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ORIGINAL PAPER

Antiviral activities of extracts and phenolic components of two *Spondias* species against dengue virus

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Abstract: In recent years, the search for natural plant products to fight viral diseases has been increasing. In this work, two *Spondias* species, namely *S. mombin* and *S. tuberosa*, found in Ceará state (Brazil), and their main phenolic components were evaluated against dengue virus. *In vitro* antiviral tests were performed against type-2 dengue virus by the MTT method and standard cytopathic effect reduction assay in $C_{6/36}$ cells. Cytotoxicity was also evaluated by MTT. The presence of phenolic compounds quercetin, rutin, and ellagic acid in plant extracts was characterized by HPLC analysis. Both *Spondias* species extracts and components were nontoxic to the cells whereas rutin and quercetin displayed relevant antiviral activity with IC_{50} of 362.68 µg/mL and 500 µg/mL, respectively.

Key words: natural products, phenols, dengue, antiviral agents.

INTRODUCTION

Dengue infection is caused by four serotypes of the dengue virus (DENV 1, 2, 3 and 4), a member of the Flaviviridae family; it is generally transmitted in a cycle involving humans and mosquito vectors. The global prevalence of dengue virus has grown dramatically in recent decades. Until now, there is no specific drug to combat infection and currently the most effective prevention measures lie in controlling the mosquito (1).

Efforts have been made to evaluate the antiviral activity of a wide range of natural products, including plants, in an attempt to isolate and characterize new compounds which can inhibit virus replication or treat viral infection (2). For this reason, plant extracts and components are becoming more important as potential sources for antiviral agents. Plant extract screening has led to the detection of some effective *in vitro* viral replication inhibitors (3, 4).

Hydroalcoholic extract of *S. mombin* inhibits replication of the herpes simplex and coxsakie B viruses, responsible for painful mouth ulcers. The active compounds against these viruses were identified as geraniin and galoilgeraniin at concentrations of 50 mg/L and two caffeoyl esters at concentrations of 100 mg/L (5, 6). No antiviral activity was reported for *S. tuberosa*.

In order to find new antiviral agents to combat dengue, *Spondias mombin* and *S. tuberosa* extracts and their main components were evaluated *in vitro* against DENV-2 in $C_{6/36}$ cells.

MATERIALS AND METHODS

Cell Line

$C_{6/36}$ (cloned cell line derived from larvae of *Aedes albopictus*) was provided by Dr. Fernanda

Montenegro (Central Public Health Laboratory – LACEN, Fortaleza, Brazil) and grown at 28°C as monolayers in Leibovitz medium (L-15®, Cultilab, Brazil), supplemented with 10% tryptose phosphate broth, 1% penicillin/streptomycin (Gigco-BRL, USA) (50 U/mL), 1% amphotericin B (250 µg/mL) (Gibco-BRL, USA), and 10% fetal bovine serum (FBS, Sigma-Aldrich, USA).

Virus

Type-2 dengue virus (DENV-2), New Guinea strain, was provided by Dr. Benedito Antonio Lopes da Fonseca (Molecular Virology Laboratory, Ribeirão Preto Faculty of Medicine, São Paulo State, Brazil). The virus was replicated in $C_{6/36}$ cells to generate working stocks for seven days at 28°C. Supernatant culture was collected and centrifuged at 2,000 rpm for 15 minutes. Supernatant was again collected and stored at –80°C as virus stock until use. Virus titer, expressed as plaque-formation units per milliliter (PFU/mL), was determined by standard plaque assay on Vero cells grown in 24-well plates.

Plant Material and Phenolic Components

Plant material was collected from their natural habitats in the state of Ceará (Northeast of Brazil). Voucher specimens (*S. mombin*-34.826 and *S. tuberosa*-34.887) were deposited in the Herbarium at the Department of Biology, Federal University of Ceará (UFC), Brazil. Quercetin, rutin, and ellagic acid were purchased from Sigma-Aldrich Co. (USA).

Preparation of Extracts

Leaves of *S. mombin* (3.59 kg) and *S. tuberosa* (3.23 kg) were soaked in methanol:water (80:20) at room temperature (25-28°C) for seven days. The solution was filtered through filter paper and evaporated in a rotary evaporator. The extracts were dried over a water bath with the temperature held below 60°C.

Phytochemical Analysis of Plant Extracts

The methanol:water (80:20 v/v) extract was submitted to silica gel column chromatography with elute mixtures of increasing polarity using

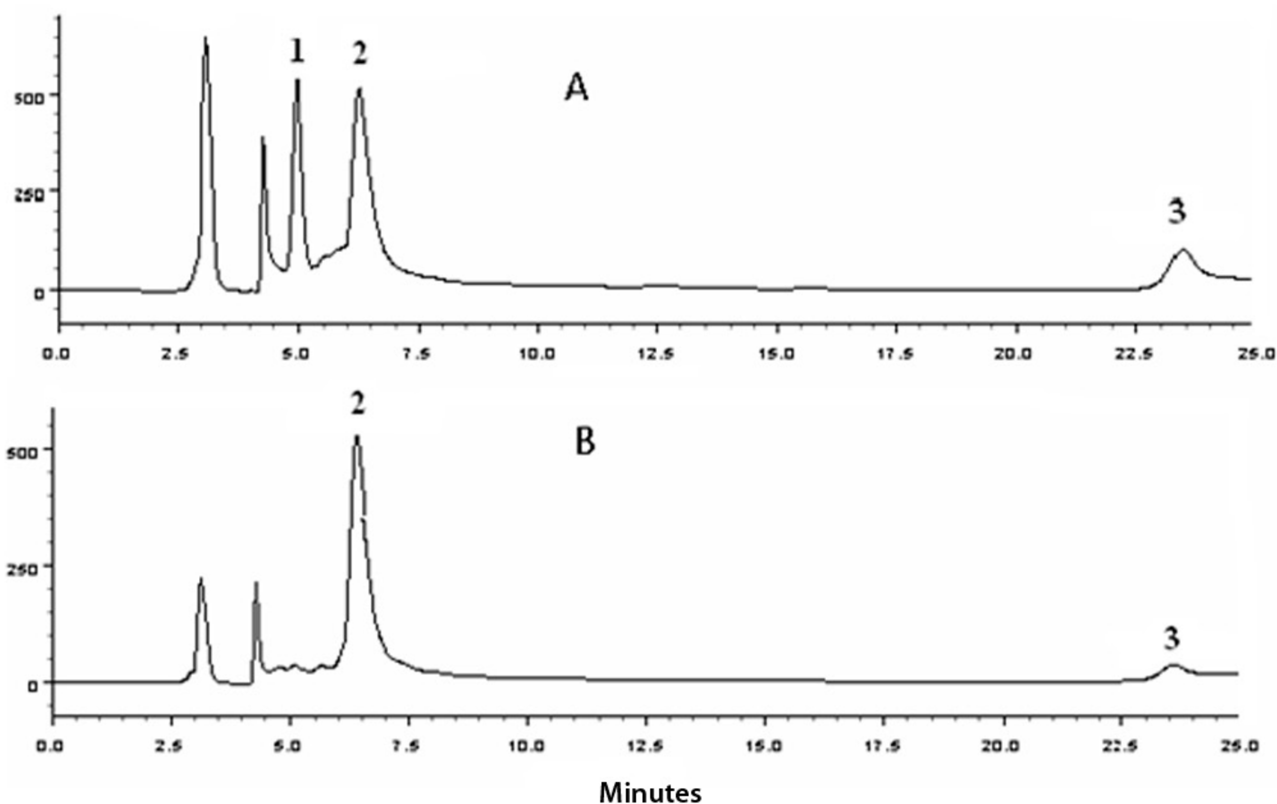

Figure 1. HPLC chromatograms of methanol extracts of (**A**) *S. tuberosa*, Hibar Lichrospher column 100RP18 4.6 mm x 25 cm, 5 mm; solvent mixture I:II [I: aqueous solution of H_3PO_4 (pH 2.8)]; (II: acetonitrile); wavelength 350 nm and (**B**) *S. mombin*. Peaks: 1 – rutin, 2 – ellagic acid, and 3 – quercetin.

hexane, chloroform, and ethyl acetate. Several 10-mL fractions were collected and after solvent evaporation, the resultant material was compared by silica gel thin layer chromatography (TLC) plates. Similar samples were joined, purified by crystallization and analyzed by infrared (IR) and nuclear magnetic resonance (NMR). The main compounds were also compared with standard compounds by TLC. IR spectra were recorded on a PerkinElmer FTIR Spectrum 100® spectrophotometer (Australia) and values expressed in cm^{-1}. NMR spectra were recorded on a Brucker Avance DRX-500® spectrometer (Germany), in MeOD.

High-Pressure Liquid Chromatography (HPLC) Analysis

HPLC analyses of extracts from *S. mombin* and *S. tuberosa* were performed using a reversed-phase column (Hibar LiChrospher®, Germany, 100RP18 4.6 mm x 25 cm – particle 5 µm) eluted at 1.25 mL/minute with a solvent mixture I:II [I: aqueous solution of H_3PO_4 (pH 2.8); II: acetonitrile] starting with 20% II/80% I until 12 minutes, then increasing II to reach 40% at 17 minutes, then with 40% II/60% I until 23 minutes, and again increasing II concentration to reach 80% at 25 minutes, with the detection wavelength set at 350 nm and 20 mL injection. A rutin standard was used to obtain calibration curves. This compound was dissolved in methanol at different concentrations (1.14 mg/mL, 0.57 mg/mL, 0.228 mg/mL, 0.114 mg/mL, 0.0456 mg/mL). Ellagic acid (standard) is also used to obtain calibration curves and it was dissolved in methanol at different concentrations (0.46 mg/mL, 0.92 mg/mL, 1.84 mg/mL).

Follow-up extractions and HPLC analysis were accomplished using the same procedure as for rutin. Recovery was determined as follows: recovery (%) = (A – B)/C x 100%, where "A" is the amount of detections, "B" is the amount of sample without added standard, and "C" is the amount of added standard. The relative standard deviations (RSD) of recoveries for the ellagic acid were 2.1 (n = 5; mean = 98). Identification of rutin, ellagic acid, and quercetin (Figure 1) in *Spondias* extracts was performed by HPLC-PDA, observing retention time (Rt) and UV-VIS spectra. The standards were prepared in methanol at 0.1 mg/mL.

Phytochemical Analysis of Plant Extracts

Chemical tests were performed following the protocols described by Matos (7), based on reactions with specific reagents for the main classes of natural products with precipitate formation or color change.

Viral Detection by RT-PCR

$C_{6/36}$ cells (2×10^5 cells/well) were infected with DENV-2 (3.5×10^5 PFU) and infectivity was confirmed by RT-PCR. The purpose of this assay was to standardize days of $C_{6/36}$ cell infection by DENV-2. Briefly, infected cells were removed each day of infection (1st to 8th days of infection) washed with phosphate buffered saline (PBS) and RNA was purified with Trizol® (Invitrogen, USA). The one-step kit (Qiagen, Germany) was used for cDNA synthesis and PCR following manufacturer's instructions. Primer oligonucleotide sequences were as follows: forward 5'-ACCATCGTGATAACA-3' reverse 3'-AGGCTGTGTCACCTA-5'. Amplification conditions were 50°C for 30 minutes and 95°C for 15 minutes, followed by 40 cycles of 94°C for one minute, 55°C for one minute, and 72°C for one minute, and a final extension at 70°C for ten minutes. The amplified product was then analyzed on 2% agarose gel.

Cytotoxicity Assay

Cell viability was evaluated by the MTT method (Sigma-Aldrich, USA) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (8, 9). Briefly, $C_{6/36}$ cultures were prepared in 96-well plates (TPP, Switzerland), at 2×10^5 cells/well. After 24-hour incubation at 28°C, the culture medium was removed and cells exposed to different concentrations of the extracts, four wells per concentration prepared in 200-µL culture medium per well. Untreated controls had 200 µL of medium culture added. Cells were then incubated for seven days. The medium was removed and 50 µL of MTT solution (5 mg/mL) was added. The plates were reincubated for four hours and the MTT solution removed, 100 µL of DMSO was added to dissolve formazan crystals and the plates were gently shaken, whereby crystals were completely dissolved. The absorbance was read on ELISA equipment (Amersham Biosciences, USA) at 495 nm. The 50% cytotoxic concentration (CC_{50}) was defined as the sample concentration that reduced cell viability 50% compared to untreated controls.

Antiviral Assay

Antiviral activity was also evaluated by the MTT method. In brief, to determine inhibitory potential of the sample for DENV-2 replication, 100 μL of virus suspension (3.5 x 10^5 PFU) was added to a cell shaker for one hour at 28°C. Thereafter, the viral suspension was removed and various concentrations of samples were added and incubated for seven days. This was followed by the same procedure as described in the previous paragraph. The 50% inhibitory concentration (IC_{50}) was defined as the concentration that inhibited 50% of viral replication compared to virus controls. The selectivity index (SI), an important parameter for evaluating antiviral activity, was calculated from the CC_{50}/IC_{50} ratio. Antiviral activity was determined a according to the following formula (10):

$$\text{Antiviral activity} = \frac{(OD_t)_{DENV} - (OD_c)_{DENV} \times 100}{(OD_c)_{cells} - (OD_c)_{DENV}}$$

Where $(OD_t)_{DENV}$ is the measured absorption of the various concentrations of *Spondias* species extracts and their phenolic components in DENV-infected cells, $(OD_c)_{DENV}$ is the absorption of the untreated control DENV-infected cells, and $(OD_c)_{cells}$ is the absorption of untreated control $C_{6/36}$ cells. The compounds showing antiviral effect were submitted to standard cytopathic reduction assay.

Statistical Analysis

CC_{50} and IC_{50} values were obtained from linear regression analysis of concentration-effect curves and represent mean ± standard deviation values of three independent experiments. One way analysis of variance (ANOVA) was used to determine statistical differences followed by Tukey's multiple comparison. Statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

Chemical Composition of *S. mombin* and *S. tuberosa*

Leaves from the two *Spondias* species were soaked in methanol:water (80:20) for seven days at room temperature. The solutions obtained were then evaporated leaving the crude extracts. Extract yields of *S. tuberosa* and *S. mombin* were respectively 1.5 and 5.0%. Qualitative phytochemical analysis of *S. mombin* and *S. tuberosa* revealed the presence of phenols, hydrolysable tannins, flavones, flavonoids, leucoanthocyanidins, and saponins. Results showed both species presenting the same classes of compounds; this agrees with Corthout *et al.* (5) who reported the presence of ellagitannins in *S. mombin.*

Three main compounds were detected in *Spondias* species; structural characterization was performed by ^1H and ^{13}C-NMR spectroscopic analysis and comparison with ellagic acid, quercetin, and rutin data (11, 12). The relative percentage yields of these phenolic compounds in dried leaves for *Spondias* species were evaluated by HPLC-PDA, considering retention time (Rt) and UV-VIS spectral analysis. Identification of components was established by overlapping their peaks (retention time) and absorption spectra with those of rutin, ellagic acid, and quercetin standards. Chromatograms of *S. mombin* and *S. tuberosa* are shown in Figure 1. Rt for rutin, ellagic acid, and quercetin were respectively 5.11, 6.24, and 23.46 minutes.

Table 1. Quantities of flavonoids (quercetin and rutin) and ellagic acid isolated by HPLC in *Spondias* species extracts from northeastern Brazil

Compounds	*S. mombin* (mg/g extract)	*S. tuberosa* (mg/g extract)
Rutin	–	53.38 ± 1.71^a
Ellagic acid	41.56 ± 0.01^a	169.76 ± 0.17^b
Quercetin	2.36 ± 0.01^b	24.46 ± 0.87^c

Different letters indicate statistically significant differences between rows in the same column, $p < 0.05$.

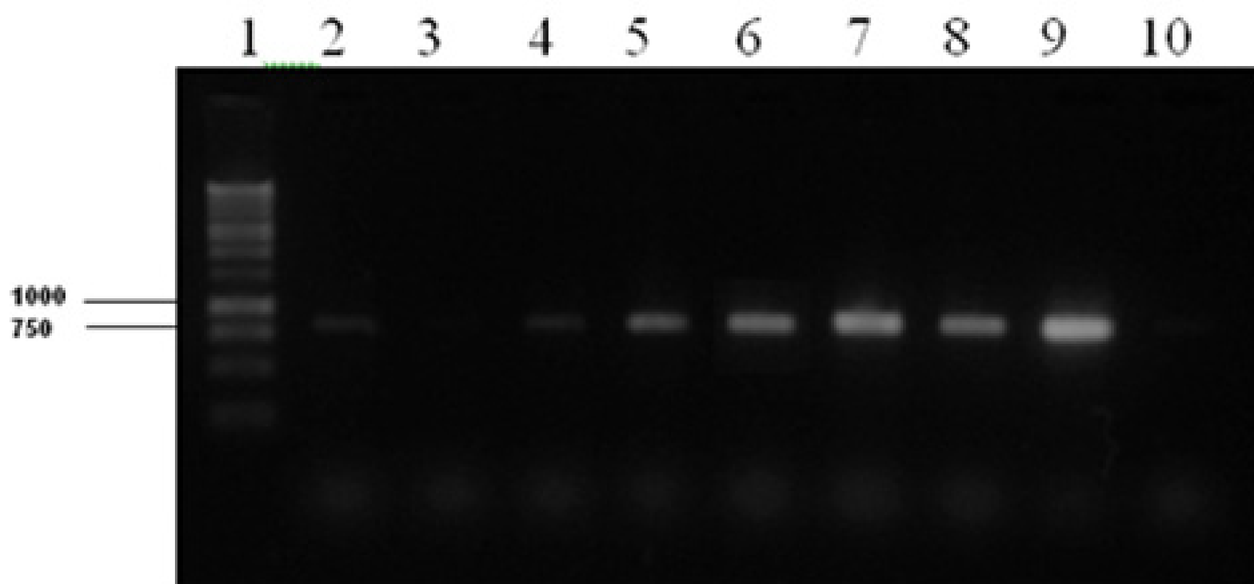

Figure 2. Electrophoresis on 2% agarose of RT-PCR over eight days of infection with DENV-2. Lane 1: molecular weight marker (1 kb, Promega, USA); lane 2: DENV-2 first day; lane 3: DENV-2 second day; lane 4: DENV-2 third day; lane 5: DENV-2 fourth day; lane 6: DENV-2 fifth day; lane 7: DENV-2 sixth day; lane 8: DENV-2 seventh day; lane 9: DENV-2 eighth day; lane 10: $C_{6/36}$ cells.

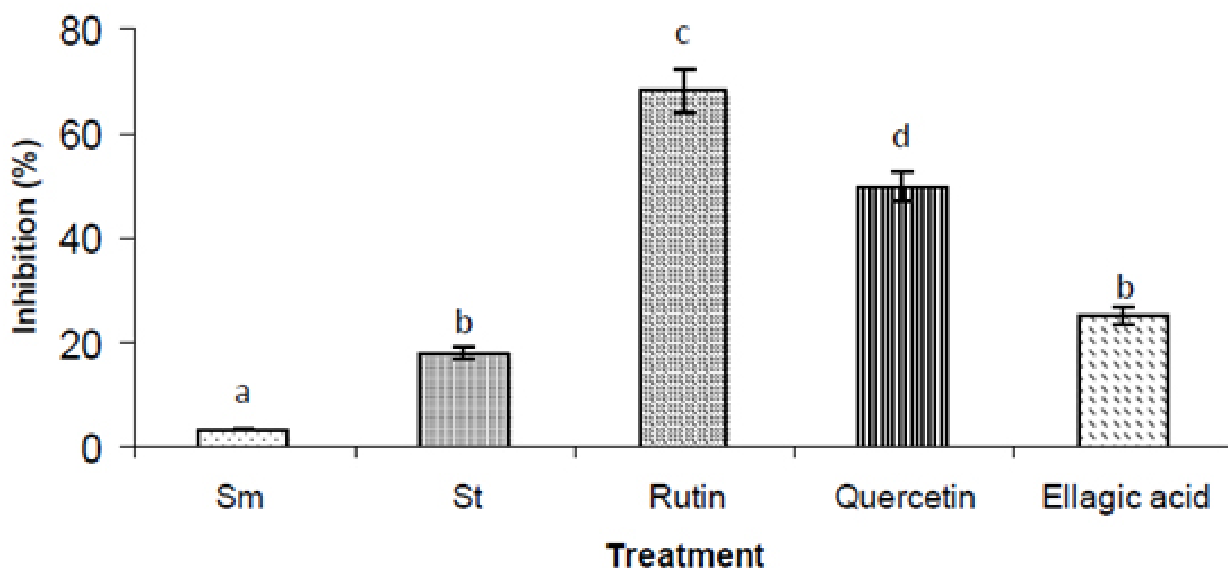

Figure 3. Inhibitory potential (%) of *Spondias* extracts and their flavonoids in DENV-2 (3.5×10^5 PFU) infected $C_{6/36}$. Sm: infected cells treated with 500 µg/mL of *Spondias mombin*; St: infected cells treated with 500 µg/mL of *Spondias tuberosa*; rutin: infected cells treated with 500 µg/mL of rutin; quercetin: infected cells treated with 500 µg/mL of quercetin; ellagic acid: infected cells treated with 500 µg/mL of ellagic acid; ANOVA and Tukey's multiple comparison. Different letters indicate statistically significant differences at $p < 0.05$.

The regression equation for rutin is Y = 25150017.88X + 203139.00 (R^2 = 1.000); quantification limit 0.1 µg/mL; detection limit 0.04 µg/mL; relative standard deviations (RSD) less than 2.0%. The regression equation for ellagic acid is: Y = 25916407.4534X + 309482.00 (R^2 = 0.9998); quantification limit 0.1 µg/mL; detection limit 0.04 µg/mL; RSD less than 2.0%. Each flavonoid peak was quantified using the rutin and ellagic acid linear regression equations. In these *Spondias* species extracts, rutin, ellagic acid, and quercetin were detected in different yields. Quercetin (2.36 ± 0.01 mg/g) and ellagic acid (41.56 ± 0.01 mg/g) were found in *S. mombin* and rutin (53.38 ± 1.71 mg/g), quercetin (24.46 ± 0.87 mg/g), and ellagic acid (169.76 ± 0.17 mg/g) in *S. tuberosa* (Table 1).

Cytotoxic Effect and Antiviral Activity

The cytotoxic effects of *S. mombin* and *S. tuberosa* extracts and related phenolic compounds were expressed by their CC_{50} values obtained by MTT method. No cytotoxicity was observed with any of these compounds at concentrations up to 1000 µg/mL, allowing us to estimate the CC_{50} for all compounds as greater than 1000 µg/mL. Extract cytotoxic levels were evaluated before carrying out the antiviral tests and they did not show toxicity towards $C_{6/36}$ cells.

As per the RT-PCR results, virus replication

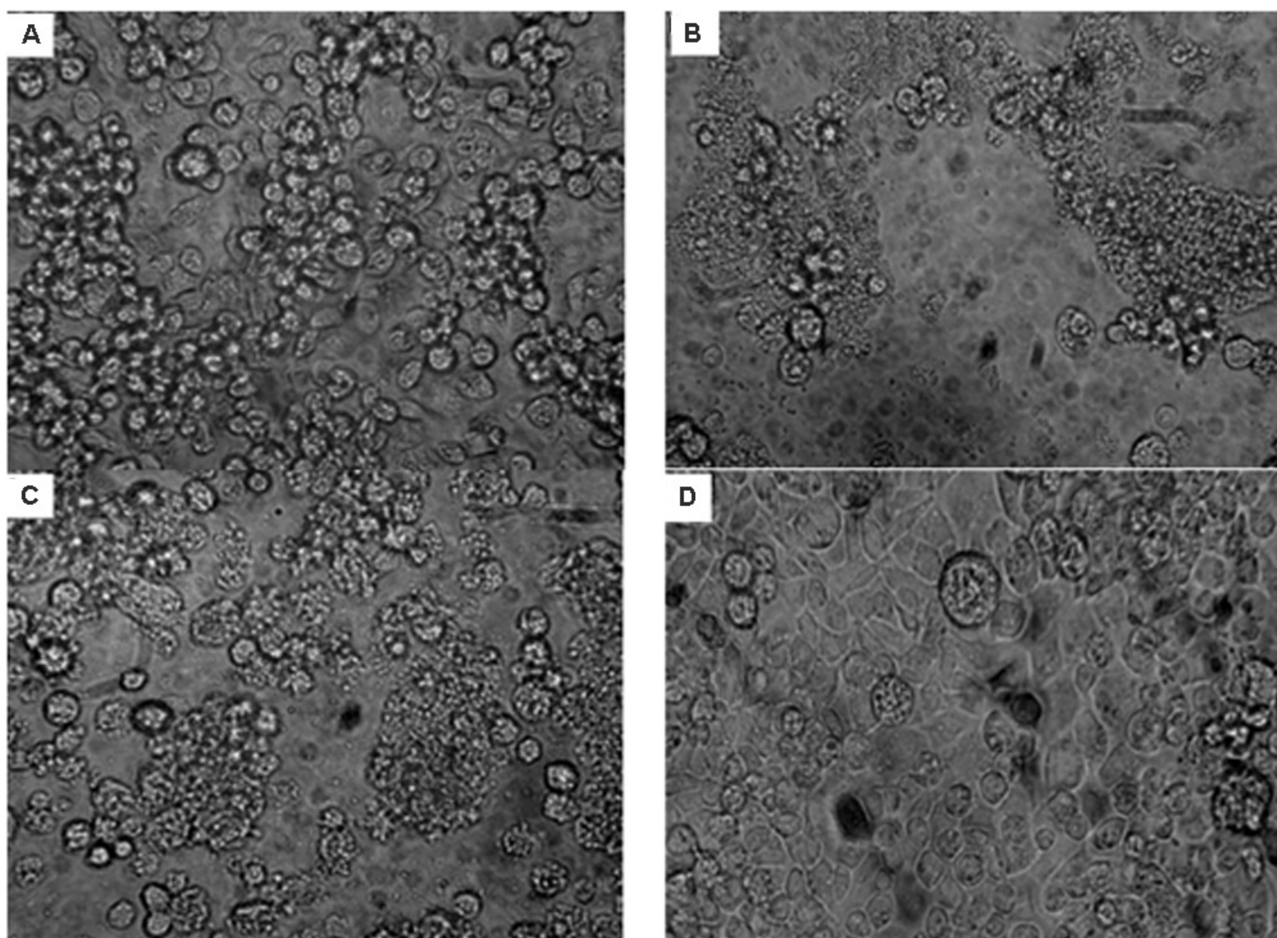

Figure 4. Inverted microscopic photographs of the cytopathic effects of dengue virus on $C_{6/36}$ *Aedes albopictus* infected cell cultures. (**A**) Uninfected control $C_{6/36}$ cell cultures, with normal cellular morphology. (**B**) Cell cultures infected with DENV-2 (3.5 x 10^5 PFU), showing advanced infection stage with large cell mass disrupted over seven days of infection. (**C**) Cells cultures infected with DENV-2 and treated with quercetin (500 µg/mL) with low cell damage over seven days of infection. (**D**) Cells cultures infected with DENV-2 treated with rutin (500 µg/mL) over seven days of infection, showing only an initial disorganization in groups of infected cells.

could be detected on the fourth day. However, to optimize experiments, antiviral assays in this study were performed on the fifth day of infection (Figure 2). *S. mombin* and *S. tuberosa* extracts and components rutin, quercetin, and ellagic acid on DENV-2 in $C_{6/36}$ cells inhibited virus replication respectively by 3.31, 17.98, 68.42, 50 and 25.02 (Figure 3) in a concentration of 500 µg/mL. Comparing IC_{50} values, rutin was more active against DENV-2 with an IC_{50} of 362.68 ± 0.04 µg/mL and SI of 2.75 than quercetin with an IC_{50} of 500.00 ± 0.01 µg/mL and SI of 2. Quercetin and rutin were also evaluated by standard cytopathic reduction assay inhibiting cell disruption (Figure 4).

There are few works studying natural products against the dengue virus; for example, Parida *et al.* (13) evaluated the antiviral action of neem (*Azadirachta indica*) extract and isolate azadirachtin against DENV-2 infected $C_{6/36}$ cells. The extract showed an inhibitory effect against dengue virus at high concentrations (1.897 mg/mL), when evaluated by cytopathic effect inhibition. Azadirachtin did not show antiviral action. Talarico *et al.* (14) investigated the action of two sulfated polysaccharides from red algae against four dengue virus serotypes using different cell lines. The authors found differential susceptibility of vertebrate and invertebrate cells to the inhibitory action of compounds against DENV. With respect to host cell type, the polysaccharides were active inhibitors of DENV-2 and DENV-3 multiplication in Vero cells and in HepG2 and foreskin PH cells, but were inactive in mosquito $C_{6/36}$ cells. There were no significant differences in DENV multiplication levels in these vertebrate and invertebrate cell lines, confirming that differential susceptibility of virus serotype or host cell is not due to virus growth ability. Sulfated galactomannans at 347 µg/mL reduced viral titer in immunofluorescence assays using DENV-1 in $C_{6/36}$ cells (15).

Antiviral activity is one of the cited biological activities displayed by flavonoids. Quercetin inhibited HIV-1 integrase which mediates the insertion of viral DNA into host cellular DNA and is essential for viral replication and virion production (16). Quercetin and its glycoside (quercetin 7-rhamnoside) were tested against porcine epidemic diarrhea virus. Quercetin 7-rhamnoside was more active than quercetin with an IC_{50} of 0.014 mg/mL (17). Tao *et al.* (18) showed *in vitro* the anti-HIV and anti-HSV activities of sodium rutin sulfate, obtained synthetically by modifying natural flavonol glycoside rutin. Kang et al. (19) demonstrated the role of ellagic acid as a therapeutic agent against the hepatitis B virus.

In summary, the results of this study revealed the presence of quercetin, rutin, and ellagic acid in two *Spondias* species. This is the first study to report quercetin and rutin in these species. The evaluation of antiviral activity against DENV-2 in $C_{6/36}$ cells suggests that rutin and quercetin have potential for the development of an anti-DENV agent. Further studies are required using other cell lines and *in vivo* assay to corroborate the effectiveness of these flavonoids against dengue virus.

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CONFLICTS OF INTEREST

There are no conflicts of interest.

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