Full Length Research Paper

In vitro cytotoxicity and biological activities of Genipa americana (Rubiaceae) ethanolic extracts

Paola Silva Campos Codignoto1, Shirley Batista de Araújo1, Natan Munhoz Bastos1, Thais de Oliveira Fernandes4, Tamiris Arielle Santana Barbosa4, Carlos Eduardo Dias Igidio2, Fernando Faustino3, Maria Judite Bittencourt Fernandes1 and Aline Oliveira da Conceição2*

1Centro de Pesquisa e Desenvolvimento de Sanidade Animal – Instituto Biológico, São Paulo/SP, Brazil.
2Departamento de Ciências Biológicas – Universidade Estadual de Santa Cruz, Ilhéus/BA, Brazil.
3Departamento de Ciências Exatas e Tecnológicas – Universidade Estadual de Santa Cruz, Ilhéus/BA, Brazil.
4Departamento de Ciências Agrárias e Ambientais – Universidade Estadual de Santa Cruz, Ilhéus/BA, Brazil.

Received 22 December, 2016; Accepted 6 February, 2017

This study aimed to determine the in vitro toxicity of Genipa americana and its antiviral and antimicrobial activities. Due to lack of knowledge on G. americana validation as medicinal plant, biological studies were performed. For the cytotoxicity experiments, decreasing concentrations of ethanolic extracts of G. americana branches, fruits, and leaves were added to a confluent monolayer of Vero cells. After 72 h, it was determined, the first concentration of each extract in which any cellular change was observed (maximum non-cytotoxic concentration- MNCC). The MNCC of fruit and branches extracts was of 0.5 and 0.25 mg/mL, respectively. The leaves extract did not show cytotoxicity in the tested dilutions. The antiviral activities of G. americana extracts were tested on the suid (SuHV-1) and equine (EHV-1) alpha herpes viruses through reduction of their viral titers. The fruit extract presented a viral inhibition on EHV-1 of 86%, but it was not effective for SuHV-1. Instead, the leaves and branch extracts showed antiviral activity against SuHV-1, with a viral inhibition of 73 and 79%, respectively, but no inhibition on EHV-1. The antimicrobial action on Escherichia coli and Staphylococcus aureus was performed by the agar diffusion and the microdilution methods; and by agar diffusion method against Candida albicans, Candida parapsilosis, Candida glabrata and Candida krusei. The fruits extract showed inhibition of bacterial growth for E. coli and S. aureus with a bacteriostatic effect on E. coli and a bactericidal effect on S. aureus. There was no effect on any Candida species. Ethanolic extract of G. americana fruits seemed to be a promising source of phytotherapeutic drugs with a spectrum of action on diverse microorganisms in non-toxic concentrations.

Key words: Alphaherpes virus, medicinal plant, antimicrobial, antiviral, cell line, broad spectrum.

INTRODUCTION

In several countries, for generations, medicinal plants have been widely used. However, it has been only for the last two decades that plant as a medicine has been integrated in allopathic therapies in public health assistance (Santos et al., 2011). Easy access and low cost of natural origin products should be recommended...
as a therapeutic source, mainly in low-income communities (Brasileiro et al., 2008). However, the plants used in any herbal preparation require strict quality control since it may exhibit toxic and/or variable chemical composition. Thus, their toxicity should be checked beforehand. The current toxicology methods employed for the initial determination of toxicity or screening of these substances are in vitro assays using cell cultures, the so-called cytotoxicity assays. The toxicity may be assessed via several forms such as morphology, incorporation of stains or radioactive nucleotides. These methods are increasingly being used to replace the animal testing together with their validation, standardization and reproducibility (Eisenbrand et al., 2002; Cos, 2006; Rispin et al., 2006).

Investigation and establishment of new compounds as a basis for medicine and/or drugs remains essential and necessary, despite the control and effective treatment of many diseases. For this, an increasing number of phyto-studies have found molecules of biological and/or therapeutic potential (Rates, 2001; Clardy and Walsh, 2004; Balunas and Kinghorn, 2005; Cos, 2006). This is especially so in the field of veterinary medicine, with the rise in the interest about the use of the medicinal plants in recent decades, which resulted in scientific studies to corroborate natural products recommendations (Viegi et al., 2003; Pieroni et al., 2006; Martínez and Luján, 2011) which turns the study involving animals pathogens of great interest.

Polymicrobial diseases, which involve two or more microorganisms that act synergistically, or in succession, mediating complex disease progress are a point of concern. This is because intermicrobial interactions and environmental cues determine infection outcomes, such that specific microbial populations under certain conditions may enhance or predict disease progression (Bakaletz, 2004; Peters et al., 2012). Therefore antimicrobial agents can act directly on the micro-organisms without affecting normal host cell and with a selective toxicity independent if they are synthetic or derived from plants (Cos, 2006; Samy and Gopalakrishnakone, 2010). Moreover, the synthetic drugs lead to emergence of resistance strains. Thus, medicinal plants would be an alternative to overcome this and they could either substitute or complement synthetic drugs (Chattopadhyay and Naik, 2007; Son et al., 2013).

These novel natural substances may be tested through in vitro bioassays that provide rapid and effective mechanisms of screening (Houghton, 2000; Cos, 2006; Samy and Gopalakrishnakone, 2010). Regardless of which bacterial, yeast, or viruses are the target of this search, concomitant or previous assays of the toxicity of these compounds should be carried out to guarantee and provide a safe and effective use, and consequent safety and reliability (Houghton, 2000; Eisenbrand et al., 2002; Cos, 2006).

Studies on the Rubiaceae family demonstrated a variety of chemical constituents of medicinal importance. In traditional medicine, several species from this family have been used as bronchitis, asthma, pneumonia, anti-rheumatic, emetic, purgative, diuretic, anti-inflammatory, antiviral and anti-oedematogenic agent (Lorenzi and Matos, 2002; Dias, 2013). Specifically, Genipa americana, an edible fruit tree, besides being used for comfitsures and alcoholics beverages, is commonly used in traditional medicine to treat anemia, jaundice, asthma, and liver and spleen diseases (Lorenzi and Matos, 2002; Moreira et al., 2002). In vitro studies of G. americana fruit showed a broad spectrum antibacterial activity (Barbosa, 2008) and an interference on trophoblast-like cells proliferation but not on differentiation (Conceição et al., 2011). Thus, it is prudent to investigate this much used species for its in vitro toxicity and to evaluate its anti-viral and anti-microbial potential.

MATERIALS AND METHODS

G. americana L. (Rubiaceae) branches, fruit and leaves were collected through botanical field work in January 2006, in Rio de Engenho district, Ilhéus, Bahia, Brazil. Plant habitat was characterized by argillaceous ground, a humidified environment, and cacao culture, in the Brazilian northeastern hieroglyphic forest (Mata Atlantica forest). Plant collection coordinates were 14° 51' S and 39° 04' W. The voucher specimen was identified and deposited in the herbarium of the Universidade Estadual de Santa Cruz, under identification number of HUESC13.923. The study was conducted following Brazilian access to nature law (permission number: IBAMA no. 02001.001749/2012-31).

Preparation of ethanolic extracts

Plant material was dried for 8 h at 28°C protected from the light. Dry plant material was reduced to a powder with mechanical knives. A 10 g sample of dried and powdered plant material was macerated in 100 mL ethanol for 24 to 48 h through direct contact with solvent and with mechanical agitation. The marc was filtered through Whatman number 1 filter paper and evaporated to dryness under reduced pressure. Each plant material was soaked in recovered solvent once more to make a new extraction and further lyophilized. For bioassays, firstly a stock solution of each extract was made diluting 100 mg in 100% sterile dimethyl sulfoxide (DMSO Sigma®). Then, this stock solution was diluted 100 mg in 100% sterile dimethyl sulfoxide (DMSO Sigma®). Then, this stock solution was diluted in Minimum Eagle Medium (MEM, Atenát®) to yield concentrations of 4, 1 and 0.8 mg/mL, respectively of fruits (FE), branches (BE) and leaves (LE)

*Corresponding author. E-mail: aoconceicao@uesc.br.

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ethanolic extracts. These extracts were filtered with 10 μm pore filter, distributed in aliquots, and stored at -20°C.

Cell line
The Vero cell line from green monkey kidney (ATCC CCL-81) was kindly provided by Instituto Butantan. The cells were maintained in MEM with fetal bovine serum 8% in 37°C incubator, 5% CO2. For the experiments, cells were subcultured on the previous day in sterile and disposable 96-microplates (3.0 x 10^4 cells/well).

Viruses
The suid alphaherpesvirus type-1 (SuHV-1) EMBRAPA: BRM43A 3, 00588 and equid alphaherpes virus type-1 (EHV-1) A4/72 Brazilian strains were used in this study.

Cytotoxicity assay
The morphological method was applied here. Extracts diluted in MEM (two base) from their stock solutions were added to the cell microplates. The changes or cell death were visualized at inverted light microscope every day up to 72 h using as a parameter, the control (cells without extract). The first concentration of the extract that did not generate cell morphological changes was called the maximum non-cytotoxic concentration (MNCC) and it was used in antiviral assays.

Antiviral assay
The antiviral activity of the extracts was based on the inhibition of cytopathic effect and consequent reduction of viral titer (Barros et al., 2012). Extracts were considered positive and promising when the viral titer of treated (extracts) and infected cells had a statistical significant reduction in the viral titer as compared to control infected cells (without addition of the extracts). Foscarnet (Sigma-Aldrich, Brazil) at 200 μg/mL was used as control. The statistical difference between treatments was accessed by t test considering 95% confidence interval (p<0.005).

Antibacterial and antifungal assays
For this study, American Tissue Culture Collection (ATCC) bacteria (E. coli, INQCS-00325-ATCC-35218; S. aureus INQCS-00015- ATCC-25923) and yeast (C. albicans, ATCC 14057); C. krusey, ATCC 6258; C. parapsilosis, ATCC 22018; and C. glabrata, ATCC 29301) were used. Bacteria were obtained from Collection of Reference Microorganisms on Health Surveillance (CMRVS, FIOCRUZ-INQCS), Rio de Janeiro, Brazil. Yeasts were kindly provided by Dr. Sydney Hartz Alves from Mycological Research Laboratory (LAPEME) at Federal University of Santa Maria. These micro-organisms were of clinical interest.

Agar gel diffusion (AGD) and microdilution techniques were used for both antibacterial and antifungal evaluation. For AGD, the well variant was used. For that, bacterial and yeast inoculums were prepared at an equivalent of 0.5 to 1.0 of McFarland scale (approximately cell density of 1.5x10^8 CFU/mL) and spread with sterile swab all over the Petri dishes containing nutrient or Sabouraud dextrose agar. Solutions of 0.125, 0.25, 0.5 and 1.0 mg/mL were prepared with NaCl 0.9% solution immediately before the test. After bacterial and yeast culture in Petri dishes, holes with 6 mm diameter were done in the agar where 50 μL of samples and controls were applied. Controls consisted of chloramphenicol (50 μg/mL), ketoconazole (50 μg/mL), chlorhexidine (1%) and NaCl 0.9%. The plates were incubated overnight at 37°C in a humidified chamber to avoid evaporation of the medium. All tests were repeated at least three times.

To analyze the antibacterial activity of the samples, inhibition halo of ≥ 14 mm at ≤ 100 mg/mL were used as criteria to consider a sample as antibacterial. To analyze the antifungal activity, inhibition halo ≥ 10 mm was used as criteria to consider the extract as antifungal (Pedroso et al., 2014).

For microdilution technique, a serial dilution (10^1 to 10^5) of both bacteria was done in brain heart infusion broth (BHI). After 24 h at 37°C, 10 µL of the serial dilutions were plated in nutrient agar plates and incubated at 37°C for another 24 h. Thereafter, bacterial colonies were counted. To perform antibacterial test, the latest dilutions having ≥100 CFU were chosen (E. coli = 10^3; S. aureus = 10^5). For that, 100 μL of extract and 100 μL of bacterial inoculum were distributed in 96 well plates in triplicates. Controls consisted of chloramphenicol (100 μL CHLOR+ 100 μL inoculum), inoculum (100 μL inoculum+100 μL BHI), plant extracts (100 μL extract+100 μL BHI) and BHI (200 μL) only. Extracts were prepared from 10 mg/mL starting solution and diluted in BHI giving final solutions of 0.125, 0.5 and 1.0 mg/mL. After 24 h incubation at 37°C, the optical density was measured by spectrophotometry at 450 nm wave length to determine the minimal inhibitory concentration (MIC). Background was reduced subtracting optical density of plant extract without inoculum and BHI only. Then, the subculture was done in nutrient agar to visualize bacterial growth and determine the minimal bactericidal concentration (MBC).

RESULTS AND DISCUSSION
The initial focus of this study was to investigate the in vitro toxicity of extracts of G. americana prior to antiviral assay. Here, the evaluation was based on cellular morphological changes where higher MNCC values mean less cytoxicity, and lower value means the greater cytotoxicity. From the cellular morphological alterations caused by extracts and visualized at inverted cell microscope, the maximum non-toxic concentration of each extract (MNCC) was determined. The MNCCs of FE and BE extracts were of 0.5 and 0.25 mg/mL, respectively. The LE extract showed none cytoxicity in all tested dilutions. Thus, the concentration of 0.2 mg/mL was established for use in antiviral assays. The toxic alteration on cell monolayer ranged from cell death with total monolayer destruction to formation of vacuoles and cellular condensation (Figure 1). While all extracts presented low cytotoxicity on Vero cells, special attention should be paid to FE extract which contributes to considering G. americana fruit extract as a promising phytotherapeutic agent.

Antiviral therapy is more effective on human herpes viruses, nevertheless the animal herpes viruses can serve as models and to better understand this family of virus through in- vitro and vivo experiments (Field et al., 2006; Son et al., 2013). In the case of equine alphaherpes virus infections, the use of antivirals combined with other measures is beneficial and well-
Figure 1. Cytotoxic evaluation of *G. americana* on Vero cells. A) Cell control (without extract); B) Cells with ethanolic extract of *G. americana* fruit (FE) at 4 mg/mL. Image obtained with inverted light microscope at 200x.

Table 1. *G. americana* extracts antiviral activities against suid (SuHV-1) and equine (EHV-1) alphaherpes viruses.

<table>
<thead>
<tr>
<th>Ethanolic extracts</th>
<th>Viral inhibition (%)</th>
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<tbody>
<tr>
<td></td>
<td>EHV-1</td>
</tr>
<tr>
<td>Branches (BE)</td>
<td>0</td>
</tr>
<tr>
<td>Fruits (FE)</td>
<td>86*</td>
</tr>
<tr>
<td>Leaves (LE)</td>
<td>0</td>
</tr>
<tr>
<td>Foscarnet (0.2 mg/mL)</td>
<td>0</td>
</tr>
</tbody>
</table>

*p<0.005 by *t* test.

studied (Garré et al., 2007; Vissani et al., 2016).

The antiviral evaluation of the extracts is described in Table 1. Only FE showed a significant inhibition to EHV-1, around 90% (*p* < 0.005). The LE and BE extracts were not inhibitors. On the other hand, BE and LE extracts inhibited 79 and 73% of SuHV-1 effect, respectively, while FE demonstrated any antiviral activity to SuHV-1. The Foscarnet, synthetic antiviral agent, was used as positive control at non-toxic concentration of 0.2 mg/mL. The strain of EHV-1 was shown to be resistant to it while the SuHV-1 strain was susceptible with a reduction of 2 log in its viral titer equivalent to 99% of inhibition. Garré et al. (2007) studying diverse nucleoside analogues and foscarnet also verified that this last is not appropriate for treatment of infected horses because it was the least effective and with differences between the isolates.

The suid alphaherpes virus is widely studied as a model for herpes virus biology, but there are diverse studies on antivirals from medicinal plants aiming in the future to decrease the infection in addition to vaccination and the negative impact of this disease on pig industry (Pomeranz et al., 2010; Son et al., 2013). For suid alphaherpes virus, less intensive antiviral activity was seen with the BE and LE extracts. In contrast, the foscarnet was active against this virus with 99% of inhibition. These results reinforce the differences between animal viruses’ models.

The procedure to study antibacterial and antifungal agents focusing on their growth can be done with different methods (Cos, 2006). Here, we used qualitative and quantitative techniques against two important Gram negative and positive bacteria. Ethanolic extracts from *G. americana* leaves, brunch and fruit showed variability between the two types of bacterial used. *E. coli* and *S. aureus* were resistant to both leaves and brunch extracts. Conversely, *E. coli* and *S. aureus* was shown to be sensitive to FE extract (Table 2 and Figure 2). Through AGD halo of inhibition was formed at 1 and 0.5 mg/mL, for *E. coli* and *S. aureus*, respectively. Through MIC/MBC, a bacteriostatic effect of *E. coli* and bactericidal effect of *S. aureus* at ≥0.05 mg/mL was remarked.

The antibacterial action against *E. coli* at ≥ 1 mg/mL (Table 2) differed from negative results found by Gonçalves et al. (2005). We consider our results promising, since the AD technique is more sensitive than disc impregnation used by Gonçalvez et al. (2005). It was also possible to establish a dose dependent response, and this technique is less susceptible to intrinsic errors. Besides, the results was shown to be still more promising with MIC and MBC techniques since bactericidal effect of ethanolic extract from *G. americana* fruit was clearly seen.

In conclusion, due to the presence of steroids found in a prior study (Conceição et al., 2011) and literature report
Table 2. Antibacterial activity of ethanolic extract of *G. americana* by agar diffusion technique against *E. coli* and *S. aureus*.

<table>
<thead>
<tr>
<th>mg/mL</th>
<th><em>E. coli</em> (ATCC-35218)</th>
<th><em>S. aureus</em> (ATCC-25923)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LE BE FE</td>
<td>LE BE FE</td>
</tr>
<tr>
<td>10</td>
<td>R R 14 ND ND 18</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>R R 13 ND ND 14</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>R R 13 R R 11</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>R R R R 15</td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>R R R R R R</td>
<td></td>
</tr>
<tr>
<td>0.125</td>
<td>R R R R R R</td>
<td></td>
</tr>
<tr>
<td>CHLOR</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
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LE – leaves; BE – brunch; FE – fruit; CHLOR – chloramphenicol. Inhibition zone diameter is expressed in millimeters (mm). The results represent the media of two to four experiments. R– resistant; ND– not done.

Figure 2. Minimal inhibitory concentration (MIC) (A and B) and minimal bactericidal concentration (MBC) (C) of *G. americana* fruit ethanolic (FE) extract against *E. coli* (A) and *S. aureus* (B, C).

related to antimicrobial activity (Taleb-Contini et al., 2003), it seems that steroids found in *G. americana* fruit ethanolic extract play an important role in microbial fight. Hence, this work emphasizes the importance of studies on medicinal plants with the purpose of obtaining no cytotoxic new molecules with biological and therapeutic potential, useful for the prevention or treatment of polymicrobial diseases.

Conflict of interest

All the authors have not declared any conflict of interest.

ACKNOWLEDGEMENTS

This work was financially supported by Universidade Estadual de Santa Cruz, Fundação de Amparo a Pesquisa da Bahia, and

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