate. T.S.S. and M.D.F.M. conducted experiments of antiviral and virucidal. T.S.S., T.E.S.G. and L.A.C. conducted experiments of virus “growing” and pre-purification. T.S.S., M.D.F.M. and L.A.C. conducted the virus purification. L.A.C. and D.F.F. performed sample preparation for TEM. T.S.S., R.C.A., M.R.S. and D.F.F. conducted the image acquisition and analysis by TEM and wrote the manuscript. T.S.S., R.M.K., R.C.A., M.R.S. and D.F.F. critically revised the manuscript. All authors reviewed the manuscript.

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Figures

Figure 1

A) Cytotoxic effect: The cytotoxicity of the substances was determined by the “dye-uptake” technique, which consists of the incorporation of neutral red dye by living cells, where the substances were applied in an initial concentration of 2 mg / mL and then, serial dilutions were made up to the last well of the column in the plate 96-wells. The values obtained in the reading were transformed into percentage of viable cells in each concentration of the cells treated with the substances compared to the control cells

(untreated). The lead gray curve represents Punicalagin, black represents Ethanol extract of Pomegranate and gray represents Ribavirin. Y axis, percentage of viable cells (Vero); X axis, different concentrations of the substances. B) Antiviral activity: Confluent Vero cells were infected with a multiplicity of infection (MOI) of 0.1 for 1 hour. After adsorption, culture medium was added without the substances in the Viral Control and with the substances at the concentrations indicated in the experiment. After 24 hours, the supernatants were collected and titrated by the TCID₅₀ titration method. The lead gray curve represents Punicalagin, black represents Ethanol extract of Pomegranate and gray represents Ribavirin. Y axis, virus titer; X axis, the different concentrations of the substances. C) Virucidal Activity - In order to evaluate if the tested substances have a “virucide” effect, that is, they interact directly with the viral particle, the substances (at the concentration of twice the value that obtained the best activity) were incubated with the virus. at a final concentration of approximately 100 PFU/mL for 1 hour at 37 °C. Soon after this treatment this inoculum was titrated by plaque assay. Vero cell monolayers were treated with the substances at the concentration of 200 µg/mL: X-axis, the substances, Ethanol extract of Pomegranate, Punicalagin and Viral Control (CV). Y-axis, percentage of virus titer.

Figure 2

A) Immunofluorescence Image - Cells were treated with Pomegranate Crude Extract, along with Mayaro virus according to the virucidal experiment. As we had already noted the Ethanol extract of Pomegranate had a significant positive effect on virus replication: A1) Cell Control, A2) Virus Control, A3) Ethanol extract of Pomegranate. Nucleus? staining in blue with DAPI and green with Sybr Green anti-Mayaro IgG antibody. B) Transmission Electron Microscopy - Ethanol extract of Pomegranate was used at a concentration of 200 µg/mL with a suspension containing purified MAYV and then incubated for 1 hour at 37 °C. The sample was placed on a 300-mesh carbon film grid, then counterstained with uranyl acetate. Subsequently it was analyzed in the electron microscope: B1) Purified Virus, B2) Virus Control, B3) Raw Pomegranate Extract. “Note: Image measurement bar is 100 nm in length.”

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