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Research Article

Evaluation of *Clusia Fluminenses* Extract for Inhibitory Effect against DENV-2 *in Vitro* and Acute Toxicity in BALB/C Mice

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ABSTRACT

The emergence of Dengue cases in recent years in Brazil and worldwide has stimulated antiviral substances to treat this disease. This work aims to evaluate cytotoxicity and antiviral activity against Dengue type 2 (DENV-2) of *Clusia fluminensis* extract through *in vitro* tests and evaluate acute toxicity in BALB/c mice. The *C. fluminensis* acetone extract of stems (CFMCAc) was tested on Vero cells to evaluate cytotoxicity (CC_{50}), virucidal inhibitory activity (EC_{50}), and your synergism against dengue. BALB/c mice were divided into four groups, CFMCAc (500 mg/kg); CFMCAc (200 mg/kg); 1% DMSO and 1% PBS for evaluation of acute toxicity. In the CC_{50} assay, *C. fluminensis* extract showed satisfactory results above 200 µg/mL. At EC_{50} evaluation, the extract inhibited the virus, above 90%, at a 2.5 µg/mL concentration. Only histological changes were observed in the animal studies presenting spleen hyperplasia without tissue injury in the groups that received 1% DMSO (vehicle) and CFMCAc-500 mg/Kg. *C. fluminensis* stems crude extract in acetone presented good responses as antiviral. Therefore, it is promising future assays in the search for new treatment against dengue.

Keywords: Antiviral; Acute toxicity; *Clusia fluminensis* plant; DENV-2; BALB/c mice

INTRODUCTION

Dengue fever is a neglected tropical disease with the etiological agent Dengue virus (DENV). This virus is transmitted mainly by hematophagous insects of the *Aedes aegypti* and *Aedes albopictus*, distributed in tropical regions with about 50 to 100 million annual cases in the world [1,2]. The disease has complex pathogenesis, with Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS) being the most severe manifestations, corresponding to about 500 000 cases of dengue in the world [2]. International Committee on Taxonomy of Viruses [3]. DENV belongs to the *Flaviviridae* family of the genus *Flavivirus*, with four well-studied distinct serotypes (DENV-1, -2, -3 -4) [2]. In 2013, the new virus serotype (DENV-5) was confirmed after analyzing of serum samples from patients in Malaysia in 2007 [4]. The DENV viral particle contains 50 nm in diameter, and a 10 723 nucleotides RNA genome from a single Open Reading Frame (ORF) synthesizes a viral polyprotein [5]. This molecule gives rise to the structural proteins of the C-capsid, the viral envelope E, and M membrane, as well as seven non-structural proteins (NS1, NS2A, SN2B, NS3, NS4A, NS4B, NS5) [5,6]. Non-structural proteins have activity related to viral replication [2,7] in the immune response in the acute phase of infection [8,9] and the performance as serological markers for diagnosis[10]. Even today, there are no drugs approved by regulatory agencies to treat the pathology of DENV. Treatment is carried out through life-support measures (e.g., 0.9% Sodium Chloride solution), and in more severe cases, by blood transfusion due to plasma extravasation [11,12]. Thus, several compounds are being investigated to obtain substances with activity against DENV, which are effective against the virus, safe and present low toxicity in different conditions [13]. Plants are an alternative in the search for substances with antiviral activity, Brazil being one of the countries with the highest biodiversity of plant species in the world [14]. The *Clusia* genus belongs to the *Clusiaceae* family and includes species of large distribution in tropical and subtropical regions, with about 800 species cataloged [15].

The *Clusia* genus covers plants found on the Brazilian coast, which in previous studies have shown anti-ulcerative [16], anti-inflammatory [17], anti-HIV [18] activities. Analysis of extracts of *Clusia* species evidenced the presence of flavonoids and terpenoids [17,19], which are molecules with unusual anti-DENV activity already observed in previous studies [16,19,20]. Several studies have shown the action of flavonoids present in plants such as *Boesenbergia rotunda, Distictella elongate, Houttuynia cordata, Andrographis paniculata*, and *Momordica charantia*, with significant activity on DENV [21-24]. Among them, we highlight the flavonoids of *Cryptocarya chartacea,* which could inhibit above 90% of the viral activity, DENV-2, with EC_{50} of 1.8 µM [25]. These characteristics prompted us to analyze *Clusia fluminensis* extract against DENV-2, given the presence of flavonoids already described in the literature [19] and previous studies of the group with extracts of species this genus and their excellent availability in tropical regions [17,26]. The main objective of this study is to evaluate the activity of *Clusia fluminenses* extract against DENV-2 in assays of inhibition of antiviral activity and virucidal effect, as well as to analyze the acute toxicity of the extract in BALB/c mice. Future research aims to investigate the biotechnological potential of the extract components and the interaction of its active principles with the virus in the search for promising treatments against dengue since there are no specific regulated treatments against this disease.

MATERIALS AND METHODS

Extract of *Clusia fluminensis*

Stems of staminate individuals of *C. fluminensis* were collected at Forte Barão do Imbuhy, Niterói, RJ, Brazil, according to the authorization of the 21st Brazilian Army Artillery. Fertile specimens were identified by Dr. Marcelo Guerra Santos and a voucher specimen has deposited the herbarium of the Universidade do Estado do Rio de Janeiro, Faculdade de Professores (UERJ-FFP) registered under the number 9213. The activities are registered in the National Management System for Genetic Heritage and Associated Traditional Knowledge - SISGEN, under 010415/2013-0.

The plant material was dried at 40°C, fractionated, and extracted using acetone for 15 days by static maceration. The solvent was evaporated under reduced pressure, resulting in the crude acetonic extract of stems (CFMCAc). The mentioned steps were performed in the Structural and Functional Botany Laboratory at Universidade Federal Fluminense (UFF).

Virus and cells

The DENV-2 used in viral titer of 10^6 PFU/mL was provided by the Laboratory of Molecular Virology from Universidade Federal do Rio de Janeiro (UFRJ), Rio de Janeiro, Brazil. The potential virulence of DENV-2 was titrated by plaque assay using Vero cells (African green monkey kidney).

Cells were incubated in 6-well plates at 90% confluence (6 x 10^5 cells/well) in DMEM medium supplemented with 5% FBS in an atmosphere of 5% CO_2 at 37°C. After cell growth, viral supernatant was removed and diluted in DMEN in 2% PBS and added to the wells. The inoculated cells were subsequently incubated to allow the adsorption of the virus for 2 hours at 37°C. Then, 3% Carboxymethyl

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Cellulose (CMC) was added to the plates were incubated at 37°C for 3 to 5 days with daily observations or until the formation of the plate. The plates were visualized after CMC removal and staining with a crystal violet solution, and the viral titer was expressed as a plaque-forming unit (PFU/µL).

Vero cells ATCC-CCL-81 lineage were used for the in vitro tests and maintained by incubation CO2 at 37°C in 75 cm^2 culture flasks, provided by Fundação Oswaldo Cruz, Rio de Janeiro, Brazil (FIOCRUZ). These cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, cat. no 11960) supplemented with 5% Fetal Bovine Serum (FBS; Invitrogen), two mmol L−1 L-glutamine (Invitrogen, cat. no. 25030). Antibiotics were added at a final concentration of 50 units/mL penicillin and streptomycin (Invitrogen, cat. no. 15070).

Cytotoxicity assay (CC_{50})

Vero cells were incubated in 96-well plates for 24 to 48 hours until reaching a 90% confluence in DMEM medium supplemented with 5% FBS in a CO_2 atmosphere at 37°C.

C. fluminensis acetone extract (CFMCAc) was diluted in 1% DMSO and divided into aliquots at 50, 100, 200, and 400 µg/mL concentrations. These aliquots were added to a 96-well plate containing Vero and then incubated for a period of 72 h at 37°C under 5% CO_2. Then, the cells were submitted to the MTT method and evaluated on a microplate reader to determine cell viability as described in the method. Therefore, 1 mg/mL MTT (3- (4,5-dimethylthiazol-2-yl) -2,5-bromophenyltetrazolium) was added to each well, and the plates were pre-incubated under the above conditions for four hours. Finally, 100% DMSO was added for the solubilization of the crystals.

The analysis was done by spectrophotometry in 545 nm absorbance. It was considered as 100% cell viability, the wells no extract and only with DMEM medium. The presence of formazan crystals turns the solution purple, being a criterion for cell viability and analysis of the biological impact on Vero cells' cytosol and plasma membrane. The test was repeated three times, with an equal number of repetitions for each measurement. The CC_{50} test was calculated by linear regression in the Windows Office Excel program.

Evaluation of antiviral (EC_{50})

Vero cells were maintained in 24-well plates with a density of 1 x 10^5 cells/well and inoculated with DENV-2 (1 x 10^4 PFU/mL), using 0.1 MOI for a period of 2h at 37°C under of 5% CO_2. After that, the cells were treated with CFMCAc extract at concentrations of 2.5, 5, 10, 20 µg / mL, a complete medium containing 5% FBS, and then incubated for two hours at 37°C under of 5% CO_2. Treatments were performed by adding non-cytotoxic concentrations of the compounds for Vero cells after viral infection.

Subsequently, the wells were washed with Phosphate-Buffered Saline (PBS), and 400 µl of 3% Carboxymethyl cellulose was added for up to 5 days at 37°C under 5% CO_2, with the daily assessment to observe the best time to assess the formation of viral plaques, according to the protocol of [27]. Then, the plates were washed with PBS, and 10% paraformaldehyde solution was added and incubated for one hour. Then, the plates were stained with violet crystal to assess the formation of viral plaques for counting concerning the control of infected and untreated cells. The experiments were carried out in triplicate and repeated three times. The EC_{50} value was calculated using linear regression using the Windows Office Excel program.

The Vvirucidal activity of CFMCAc

The CFMCAc extract at a concentration of 5 µg / mL was incubated with DENV-2 (MOI from 0.1 to 1 x 10^4 PFU / mL) under temperature conditions of 37°C and 4°C for two hours. The temperature of 4°C was used as a control, as there is great difficulty or no binding of molecules to viral proteins that do not allow the virucidal effect, according to studies by [28]. After incubation with DENV-2, the content was added to a 24-well plate with cell density in each well of 1 x 10^5, with analysis being determined by plaque assay as described above. The test was performed in triplicate and repeated three times.

Evaluation of the additive effect of inhibiting viral activity with ribavirin and CFMCAc

CFMCAc extract was submitted to the ribavirin synergism assay. The procedure started with the adsorption of DENV-2 (1 X 10^4 PFU/mL) to the 24-well plate with a density of 1 x 10^5 cells/well for 2 hours at 37°C under 5% CO_2. Cells were submitted to the following treatments: CFMCAc 10 µg/mL; CFMCAc 0.5 µg/mL; Ribavirin 5 µM; Ribavirin 0.5 µM; 10µg/mL CFMCAc + 5 µM Ribavirin; CFMCAc 0.5 µg/mL + 0.5 µM Ribavirin. The plates were incubated under the above conditions for five days, with a daily assessment to determine the best time to stop the test. The additive effect of inhibiting antiviral activity was evaluated by the plaque assay, as previously described. The test was repeated three times, with each analysis in triplicate.

Acute toxicity assay in BALB/c mice

BALB/c mice (n = 30) females, aged around 90 days weighing between 20-30 g. The animals were divided into four groups (G1 to G4) with six animals each (n = 6/each), kept in an environment with exhaustion, light/dark cycle every 12 hours for 22-24°C in the Laboratory of Virology, Institute of Biology, Universidade Federal Fluminense. The Ethics Committee approved the Use of Animals (CEUA) of UFF by registry 311 on 9/19/2017.

The extract CFMCAc was submitted to the acute toxicity test. The administration of the substances in animals was done by gavage, a single dose of 100 µL divided into G1. CFMCAc-500 mg/kg; G2. CFMCAc – 200 mg/kg; G3. 1% DMSO and G4. 1% PBS. The mice were monitored for 14 days and weighed in two moments: on day zero (D0), before administering the substances, and on the fourteenth day of the experiment (D14).

Blood was collected on D0 and D14 for biochemical analysis of Blood Urea Nitrogen (BUN), creatinine, and Alanine aminotransferase (ALT) parameters. The animals were submitted to euthanasia using ketamine and xylazine (ratio 2:1) on the 14th day. The organs, kidney, liver, heart, and spleen were removed for histological analysis. The organs were preserved in 10% Carson formalin and processed in alcohol solution (70%, 80%, 95%, and 100%) and xylol 100% with inclusion in paraffin. Microtomes (LEICA) were cut with a thickness of 5 µm, and the tissues were stained with hematoxylin and eosin (H&E). The histological analysis was performed for the groups CFMCAc-500 mg/kg, 1% DMSO, and 1% PBS by optical microscopy (LEICA) under 200X increase, 11.7 ms saturation of 0.6 in the Immunobiology Department of the Universidade Federal Fluminense.

Statistical analyses

We used the program GraphPad Prisma 5 and GraphPad InStat software inc. One-way ANOVA with Tukey's correction and the t-test with Welsch's correction was used in the antiviral activity, virucidal and biochemical assays, with p-value < 0.05 and 95% confidence.

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RESULTS

Cytotoxicity vero cells assay (CC_{50}) and antiviral activity assay (EC_{50}) *in vitro*

The extract's linear regression curves can be seen in figure 1a. The evaluation of antiviral activity against DENV-2 *in vitro* was performed for four concentrations of the CFMCAc extract: 2.5, 5, 10, and 20 µg / mL. The results showed that the CFMCAc extract inhibited DENV-2 replication at all concentrations in values above 50%. The following values of potential inhibition of viral activity were observed in relation to the untreated control: 70% (SD = 11.6) at the 2.5 µg / mL; 85% (SD = 8.6) at the 5.0 µg / ml; 91% (SD = 3.3) at the 5.0 µg / mL, and 96% (SD = 1.0) at the 20 µg / mL, which was the one with the best inhibitory percentage (Figure 1b). The number of syncytia per plate was 145; 86; 39, and 14 for 2.5, 5, 10, and 20 µg/mL concentrations, respectively.

In our analysis with the extract in test *in vitro*, the virucidal activity and evaluation of the additive effect to inhibit the activity with ribavirin drug we obtained the EC_{50} = 1.50 µg /mL and the CC_{50} = 330.11 µg /mL with a SI = 220. According to FDA, an SI equal to or greater than 120 is considered a safe drug. The CC_{50} value obtained revealed that the extract is safe for evaluating acute toxicity in BALB/c mice.

The virucidal effect with the 5 µg/mL of the CFMCAc extract at temperatures of 37°C and 4°C presented the results of 73% (SD = 5.7) and 39% (4.9), respectively (Figure 2a). The cytopathic effect and the ability of the extract to eliminate it were observed at a temperature of 37°C.

CFMCAc extract had no additive effect when combined with ribavirin. The CFMCAc at 10 µg/mL concentration, associated 5 µM ribavirin, was 46% (SD = 2.4) inhibition potential on viral

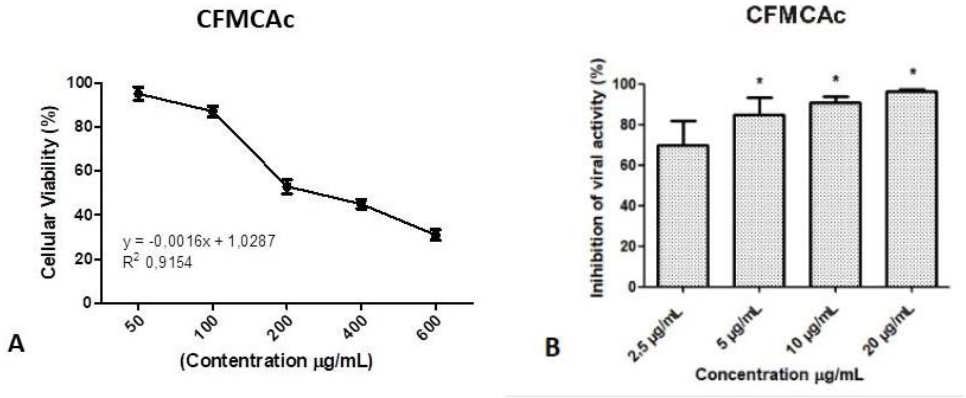

Figure 1: (a). Cytotoxicity curves of CFMCAc extract at the concentrations of 50, 100, 200,400 µg/mL, with cell viability values equal to and 98%, 88.1%, 59.8%, and 43.3%, respectively. Cytotoxicity was assessed using MTT colorimetric assay and the CC_{50} value (330.11 µg/mL) was calculated using linear regression. The cells were incubated at 37 °C under 5% CO_2.
(b). Inhibition of the antiviral activity of CFMCAc extract with DENV-2 in concentrations of 2.5, 5.0, 10, 20 µg/mL., with a percentage of inhibition equal to 70%, 85%, 91%, and 96%, respectively. A density 1 x 10^5 cells/well, inoculated with DENV-2 (1 x 10^4 PFU / mL), using MOI of 0.1 at 37° C under of 5% CO_2. After incubation with extract, the plaques assay was performed by syncytium counting under the inverted optical microscope. The EC_{50} (1.50 µg/mL) value was calculated using linear regression (*p < 0.05).

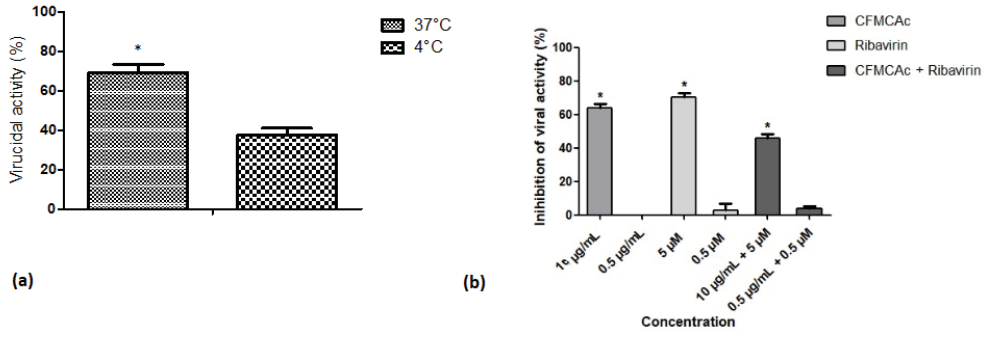

Figure 2: (a). Virucidal effect of CFMCAc extract at the concentration of 5 µg/mL against DENV-2 at 37°C and 4 °C, with resultsequal to 73% and 39%, respectively. The plaques assay was performed by syncytium counting under the inverted optical microscope.
(b). Analysis of the association effect of CFMCAc extract (concentrations 0.5 µg/ml and 10 µg/ml) and ribavirin (concentrations 0.5 µM and 5 µM) against DENV-2 *in vitro*. The potential for inhibiting viral activity for the extract, ribavirin and the additive effect of both were equal to 63.3%, 70.6%, and 46%, respectively (*p < 0.05).

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replication. The extract and ribavirin using the same concentrations showed inhibition of viral activity when incubated separately had a viral activity inhibition of 63.3% (SD = 2.5) and 70.6% (SD = 2.3), respectively. No significant effects were observed at concentrations of 0.5 µM ribavirin and 0.5 µg/mL extract (Figure 2b).

Acute toxicity assay in BALB/c mice

Weight, Biochemical Analysis and histology: The weight of the animals between the average values of D0 and D14 were for the groups CFMCAc 500 mg/kg: 24.7 g - 25.3 g; CFMCAc 200 mg/kg: 26.0 g - 26.7 g; 1% DMSO: 25.4 g - 26.0 g, and PBS: 25.0 g - 25.2 g. The weight of animals between days D0 and D14 was observed a change of 10%, with no significance between groups ($p > 0.05$), as shown in figure 3.

Biochemical analyzes of BUN (Figure 4a) and the ALT (Figure 4c) did not have any significant changes in all groups in D0 and D14 ($p > 0.05$). The values obtained for creatinine in D0 and D14 were equal to 0.6 and 0.1 mg/dL for the CFMCAc 500 mg/kg and 0.7 and 0.2 mg/dL for the CFMCAc 200 mg/kg that showed a significant reduction ($p < 0.05$) (Figure 4b).

Histological analyzes did not show significant changes in the samples of the liver, kidney, and heart organs in all groups studied. The spleen presented a change in the groups CFMCAc 500 mg/kg and 1% DMSO with white pulp hyperplasia and activation in the germinal center (Figures 5a, 5b, and 5c).

DISCUSSION

Previous studies with the species evidenced the presence of terpenoids, benzophenones, and flavonoids. It has been shown [17] the presence of flavonoids in *C. fluminensis* methanol and acetone extracts of leaves, fruits, and stems, this last one, specifically showed 9.12% of flavonoids of flavone and flavanol types.

Flavonoids represent one of the main components of *Clusia* species. These substances showed activity against the DENV in previous studies, as in the previously cited experiment as shown by [29], to determine the antiviral action of four flavonoids against DENV-2. In other work as shown by [30] have evidenced the antiviral action of isolated flavonoids from *Dacrydium* plant swing over the NS protein of DENV-2 with a value of EC_{50} near 3.12 µM.

This work made it possible to evaluate the cytotoxicity of *C. fluminensis* extract in Vero cells and subsequently in BALB/c mice. The lower toxicity is intrinsically related to the use of different solvents since solvents with more hydrophilic or more hydrophobic characteristics were employed, allowing differentiated extraction of compounds. In extracts of *C. fluminensis*, the extraction with more polar solvents, such as acetone and methanol, led to less toxic extracts than those extracted with hexane, more apolar.

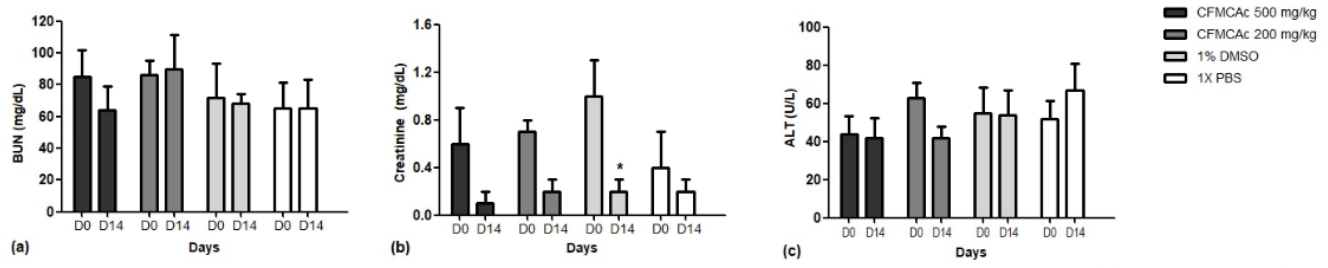

Figure 3: Analysis of the weight of the mice BALB/c in the D0 and D14 days using the average of the animals ($n = 6$) by the substances CFMCAc 500 mg/kg, CFMCAc 200 mg/kg, 1% DMSO,1X PBS ($p > 0.05$).

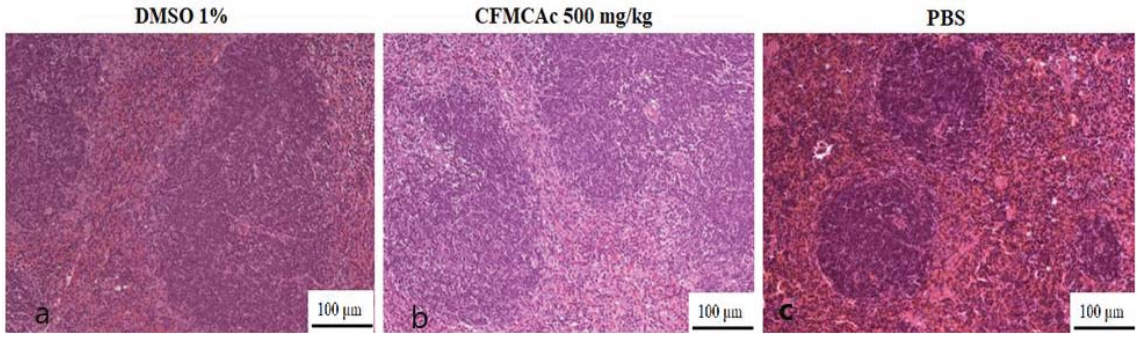

Figure 4: Biochemical analyses.
(a) BUN
(b) Creatinine
(c) ALT. Creatinine for groups CFMCAc 500 mg/kg, CFMCAc 200 mg/kg, 1% DMSO, 1X PBS on days 0 (D0 and14 (D14). Only creatinine had a reduction between D0 and D14 of 0.6 to 0.1 for the CFMCAc 500 mg/kg group and from 0.7 to 0.2 for the CFMCAc 200 mg/kg group (*$p < 0.05$).

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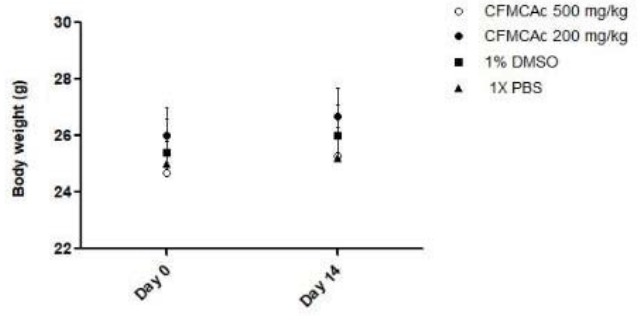

Figure 5: Spleen histology images of the groups
(a). 1% DMSO.
(b). CFMCAc 500 mg/kg showed a change as hyperplasia in the white pulp and activation in the germinal center.
(c). 1X PBS (normal control). The histological analysis was performed by optical microscopy (LEICA) under 200X increase, 11.7 ms, and saturation of 0.6.

Other studies corroborate the variation of toxic effects in cellular lineages according to the part of the extracted plant and the solvent used. The work showed [18] that extracts of leaves of *Clusia* species extracted with methanol showed greater cellular viability than those extracted with acetone and other parts of the vegetable.

The analysis of the antiviral activity, through the determination of EC_{50}, was performed for the acetonic extract of *C. fluminensis* stems (CFMCAc). The test of antiviral activity inhibition presented a great result with inhibition up to 97% in DENV-2 in the concentration of 20 µg/ml and a value EC_{50} equal to 1.50 µg/ml, indicating that a low concentration of the extract can inhibit DENV-2, under 0.1 MOI. Although previous studies have not been carried out with this extract on other viruses [31], it is known that the acetone extract of this species has a significant concentration of flavonoids and terpenoids, compounds with antiviral activity documented on DENV-2 [19].

In addition to the flavonoids, other compounds such as coumarins, terpenoids, and polyphenols extracted from plants presented activity against DENV-2, as described in other works. Studies documented the activity of isolated compounds on different extracts of the plant *Arrabidaea pulchra* with values of CC_{50} above 200 mg/mL and EC_{50} values equal to 3.2 µm for ursolic acid, a terpenoid, and from 3.4 µM to the *Verbacosida polyhay*, revealing a good activity against DENV-2 [32].

The CFMCAc extract showed a considerable virucidal effect, ranging from 73% at 37°C. Studies conducted by De Oliveira *et al.* [6] demonstrated that a great diversity of compounds present in the extract of *C. fluminensis* shows that interaction between substances can interfere with the final effect on the viral particle, which can justify the virucidal activity. Furthermore, studies have shown that the DENV particle is susceptible to conformational changes when subjected to temperatures above 35°C, thus increasing the composition with the virus [28]. Studies by [33] demonstrated that flavonoids, such as quercetin and baicalein, flavonol, and flavone, can interact with domains I and II of protein E, respectively. The flavonoids identified in CFMCAc are flavonol and flavone types expressed as rutin, quercetin heteroside [17].

CFMCAc extract, due to its good antiviral response, was subjected to the evaluation test of the antiviral activity in association with ribavirin. Ribavirin is used to treat Hepatitis C (HCV) virus infections [20], and some studies have shown their inhibitory effect on DENV. The work is shown by [34] that the association of ribavirin with mycophenolic acid could enlarge the antiviral activity over the DENV-2 compared to the individual use of the LLC-MK2 cell compound. Another study showed that the association of ribavirin with α-glucosidase presented an antiviral activity on the DENV [35].

However, the CFMCAc extract did not present a synergistic or additive effect of the antiviral activity associated with ribavirin, showing a more negligible effect than that observed when the CFMCAc extract or ribavirin was incubated individually in wells with DENV-2 infected cells. Although a significant percentage of the extract compounds of flavonoids present an excellent action on DENV-2, other extract compounds may have interacted with ribavirin and interfered with their inhibitory action on viral replication, resulting in a reduced effect antiviral activity. When the extract and ribavirin do incubate separately, the antiviral activity becomes more effective, indicating that compounds of the extract, when associated with ribavirin, can interfere in the total effect, and not show additive effect.

The BALB/c mice lineage is one of the most used for acute toxicity tests for its therapeutic response, extended periods of life, good responses in the production of monoclonal antibodies, and easy manipulation compared to other lineages [36].

The ALT enzyme is an essential parameter of liver damage associated with hepatitis and cirrhosis. The creatinine and BUN are parameters for the measurement of renal metabolism. In the present study, BALB/c mice did not indicate hepatic damage between the evaluated groups. An increase in the parameters in the group administered with an exogenous substance compared to the controls (1% DMSO and 1X PBS) was not considered significant and is following the observed in other studies of acute toxicity with plants in BALB/c mice [37-39]. The reference values for the biochemical parameters in BALB/c mice are not well standardized. Different experimental and behavioral conditions are variables (such as age, sex, genetic variety, among others) that can interfere with the observed results [39].

In their studies, the reference values for biochemical parameters in BALB/c mice for BUN are between 20-45 mg/dL and for creatinine of 0.2-0.7 mg/dl, values close to those obtained in this work [40]. The work shown by [41] found reference values for BALB/c mice for ALT of 36.5 ± 7.45, values close to that found in our studies for untreated groups.

The variations observed between days 0 and 14 in some biochemical parameters for CFMCAc at 200 and 500 mg/Kg were reduced. These variations may be due to the natural metabolic process of eliminating the substance by the mice organism, decreasing its plasma concentration over time. This characteristic was evident for the CFMCAc extract, where the ALT values were higher in the lowest concentration administered to the animals (D0: 62.5 U/L; D14: 44 U/L).

The weight body between the groups was not significant ($p < 0.05$), obtaining a minimum increase of 10% of weight when compared to the initial [42].

The results of the histological analysis showed no changes in the kidney, liver, and heart in all groups. The results corroborated the studies conducted by [39] with the extract of the *Euphorbia hirta* and as shown by [36], where they evaluated the effect of the sesquiterpene *Zerumbone*.

The spleen tissue showed activation of the germination center and hyperplasia of the white pulp, characteristic of cellular proliferation. This result was observed in CFMCAc 500 mg/kg and 1% DMSO groups to the detriment of the group administered with 1X PBS saline solution. This observation allows the assumption that the white pulp hyperplasia may be due to the presence of the solvent DMSO instead of the extract. Since the architecture of the spleen for CFMCAc 500 mg/kg and 1% DMSO groups were quite similar in structural terms, the tissue has not presented damage at the cellular level, besides the absence of changes in the PBS group. Some studies revealed the dynamic organization of the germination center of the spleen, revealing that the same is variable between the light and dark areas of the tissue, with the cellular proliferation of lymphocytes B variable in up to 72 hours, at different locations of the organ. An immunohistochemical study for surface proteins of the spleen could be more specific to determine the presence of some component of the extract that could induce this proliferation. However, tissue cells did not present morphological alterations [43].

CONCLUSION

The results obtained by *Clusia fluminensis* acetone extract of the stems (CFMCAc) in the tests done *in vitro* and *in vivo* showed sound antiviral and virucidal effects against the DENV-2. In BALB/c mice, it presented a low level for acute toxicity according to [44]. These characteristics make this extract promising for future assays for analyzing its components and evaluating the binding of its active principles to the virus in the context of research into new dengue treatment options.

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