Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Disease

journal homepage: www.elsevier.com/locate/apjtd

Document heading doi: 10.1016/S2222-1808(14)60635-6 © 2015 by the Asian Pacific Journal of Tropical Disease. All rights reserved.

Ethanol extracts of *Cassia grandis* and *Tabernaemontana cymosa* inhibit the *in vitro* replication of dengue virus serotype 2

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PEER REVIEW

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Comments

The study shows that the ethanol extracts obtained from *C. grandis* or *T. cymosa* inhibit the replication of dengue virus with a selectivity that is higher for DENV-2/NG strain than for DENV-2/16681 strain. The experiments also indicate that the extracts may affect both, the internalization process and steps subsequent to the internalization of the virus. The experiments were well conducted.
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ABSTRACT

Objective: To determine the antiviral activity of ethanol extracts derived from *Cassia grandis* leaves and *Tabernaemontana cymosa* bark against two dengue virus (DENV) serotype 2 strains DENV-2/NG and DENV-2/16681 in two cell lines susceptible to infection, VERO and U937.
Methods: The cytotoxic concentration 50 (CC_{50}) was assessed using the MTT method, and the effective concentration 50 (EC_{50}) was determined using the technique of inhibiting the production of infectious viral particles by the plating method. Further testing of dose-response inhibition was performed, and three experimental approaches were evaluated (pre-, trans- and post-treatment) to determine the effect of the extracts according to the time of administration. Finally, a preliminary phytochemical analysis for both extracts was performed.
Results: The cytotoxicity of the extracts was low (CC_{50}>300 μg/mL), and the U937 cell line was more sensitive to the antiproliferative effect of both extracts. When the virus strain-dependent selectivities of the extracts were compared, it was found that both extracts were more selective in cultures infected with the DENV-2/NG strain than in those infected with the DENV-2/16681 strain. A dose-dependent inhibitory effect of the extracts was not observed in any of the evaluations. Finally, the highest inhibition was detected with the post-treatment approach with the *Tabernaemontana cymosa* extract (99.9% in both cell lines).
Conclusions: A therapy with compounds derived from these extracts would inhibit viral replication and affect steps after viral internalization.

KEYWORDS
Dengue virus, *Cassia grandis*, *Tabernaemontana cymosa*, Antiviral activity

1. Introduction

Dengue virus (DENV) belongs to the Flaviviridae family, genus *Flavivirus*. It is transmitted by mosquitoes of the *Aedes* genus, and *Aedes aegypti* is its main vector[1]. DENV causes two clinical forms of the same disease, which are known as dengue (cases with and without warning signs) and severe dengue (which includes cases with plasma extravasation and fluid accumulation, respiratory failure, severe bleeding and severe compromise of organs). It is estimated that there are more than 2 500 million people at risk of infection worldwide. Each year, there are 50 to 100 million dengue infections and 500 000 severe dengue infections, causing a mortality rate of up to 2.5%[2].

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Foundation Project: Supported by COLCIENCIAS-Administrative Department of Science, Technology and Innovation, and National Program of Science and Health Technology, project code 1115-4932-6092 of 2009.

Article history:
Received 7 Apr 2014
Received in revised form 14 Apr, 2nd revised form 20 Apr, 3rd revised form 28 Apr 2014
Accepted 3 Jun 2014
Available online 23 Jul 2014

DENV has a genome of positive-sense RNA[3], with an approximate size of 11 kb. It encodes a polyprotein that produces three structural proteins, capsid, pre-matrix/matrix and envelope, and seven non-structural (NS) proteins corresponding to NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5, each of which has specific functions in viral replication[4]. Variations in genes coding for the different proteins in DENV allow for the classification of the virus into four serotypes (DENV-1 to DENV-4), and each serotype may also be divided into genotypes. In turn, each genotype is classified into different viral strains that share a common ancestor but differ in certain nucleotide substitutions. Although the simultaneous circulation of the four serotypes is common in endemic regions, serotype 2 is the most prevalent in the world, with some genotypes more virulent than others[5,6].

The susceptibility of the host to viral infections varies according to the serotype and genotype or according to the cell type in which DENV performs its replication cycle. This phenomenon is due to multiple factors, including the presence of different receptors in each cell line, the binding affinity between the virus and the receptor on the cell surface, and the different mechanisms that viral serotypes used for transport into or replication inside the host cell[7,8]. Additionally, differences in infection characteristics among viral strains have been reported, and it is hypothesized that these differences may determine, in part, the response to compounds with potential antiviral activity[9]. By other hand, recently has been reported that the infection of different types of cells with different viral strains differentially modifies the expression of certain proteins, which can, in turn, facilitate infection or affect the antiviral response[10].

Because there is no specific drug for the treatment of this disease, several years ago, the World Health Organization proposed that research groups focus part of their research on the search for and identification of a safe and effective antiviral, it natural or licensed for another use[11]. However, despite the work of many research groups worldwide[12], thus far, there is no available medicine to treat this disease. Most of these studies have focused on the inhibition of one of the steps of the viral replication cycle *in vitro*[13] and *vivo*[14]. Such steps include viral entry into the host cell, the fusion of protein E with the cell membrane, the maturation process of each viral protein, and RNA synthesis and viral protein synthesis[15].

Based on the fundamental fact that native communities have traditionally used medicinal plants for the treatment of the different diseases found in their villages, much of the medicines currently sold for the treatment of infectious diseases were discovered from the initial evaluation of extracts derived from natural products. This initial evaluation then allows the extracts to be moved to a bio-guided fractionation phase for the final identification of specific compounds. In this sense, the activity of a wide variety of plants has been assessed against DENV[16]. In the field of extract evaluation, for example, the antiviral activity of methanol extracts of the *Momordica charantia* and *Ocinum sanctum* plants against DENV-1 has been reported[17]. There are also reports of ethanol extracts of the plant *Distictella elongata*[18] and crude extracts from *Quercus lusitanica* acting against DENV-2 infections[18,19]. Colombia has approximately 130 000 different plant species, providing a huge floral diversity that allows for the exploration for therapeutic potential against tropical disease pathogens. However, there is only one report that shows an inhibitory effect of essential oils derived from the *Lippia alba* and *Lippia citriodora* plants native to Colombia against the replication of all four DENV serotypes[20].

Considering dengue research priorities, the great diversity of flora that exists in Colombia, and the differential selectivity a compound may have depending on the viral strain or cells evaluated, the aim of this work was to determine the antiviral activity of two ethanol extracts derived from *Cassia grandis* (*C. grandis*) and *Tabernaemontana cymosa* (*T. cymosa*) plants (collected in the Colombian Caribbean region) in two cell lines susceptible to infection (VERO and U937) with the two reference DENV serotype 2 strains DENV-2/NG and DENV-2/16681.

2. Materials and methods

2.1. Obtaining extracts from plant material

Plant selection was based on the results of an ethnobotanical survey conducted in the city of Cartagena (Colombia) and on previous reports of antiviral activity of these plants against other viruses causing febrile symptoms (unpublished results). The different parts of each plant were identified in the herbarium of the Botanical Garden Guillermo Piñeres (Cartagena, Colombia): *C. grandis* Voucher No. JBC 1452 and *T. cymosa* Voucher No. JBC 3243. The plant material was macerated with 90% ethanol overnight, and the resulting extract was filtered and concentrated in a rotary evaporator. The dry ethanol extract was resuspended in an ethanol 0.1% distilled water solution and stored at -70 °C until further use. For the phytochemical screening of each extract, identification tests of different secondary metabolite groups (alkaloids, tannins, polyphenols, triterpenes, sterols, coumarins, saponins, glycosides, flavonoids and quinones) that have been previously reported were performed[21].

2.2. Maintenance of viruses and cells

VERO epithelial cells (*Cercopithecus aethiops*) were obtained from ATCC. Cells of monocytic origin (U937) were donated by Dr. Jaime Castellanos of the Virology Group of El Bosque University (Bogotá, Colombia). C6/36HT cells (originated from *Aedes albopictus* mosquito larvae) were donated by Dr. Guadalupe Guzmán of the Department of Virology at the Pedro Kouri Institute (La Habana, Cuba). VERO and U937 cells were maintained in DMEM supplemented with 2% FBS and incubated at 37 °C under 5% CO_2. C6/36HT cells,

used for the production of viral stocks, were maintained in DMEM supplemented with 10% FBS at 34 °C. Viral reference strains belonging to serotype 2 were strain DENV-2/NG, originally isolated from a patient with dengue[22], and strain DENV-2/16681, isolated from a patient with hemorrhagic dengue[23]. Both strains were donated by Dr. Jorge Osorio of the Department of Pathobiological Sciences, University of Wisconsin (Madison, WI, USA).

2.3. Determination of selectivity index (SI)

The SI of each ethanol extract was determined from the relationship between the cytotoxic concentration 50 (CC_{50}) and the effective concentration 50 (EC_{50}). To calculate the CC_{50}, the cytotoxicity of the extracts on cell lines was established using the MTT method. To calculate the EC_{50}, the supernatants obtained from cultures treated with the ethanol extracts or left untreated were titrated using the plating technique. Both methods have been previously described[13].

2.4. Determination of CC_{50}

First, 2.5×10^4 VERO or U937 cells were plated into 96-well plates for 24 h. Serial dilutions of the ethanol extracts (1 000 μg/mL to 7.8 μg/mL) were made. After the cells were incubated with the extracts for 48 h, MTT solution (0.5 mg/mL) was added, and the cells were incubated for an additional 3 h at 37 °C. Finally, dimethyl sulfoxide was added, and the absorbance was measured in a Bio-RadMicroplate Reader Benchmark® at 450 nm. Cultures without extract were processed as viability positive controls. The CC_{50} was calculated as the concentration of extract that reduced cell viability by 50% using regression analysis (Probit software). Each experimental condition was evaluated in triplicate in two independent experiments (n=6).

2.5. Determination of EC_{50}

To calculate the EC_{50}, 2.5×10^4 VERO or U937 cells were plated into 96-well plates for 24 h. After 24 h, serial dilutions of the ethanol extracts (1 000 μg/mL to 7.8 μg/mL) were made and mixed with strains DENV-2/NG and DENV-2/16681 (MOI:1). This solution was then added to the cell monolayer for 2 h. At 2 h post-inoculation, the solution was removed, and the same serial dilutions of the ethanol extracts (without the virus) were added and incubated for 24 h. After this time, the supernatants were collected for titration by plaque assay. For titration, 5×10^4 VERO cells were plated into 24-well plates, and serial dilutions of the collected supernatants (starting at 1×10^{-1} to 1×10^{-5}) were added to the cell monolayer. After 2 h post-inoculation, the supernatant was removed, and semisolid medium consisting of 1.5% carboxymethylcellulose prepared in antibiotic/antimycotic DMEM supplemented with 2% FBS was added. The plate was then incubated at 37 °C in 5% CO_2 for 7 d. After this time, the cells were fixed with 4% paraformaldehyde prepared in PBS and stained with 1% crystal violet. Subsequently, the number of plaques per well was counted, and the plaque-forming units per milliliter (PFU/mL) were determined. The EC_{50} was calculated as the concentration of extract capable of reducing plaque formation by 50% using regression analysis (Probit software). Each experimental condition was evaluated induplicate in two independent experiments (n=4).

2.6. Dose-response inhibition assay

In 96-well plates, 2.5×10^4 VERO or U937 cells were plated per well. After 24 h, serial dilutions of the ethanol extracts (1 000 μg/mL to 7.8 μg/mL) were madeand mixed with strains DENV-2/NG or DENV-2/16681 (MOI:1). This solution was added to the cell monolayer for 2 h. After 2 h post-inoculation, the solution was removed, and the same serial dilutions of the ethanol extracts used previously were added but, in this case, without the DENV-2 viral inoculum. The plate was then incubated for 24 h. After 24 h, cell supernatants were collected from the VERO and U937 cells treated with both ethanol extracts and infected with strains DENV-2/NG or DENV-2/16681 and were titrated by plating technique.

2.7. Determination of the effect of the extracts on the viral replication cycle

To evaluate the effect of the extracts on phases of the viral replication cycle, three previously described methodologies were used[13,24]: pre-, trans- and post-treatment. In each case, two independent experiments were conducted, each with two replicates (n=4). In addition, in all cases, 2.5×10^4 VERO or U937 cells were plated in 48-well cell culture plates for 24 h, and infections were performed with DENV-2/NG and DENV-2/16681 strains at an MOI of 1. The ethanol extract of *C. grandis* was evaluated at a concentration of 31.2 μg/mL, and that of *T. cymosa* was used at a concentration of 62.5 μg/mL.

2.7.1. Pre-treatment

This strategy allowed for the evaluation of the effect of extracts prior to infection. At 24 h after plating, the ethanol extract was added, and the plate was incubated for another 24 h. After this time, the treatment was removed, and the inoculum was added for 2 h. Subsequently, the viral inoculum was removed, and the cells were incubated in fresh medium for an additional 24 h. After this period, the supernatants were collected and stored at -70 °C until processed by plaque titration assay.

2.7.2. Trans-treatment

This strategy allowed for the evaluation of the virucidal effect of the ethanolic extracts. At 24 h after plating, a solution of equivalent fractions of each viral strain and ethanol extract was made. This solution was incubated for 1 h at 4 °C and was then added to the cell monolayer and incubated for 2 h at 37 °C. After this time, the viral inoculum was removed, and the cells were incubated in fresh medium

at 37 °C for 24 h. After this period, the supernatants were collected and stored at −70 °C until processed by plaque titration assay.

2.7.3. Post-treatment

This strategy allowed for the evaluation of the antiviral effect of the extracts in steps subsequent to the entry of the virus into the cell. After 24 h, the viral inoculum was added to the cell monolayer, and the plate was incubated at 37 °C for 2 h. The viral inoculum was subsequently removed, the ethanol extract was added, and the cell culture plate was incubated for 24 h at 37 °C. After this period, the supernatants were collected and stored at −70 °C until processed by plaque titration assay.

2.8. Statistical analysis

To determine the CC_{50} and the EC_{50}, a regression analysis was performed using the Probit software. To compare the number of infectious viral particles released from the cells treated with each extract at different treatment concentrations relative to the untreated control, the viral titers in PFU/mL were tested for normality of distribution using a Kolmogorov-Smirnov test, and after that an analysis of variance (ANOVA) was used, followed by a Tukey *post-hoc* test. Finally, to compare the number of infectious viral particles released from the cells treated under the different experimental conditions and untreated cells, student's *t*-test was used. In all cases, differences were considered statistically significant at a P value lower than 0.05 (P<0.05).

3. Results

3.1. Determination of the toxicity of the extracts on both cell lines

To determine the cytotoxicity of the extracts, an MTT assay was used. This test determined the CC_{50} (to subsequently calculate the SI) and the nontoxic concentrations to be evaluated in the dose-response assay. The CC_{50}values for the *C. grandis* extract were 805.0 µg/mL and 325.0 µg/mL in VERO and U937 cells, respectively. The CC_{50}values for the *T. cymosa* extract were 2 970.7 µg/mL and 460.6 µg/mL in VERO and U937 cells, respectively. Although the two extracts were not substantiallytoxic to either cell line, U937 cells were more susceptible to the antiproliferative effects of both extracts.

3.2. Determination of the selectivity of the extracts to DENV infection

The EC_{50} of the extracts was determined after the titration of the supernatants obtained in the inhibition of infectious viral particle production assay, and together with the CC_{50}, the SIs were calculated.

In VERO and U937 cell cultures infected with the DENV-2/NG strain and treated with the extract of *C. grandis*, the EC_{50} values were 23.4 µg/mL (CC_{50}: 805.0 µg/mL) and 7.8 µg/mL (CC_{50}: 325.0 µg/mL), respectively. In VERO and U937 cell cultures treated with *T. cymosa* extract, the EC_{50} values were 7.8 µg/mL (CC_{50}: 2 970.7 µg/mL) and 21.9 µg/mL (CC_{50}: 460.6 µg/mL), respectively. The SIs were higher than 20, in a range of 21.0 to 380.9, and the most selective was the extract of *T. cymosa* on VERO cells. When comparing the results obtained in both cell lines, it was found that *C. grandis* extract was more selective in U937 cells (SI 41.7) than in VERO cells (SI 34.4). However, the extract of *T. cymosa* was more selective in VERO cells (SI 380.9) than in U937 cells (SI 21.0).

In VERO and U937 cell cultures infected with DENV-2/16681 and treated with *C. grandis* extract, the EC_{50}values were 66.7 µg/mL (CC_{50}: 805.0 µg/mL) and 10.5 µg/mL (CC_{50}: 325.0 µg/mL), respectively. In VERO and U937 cultures treated with *T. cymosa* extract, the EC_{50}values were 23.4 µg/mL (CC_{50}: 2 970.7 µg/mL) and 46.8 µg/mL (CC_{50}: 460.6 µg/mL), respectively. The SIs were higher than 9.0, with a range of 9.1 to 127.0, and the most selective was *T. cymosa* extract on VERO cells. When comparing the results obtained in both cell lines, *C. grandis* extract was more selective in U937 cells (SI 31.0) than in VERO cells (SI12.1). However, *T. cymosa* extract was more selective in VERO cells (SI 127.0) than in U937 cells (SI 9.8).

When comparing the virus strain-dependent selectivity of the extracts, it was found that both extracts were more selective in cultures infected with strain DENV-2/NG (SIs between 21.0 and 380.9) than in cultures infected with strain DENV-2/16681 (SIs between 9.8 and 127.0).

3.3. Dose-response inhibition assay

Using the ethanolic extract of both plants, a dose-response inhibition assay was performed using nontoxic concentrations from 15.6 µg/mL to 125 µg/mL as defined by MTT assay. Similarly, the collected supernatants were titrated to obtain the number of infectious viral particles in cultures with and without treatment.

In VERO cell cultures infected with strain DENV-2/NG and treated with *C. grandis* extract, a statistically significant inhibition was found compared with the untreated control at concentrations higher than 31.2 µg/mL (inhibition percentage higher than 97.5%). However, in cultures infected with strain DENV-2/16681, the inhibition was significant only at concentrations higher than 62.5 µg/mL (inhibition percentage higher than 80.3%) (ANOVA-Tukey P<0.05) (Figure 1A). In U937 cell cultures infected with strain DENV-2/NG, the inhibition was statistically significant at concentrations higher that 15.6 µg/mL (inhibition percentage higher than 99.7%). However, in cultures infected with strain DENV-2/16681, the inhibition was significant only at concentrations higher than 31.2 µg/mL (inhibition percentage higher than 79.4%) (ANOVA-Tukey P<0.05) (Figure 1B). An inhibitory effect dependent on the extract dose used was not observed in any case.

In VERO cell cultures infected with strain DENV-2/NG and treated with *T. cymosa* extract, a statistically significant inhibition was observed compared with the untreated control at concentrations higher than 15.6 μg/mL (inhibition percentages higher that 90.5%). However, in cultures infected with strain DENV-2/16681, the inhibition was significant only at concentrations higher than 62.5 μg/mL (inhibition percentages higher than 98.9%) (ANOVA-Tukey $P<0.05$) (Figure 2A). The same behavior was observed in U937 cell cultures infected with strain DENV-2/NG, with statistically significant inhibition at concentrations higher that 15.6 μg/mL (inhibition percentages higher than 67.9%). In addition, when the cultures were infected with strain DENV-2/16681, the inhibition was significant only at concentrations higher than 62.5 μg/mL (inhibition percentages higher than 98.3%) (Figure 2B). An inhibitory effect dependent on the extract dose used was not observed in any case.

3.4. Effect of extract treatment time on infection with DENV

Three experimental strategies were used for determining the effect of the extracts when added at different times of infection. The pre-treatment strategy allowed for the evaluation of the effect of the extract on the cell. However, the trans-treatment method evaluated the virucidal effect of the extract, and finally, the post-treatment strategy evaluated the effect of the extract after the entry of the virus into the cell. For these assays, only one concentration of the extracts (*C. grandis* at a concentration of 31.2 μg/mL and *T. cymosa* at a concentration of 62.5 μg/mL) was used based on the dose-response assays.

When the cells were treated with the ethanol extract derived from leaves of *C. grandis* 24 h prior to infection with the strain DENV-2/NG (pre-treatment), a statistically significant ($P<0.05$, by student's t-test) decrease was detected relative to the control without extract. The inhibition percentages were 9.2% and 17.9% in VERO and U937 cells, respectively. When the cells were treated immediately after infection (post-treatment), the inhibition percentages were higher than 60% (61.4% and 71.9% in VERO and U937 cells, respectively). No statistically significant differences were found in cultures subjected to the trans-treatment strategy or the untreated control (Figure 3A).

When cells were treated with the ethanol extract derived from the bark of *T. cymosa* and subsequently infected with strain DENV-2/16681 (pre-treatment), a statistically significant ($P<0.05$, student's t-test) decrease was detected relative to the control without extract. The inhibition percentages were 27.6% and 12.9% in VERO and U937 cells, respectively. Likewise, a statistically significant decrease of

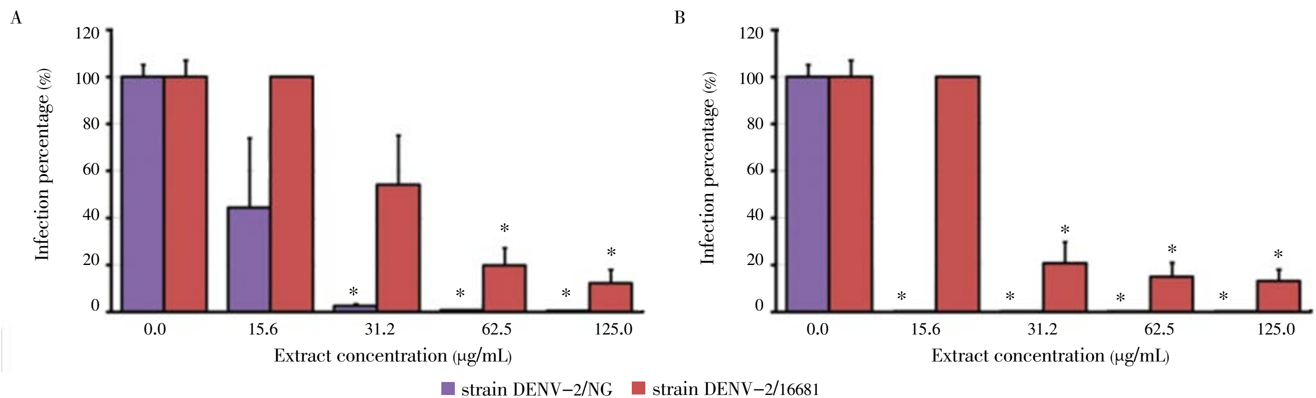

Figure 1. Inhibitory effect of the extract of *C. grandis* leaves on DENV infection.
Cells were infected with strain DENV-2/NG or with strain DENV-2/16681 at an MOI of 1 and treated with different concentrations of the ethanol extract of *C. grandis*. A: VERO cells, B: U937 cells. The asterisks indicate statistically significant differences relative to the untreated control. ($P<0.05$; ANOVA-Tukey test). The error bars represent the standard error of the mean.

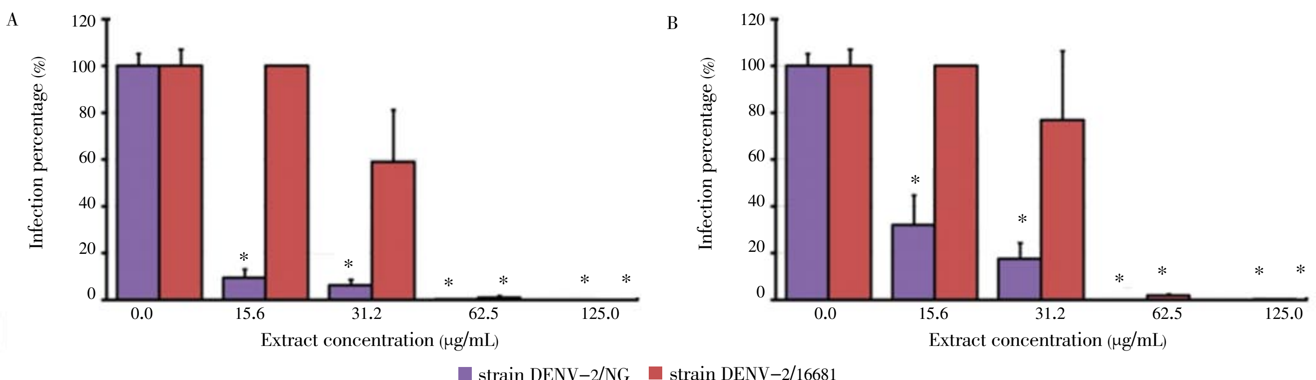

Figure 2. Inhibitory effect of the extract of *T. cymosa* bark on infection by DENV.
Cells were infected with strain DENV-2/NG or with the strain DENV-2/16681 at an MOI of 1 and treated with different concentrations of the ethanol extract of *T. cymosa*. A: VERO cells, B: U937 cells. The asterisks indicate statistically significant differences relative to the untreated control. ($P<0.05$; ANOVA-Tukey test). The error bars represent the standard error of the mean.

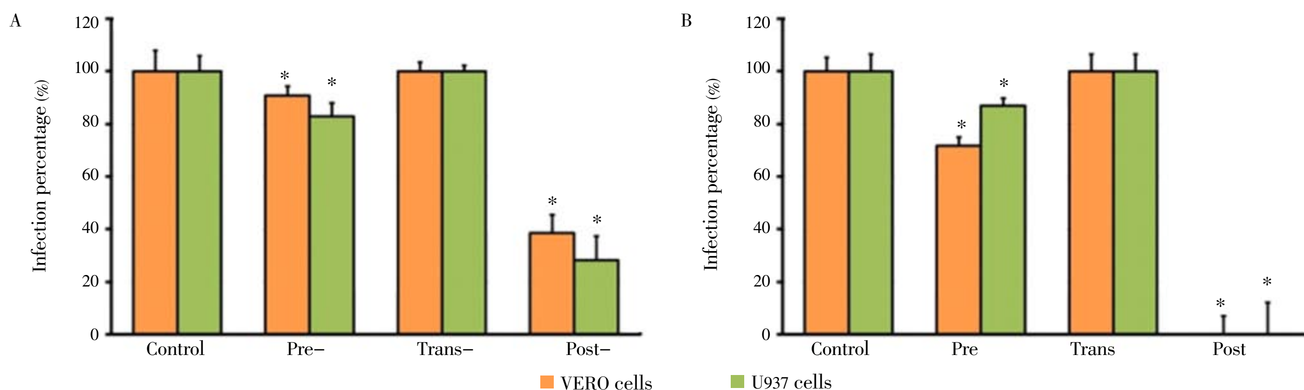

Figure 3. Antiviral effect of the extracts under the different treatment strategies.
VERO cells and U937 cells were infected with DENV-2/NG and DENV-2/16681 strains at an MOI of 1 and treated under different treatment strategies (pre-, trans- and post-treatment). A: Cells treated with *C. grandis* extract (31.2 μg/mL), B: Cells treated with *T. cymosa* extract (62.5 μg/mL). The asterisks indicate statistically significant differences relative to the untreated control. (P<0.05; student's t-test). The error bars represent the standard error of the mean.

99.9% was detected for both cell lines (P<0.05, student's t-test) in the post-treatment strategy. However, no statistically significant difference was found in cultures subjected to the trans-treatment strategy or the untreated control (Figure 3B).

3.5. Phytochemical screening

Phytochemical screening was performed to identify secondary metabolites in the extracts of both plants. In the ethanol extract of *C. grandis* leaves, large quantities of tannins, polyphenols, triterpenes, sterols, glycosides, and flavonoids were detected. However, with the techniques used, no alkaloids, coumarins or saponins were detected. In contrast, in the ethanol extract derived from the bark of *T. cymosa*, moderate quantities of alkaloids, triterpenes, and sterols were detected. Conversely, no tannins, polyphenols, saponins, glycosides, flavonoids, or quinones were identified. There were differences in the secondary metabolites present in the two extracts, especially in tannins, polyphenols, glycosides, and flavonoids, which were present in the ethanolic extract from *C. grandis* but were not detected in the ethanol extract of *T. cymosa*. In addition, there were differences in metabolites such as alkaloids, which were present in *T. cymosa* extract but were not detected in the extract of *C. grandis*. Saponins were not detected in any of the extracts (Table 1).

Table 1
Preliminary phytochemical screening of the ethanol extracts derived from *C. grandis* leaves and *T. cymosa* bark.

Secondary metabolite type	*C. grandis*	*T. cymosa*
Alkaloid	-	++
Tannins and polyphenols	+++	-
Triterpenes	+++	++
Sterols	+++	++
Coumarins	-	+
Saponins	-	-
Glycosides	+++	-
Flavonoids	+++	-
Quinones	++	-

+++: high; ++: moderate; +: low; -: not detected.

4. Discussion

Despite the serious public health problem represented by the diverse clinical forms of dengue, there is still no specific and effective antiviral therapy, and patients suffering from the disease are treated only with supportive therapy. An additional problem that arises in the search for compounds with antiviral activityis the differential selectivity they may present against different viral strains[24]. This implies that a drug that is licensed should be used depending on the form of the disease or the type of virus causing the infection, and this would present difficulties forits administration. Considering that the differential selectivity of active compounds is a poorly studied research field, and based on a previous screen performed byour group, we decided to evaluate the antiviral potential of the extracts of *C. grandis* and *T. cymosa*. This evaluation was performed in two different cell lines and against two viral strains (each associated witha different form of the disease); we also evaluated the effect depending on the time of administration.

The antiviral activity of plants belonging to the Cassia and Tabernaemontana families has been reported previously. For example, triterpenes, cycloartenols and saponins from *Cassia occidentalis* have proved to be effective at inhibiting infection by human immunodeficiency virus type 1[25]. Moreover, steroids, terpenoids and phenolic compounds from *Tabernaemontana catharinensis* exhibited a potencial viral inhibition against herpes simplex virus type 1[26]. In addition, secondary metabolites of *Cassia fistula* have been shown to have antiproliferative activity in HeLa cells[27], and ethanol extracts of *Cassia eucalyptus* inhibit the growth of *Salmonella typhi*[28]. However, no antiviral activity against DENV has been reported thus far for any of these plants.

In this study, the cytotoxicity of both extracts was first evaluated. The CC_{50}values for *C. grandis* extract in both cell lines were higher than 300 μg/mL. The cytotoxic for *Cassia angustifolia* extract had been reported in BRL-3A cells at concentrations that appear largely higher than those attainable in humans, nevertheless *in vivo* the extract did not induce any significant change after oral administration at

doses of 12-58 mg/(kg•d)[29]. This indicates that the extract is slightly toxic to both cell lines (VERO and U937). The extract of *T. cymosa* showed CC_{50} values of 2970.7 μg/mL and 460.6 μg/mL in VERO and U937 cells, respectively. This indicates that although the extract is slightly toxic to both cell lines, U937 cells are more sensitive to the antiproliferative effect than VERO cells. The cytotoxicity for *Tabernaemontana elegans* roots ethanol extract had been reported in HepG2 cell (CC_{50} 5.81) and THP-1 cell (CC_{50} 16.8)[30].

As calculated by EC_{50} and SI, the extract of *C. grandis* was more selective in U937 cells, whereas the *T. cymosa* extract was more selective in VERO cells infected with the strains DENV-2/NG and DENV-2/16681. Additionally, both extracts were more effective against infection by the DENV-2/NG viral strain than against infection by the DENV-2/16681 strain. As mentioned previously, the effect of a compound with antiviral potential may be determined in part by the type of virus causing the infection or by the cell type being studied[9]. For example, it has been demonstrated that sulfated polysaccharides isolated from seaweed, surfaceplants, marine invertebrates, and cyanobacteria exhibit higher inhibitionof DENV-2 compared withother serotypes[24], more effectively in VERO cells relative to other cell lines[31]. This differential selectivity may be due to characteristics of the cells such as the presence of receptors on the cell membrane, transport mechanisms, or replication within the cell[8]. Moreover, it is of great importance that both extracts are more effective and selective against infection with viral strain DENV-2/NG (strain isolated from a patient with dengue and associated with this less-severe type of disease) than against infection with viral strain DENV-2/16681 (strain isolated from a patient with hemorrhagic dengue and associated with severe forms of the disease). This is important because it may indicate that viral strains associated with non-severe forms of the disease could be more susceptible to a possible treatment than the strains associated with severe forms.

Finally, when the effectiveness of the extracts in relation to the time in which they were added was evaluated (pre-, trans- and post-treatment strategies), differences were found in the antiviral effect, depending on the time of addition of the extract. Statistically significant inhibition was found relative to the untreated controls in both cell lines infected with either viral strain and treated with the pre- or post-treatment strategy. However, no differences were found with the trans-treatment strategy. The differences found in the pre-treatment strategy indicate that the extracts may be affecting some or allof the steps prior to the internalization process of the viral particle into the host cell. A similar effect has been reported for essential oils derived from *Lippia alba* and *Lippia citriodora*[20]. A compound effective in this typeof strategy could be used in the future to prevent infection. However, the differences found using the post-treatment strategy indicate that the inhibition could be due to a blockage in steps subsequent to the internalization of the virus (such as protein synthesis, RNA replication or assembly of new viral particles). In addition, these differences correlate with evaluations of sulfated polysaccharides, which were shown to inhibit the replication of DENV-2 in VERO and HepG2 (hepatic origin) cells[24] and in mosquito cells[31] using the same experimental strategy. This finding is of great importance because an effective compound in such an experimental strategy could be used in the future for the treatment of the disease because it could be administered once the virus has entered the cell.

Finally, the preliminary phytochemical screening of the *C. grandis* ethanol extract showed high quantities of tannins, polyphenols, triterpenes, sterols, glycosides and flavonoids in this research. These secondary metabolites have also been reported by other authors in extracts from diverse plants belonging to the same family as *Cassia surattensis*[32]. The antioxidant activity of glycosides and flavonoids has been reported in *Cassia roxburghii* DC.[33], *Cassia alata*[34], and *Cassia spectabilis*[35]. In all cases, the presence of these metabolites has been related with the antioxidant activity of the extract and, in some cases, with hepatoprotective activity. Considering the role oxidative stress plays in dengue pathogenesis[36,37], a therapy based on compounds with reported antioxidant activity may be useful to decrease the severity of the disease. It is important to emphasize that so far, no study has evaluated compounds with antioxidant activity for the treatment of dengue. However, although the main clinical manifestation of dengue is the presence of febrile episodes, in cases of severe dengue, hepatic cells can be affected[38]. For this reason, a compound with hepatoprotective activity, as reported for the flavonoids derived from certain plants of the Cassia family[35], could be useful not only for its antiviral activity (inhibiting viral replication) but also for its effects on oxidative stress.

Moreover, the phytochemical screening of the ethanolic extract from *T. cymosa* bark contained alkaloids, triterpenes, and sterols, among other secondary metabolites. Some of these compounds have been previously reported in plants of the same family, such as *Tabernaemontana stapfiana* Britten, which contains alkaloids, flavonoids, coumarins, tannins, and saponins (associated with antibacterial activity)[39]. In addition, similar compounds have been reported in *Tabernaemontana elegans*,which containsa predominant alkaloid compound (associated with activity against *Mycobacterium tuberculosis*[40,41]) and in *Tabernaemontana catharinensis* (antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*)[42]. Furthermore, it has been reported that the alkaloids present in the extract derived from *Tabernaemontana divaricata* are effective as analgesics[43] and that the extract from *Tabernaemontana divaricata* Linn. (consisting of flavonoids, tannins, alkaloids and steroids) has antioxidant effects[44]. As mentioned above, a compound with antiviral and antioxidant activity would be of great value for the treatment of dengue.

This is the first report of an antiviral effect of ethanol extracts derived from *C. grandis* leaves and *T. cymosa* bark against infection by DENV serotype 2. Based on these findings, these extracts maybe considered potential antiviral

agents against DENV that specifically affect steps of the viral replication cycle after viral internalization. Additionally, this antiviral effect, which is directly related to a decrease in viral replication, could be enhanced by the effect of these extracts on oxidative stress (a mechanism involved in the development of severe forms of dengue). Thus, a therapy with compounds derived from these extracts would be twice as beneficial, as it would inhibit viral replication and oxidative stress in infected patients. Further studies will be needed to identify which steps of the viral replication cycle are being affected by the extracts.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

This work was funded by COLCIENCIAS-Administrative Department of Science, Technology and Innovation, and National Program of Science and Health Technology, project code 1115-4932-6092 of 2009.

Comments

Background

Arthropod-borne flaviviral diseases such as dengue are a major public health concern in the tropics. Due to the lack of an effective treatment against dengue, a large number of plants have been tested as a source of biologically active molecules that can be used to treat the infection.

Research frontiers

Due to the clinical and economic impact of flaviviral infections in the human population and the lack of an effective treatment against dengue, in the last years, a large number of papers have studied the effects of plants extracts in the search of lead molecules that could be used in the treatment of DENV or other flaviviral infections.

Related reports

Although there are several reports in the literature evaluating the effect of several plant extracts or isolated compounds against DENV, there are no reports with extracts obtained from *C. grandis* or *T. cymosa*. Then, the results presented in the manuscript are important for the community working in the area.

Innovations and breakthroughs

Although there are no innovations in the paper, the fact that this is the first report of anti-dengue virus activity in extracts obtained from *C. grandis* and *T. cymosa*, the study is important.

Applications

The results are important to continue with the fractionation of the extracts to get lead compounds with anti-dengue virus activity. For this reason, the results can be applicable in the future for the development of therapeutic options against dengue.

Peer review

The study shows that the ethanol extracts obtained from *C. grandis* or *T. cymosa* inhibit the replication of DENV with a selectivity that is higher for DENV-2/NG strain than for DENV-2/16681 strain. The experiments also indicate that the extracts may affect both, the internalization process and steps subsequent to the internalization of the virus. The experiments were well conducted.

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