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In vitro cytotoxic and anti-herpesvirus properties of jackfruit (*Artocarpus heterophyllus* Lam., Moraceae) leaf extracts

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The jackfruit tree (*Artocarpus heterophyllus* Lam.) is considered one of the most versatile trees and is used from wood production to traditional medicine. In traditional medicine, leaves are used to treat asthma, wounds and abscesses, and prevent ringworm infection. Several biological activities have been documented for jackfruit extracts confirming its popular use; however, its *in vitro* cytotoxicity and anti-herpesvirus activity are rarely discussed. The objective of this study was to explore the *in vitro* anti-herpesviral potential of *A. heterophyllus* leaf extracts at non-cytotoxic levels. Leaves were obtained from specimens located in the Atlantic forest biome. Dried and fresh ethanolic, methanolic and hexanic leaf extracts were obtained from the partition of a crude ethanolic extract. The cytotoxicity was performed on erythrocyte and kidney cell lines. The antiviral test was against equine, suid, and bovine herpesviruses. The contents of phenolic compounds were established from methanolic leaf extracts. Fresh leaf extracts did not show toxicity to erythrocytes in any concentration tested; however, dry leaf extracts showed >10% of hemolysis at ≥200 µg/ml. The maximum non-cytotoxic concentrations for Vero and MDBK cells ranged from 7.8 to 125 µg/ml. The content of phenolic compounds was 22.02% higher in fresh leaf than in dry leaf methanolic extracts. Methanolic extracts showed antiviral activity against BoHV-1 (PI of 99.20%) and SuHV-1 (PI of 94.38%). *A. heterophyllus* fresh and dry leaf extracts are remarkable as anti-herpesviruses and candidates to proceed for *in vivo* tests.

Key words: *Artocarpus heterophyllus* Lam., Jackfruit tree, antiviral activity, kidney cell-line, erythrocyte, medicinal plant.

INTRODUCTION

Artocarpus heterophyllus Lam. (Moraceae), commonly known as the jackfruit tree, is native to India and cultivated in tropical and subtropical regions (Prestes, 2000). Jackfruit is a versatile species with all its parts recommended in folk medicine for their therapeutic qualities and is also considered a functional food (Baliga

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et al., 2011; Swami et al., 2012). Specifically, the leaves are used to treat asthma, prevent ringworm infestation, heal cracking feet, wounds and abscesses (Swami et al., 2012), pain and ear problems (Morton, 1987; Elevitch and Manner, 2006).

Pharmacologically, the biological potential of *A. heterophyllus* leaves has been identified in antibacterial activity (Khan et al., 2003; Loizzo et al., 2010; Cavalcante et al., 2013), hypoglycemic effect (Fernando et al., 1990, 1991) and anti α-amylase activity (Kotowaroo et al., 2006). Moreover, the phytochemical characterization of jackfruit leaves revealed that different parts of the jackfruit tree, including its leaves, have a high content of phenolic compounds (Ojwang et al., 2018) of which antiviral (Chávez et al., 2006; Daglia, 2012) and antioxidant (San Miguel-Chávez, 2017) potentials have been associated.

The toxicology of *A. heterophyllus* extracts has also been a point of concern. A nontoxic effect of its leaves (Patel and Patel, 2011), petiole (Kurian et al., 2018), and seeds (Burci et al., 2018) has been reported. In this regard, several studies reported in the literature for *A. heterophyllus*, until now, have rarely discussed its leaves processing and solvent fractionation influence on its biological activity.

Herpesviruses constitute a common virus family causing disease in a wide range of hosts (Woźniakowski and Samorek-Salamonowicz, 2015). The Herpesviridae family encompasses viruses with similar morphological, cell tropism, and latency characteristics (Dezengrini et al., 2010; Phelan et al., 2017). The suid alphaherpesvirus type 1, for example, shares characteristics with the human alphaherpesvirus types 1, 2 and 3 (ICTV, 2019). These characteristics and the fact that they are DNA viruses make animal herpesviruses suitable for *in vitro* experimental models on human herpesviruses (Dezegrini et al., 2010) and other DNA viruses in the search for antiviral drugs (Barros et al., 2012).

Two main problems can be encountered concerning herpesvirus infection: latency and antiviral drug resistance (Coen et al., 2014). Particularly, the selection of DNA polymerase resistant to acyclovir and penciclovir (Andrei and Snoeck, 2014) makes alternative substances to override difficulties in the control of herpesvirus infection. In this context, plant metabolites have been considered as viable alternatives.

Thus, the objective of the present work is to demonstrate non-cytotoxic concentrations of dried and fresh jackfruit leaf ethanolic extract fractions with anti-herpesviral potential.

MATERIALS AND METHODS

Plant and extract preparation

Leaves of *A. heterophyllus* were collected from specimens located in the Atlantic forest biome along the BR 415 Jorge Amado Road, in Ilhéus, Bahia (14°47'33" S and 39°11"0' W) in the morning of the 20th of November, 2017. The plant material was identified by a botanist and registered under voucher number HUESC23705 at the Herbarium of the State University of Santa Cruz. In compliance with Brazilian Genetic Heritage and Associated Traditional Knowledge Management (Law 13.123/2015), the research was registered under access number A834C3A.

For dry material production, the leaves were dried under forced ventilation (Quimis® Q317M-12, Diadema, Brazil) at 50°C until constant mass. To produce extracts, the liquid-liquid extraction with clean up by precipitation at low temperature described by Goulart et al. (2008) was used. To do this, 500 g of dried and powered material was subjected to ethanolic extraction (1.0 L) for 72 h, with the mixture sonicated every 24 h for 1 h with Ultra-Sonic equipment (Unique Ultrasonic Cleaner: USC3380A, Unique, Brazil). Then, the liquid was filtered and transferred to a volumetric flask. The plant material was resubmitted for extraction two more times. All the combined filtrated materials were concentrated in a rotary evaporator (SOLAB: SL-126), providing 162.5 g of dark solid extract, called crude extract (cEtOH).

The cEtOH (10 g) was resuspended in 200 mL of EtOH/H_2O (1:2), homogenized and, after 12 h, the insoluble residue was filtered. The hydroalcoholic solution was fractionated with hexane (QHEMIS, Jundiaí, Brazil), chloroform (QHEMIS, Jundiaí, Brazil), ethyl acetate (QHEMIS, Jundiaí, Brazil) and methanol (QHEMIS, Jundiaí, Brazil) as solvents. This experimental step was performed three times. After mixing each solvent residue, fractions yielded 3.05 g (fHex), 3.87 g (f$CHCl_3$), 3.53 g (fEtAc), and 2.02 g (fMeOH).

For fresh leaf extraction, all procedures described earlier were the same and extracts yielded 2.89 g (fHex), 2.08 g (f$CHCl_3$), 2.59 g (fEtAc), and 2.56 g (fMeOH). For this sudy, only fMeOH, fEtOH and fHex were used.

A stock solution of 10 mg/ml fractions was obtained using 0.15% dimethylsufoxide (DMSO, SYNTH, Jundiaí, Brazil) in water solution solvent. Solutions were filtered with 0.22 µm pore membrane (Kasvi, São José do Pinhais, Brazil) and aliquots of 1 ml were stored at -8°C until use.

Cytotoxicity

Hemolysis assay

Fresh erythrocytes were obtained from 4 ml of equine blood collected by venipuncture and transported in an ethylenediaminetetraacetic acid (EDTA) containing tube (Vacuplast; CRAL, SP, BR). Collection was made at the Veterinary Hospital of the State University of Santa Cruz, Ilhéus, Bahia, following the Ethics Committee on Animal Experimentation guidelines and approval (CEUA-011/18). The total blood was centrifuged at 2,525×g (Rotanta 460R Hettich, Nova Analítica, São Paulo, Brazil) for 10 min and a 2% erythrocytes dilution was prepared with 0.9% NaCl solution.

The hemolysis assay followed the technique described in Fischer et al. (2003) and Barbosa et al. (2012) with adaptations. To 500 µl of 2% erythrocyte solution, 50 µL of each fraction at final concentration of 7.8, 15.2, 31.2, 62.2, 125, 250, 500, and 1000 µg/ml were added. To calculate the hemolysis percentage, a standard curve was prepared using Triton X-100 from 0.001 to 20%. The 0.9% NaCl solution was used as blank. After an incubation period of 30 min at 37°C, solutions were centrifuged at 2525×*g* for 10 min. The supernatant was transferred to 96 well plates (200 µL each well) and 545 nm wavelength absorbance was determined using Microplate Reader equipment (EZ Read 4000, Biochrom, Nova Analítica, São Paulo, BR).

The hemoglobin release percentage was determined by Triton X-100 standard curve interpolation through exponential one phase decay at 95% confidence interval. Three independent tests were

made in triplicate. Statistical analysis was performed by one-way analysis of variance (ANOVA) with Tukey's post-test using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Significance was considered at $p<0.05$.

Non-cytotoxic concentrations were considered when hemoglobin release percentage was ≤10% (Fischer et al., 2003).

Maximum non-cytotoxic concentration

African green monkey kidney (Vero, ATCC® CCL-81™) and Madin-Darby bovine kidney (MDBK, ATCC® CCL-22) cell lines were used. They were maintained in Eagle's essential medium (EM) supplemented with 10% fetal calf serum (FCS).

To determine the maximum non-cytotoxic concentration (MNCC), 3.0×10^4 cells were seeded in sterile 96 well plates and incubated for 24 h at 37°C, and 5% CO_2. After the incubation period, the supernatant was discarded and 100 µL of serial dilutions of plant fractions (7.8, 15.2, 31.2, 62.2, 125, 250, 500, and 1000 µg/ml) were added to wells in triplicate (Barbosa et al., 2012). Cell morphology was observed daily using an inverted light microscope (TCM400, Labomed, Fremont, CA, USA) for three days. The MNCC was considered at concentrations where no signs of cytotoxicity (death, wrinkling or reduction in cell confluence compared to control) were seen. The experiments were repeated three times.

Anti-herpesviral activity

Three animal alphaherpesviruses were used to test anti-herpesviral activity: equine alfaherpesvirus 1 (EHV-1) A4/72 strain, suid alfaherpesvirus 1 (SuHV-1) EMBRAPA: BRMSA 3, 00588 strain, and bovine alfaherpesvirus 1 (BoHV-1) Los Angeles strain. The equine and suid herpesviruses were inoculated in Vero cells and the bovine virus was inoculated in MDBK cells.

Twenty-four hours after seeding, Vero and MDBK cell monolayers were treated with fractions (100 µl) at their respective MNCC followed by logarithmic dilutions (10^{-1} to 10^{-7}) of each corresponding virus (50 µl). Controls consisted of untreated infected (virus titer), treated non-infected (cytotoxicity control) and untreated non-infected (cell control) cells. The tests were done in triplicate and repeated twice. The antiviral activity was determined by the reduction of virus titers through the Reed and Muench method (Reed and Muench, 1938) to establish 50% tissue culture infective dose (TCID50). The difference in viral titre between treated and untreated control cultures was expressed as the viral inhibition index (VII) (Nishimura et al., 1977). Also, the inhibition percentage (IP) was calculated using the anti-logarithmic TCID50: PI = (1 - antilogT / antilogC) × 100; where T corresponds to the virus titre treated cells and C is the viral titre of the untreated cells (Nishimura et al., 1977; Koseki et al., 1990). The effective antiviral activity was considered when IP was ≥93% (VII≥1.2) (Felipe et al., 2006).

Phenolic compounds quantification

The total phenolic compounds quantification was done using the Folin-Ciocalteau reagent (Ruiz and Alarcón, 2012). First, the standard curve of gallic acid was established. In a 10 ml tube, covered with aluminium foil to protect against light, a solution containing the Folin Ciocalteu reagent (500 µl) and $NaHCO_3$ (1 ml) was added to a 500 µL of gallic acid (Sigma-Aldrich®) at different concentrationS (5 to 25 µg/ml) in water to make up 8 ml final volume. Samples were homogenized occasionally for 25 min using UV/Vis 1600UV (Novainstruments Equipamentos Ltd, Brazil) spectrophotometer at 773 nm wavelength.

The same procedure was conducted with dried and fresh leaf MeOH fractions (500 µl) from a stock solution of 1 $mg\cdot ml^{-1}$. Methanol (500 µl) (Anidrol®), the Follin Ciocalteu reagent and $NaHCO_3$, at the same concentration earlier mentioned, were used as blank. The phenolic quantification was based on Mo^{+6} from Folin Ciocalteau oxidation by reacting with the phenol group, which confers a green color to thesample.

All analyses were done in triplicate with median and standard deviation determined. In addition, the variation coefficient percentage (CV %) of methanol was calculated. Detection limit (DL), quantification limit (QL), linear regression and determination coefficient (r^2) (Thompson et al., 2002) were also obtained. An independent T test was used to compare the phenolic content of fresh and dried fractions.

RESULTS AND DISCUSSION

A. heterophyllus has been considered as a form of treatment in various diseases. Specifically, alcoholic derived dried leaf extracts showed evidence of wound healing properties (Gupta et al., 2009). The present study reports the antiviral action of alcoholic fractions of *A. heterophyllus* dry and fresh leaves on epithelial tropic herpesviruses at non-cytotoxic levels.

First, cell damage visualization and MNCC determination were done on Vero and MDBK cells. Morphological damage consisted of wrinkling, rounding, and reduced cell confluence (Figure 1). Methanolic fraction of fresh leaf ethanolic extract (fl-fMeOH = 125 µg/ml) was the least cytotoxic fraction for both types of cells (Table 1).

The kidney derived cell lines used in this study showed different sensitivity to each extract, with MDBK cells, in general, being more sensitive. Cell transformation, characteristic of cell lines, leads to changing regulatory network and consequently cell regulation alteration (Lopes-Ramos et al., 2017) which implies a different reaction of the cell to exogenous substances. Thus, although being from the same tissue origin, it is mandatory to perform a cytotoxic assay prior to *in vitro* natural products test in each particular cell type.

The hemolytic effect of fresh and dry leaf fractions was also evaluated. Through this test, differences were seen between leaves processing (Figure 2). All fresh leaf fractions presented hemolysis percentage <10%, including the higher 1000 µg/ml. In contrast, dry leaf fractions showed <10% of hemolysis in concentrations under 200 µg/ml. Moreover, significant difference ($p<0.5$) in hemolysis percentage was observed between dried and fresh leaf fractions at 1000 µg/ml where fractions obtained from dry leaves caused 85% hemolysis and from fresh leaves fractions, only 5%.

The difference in results seen for the same solvent partition (that is methanolic extracts) but different leaf processing before extraction is important. This phenomenon may be due to the concentration of toxic substances by reduced enzymatic degradation and loss of active compounds (Costa et al., 2005).

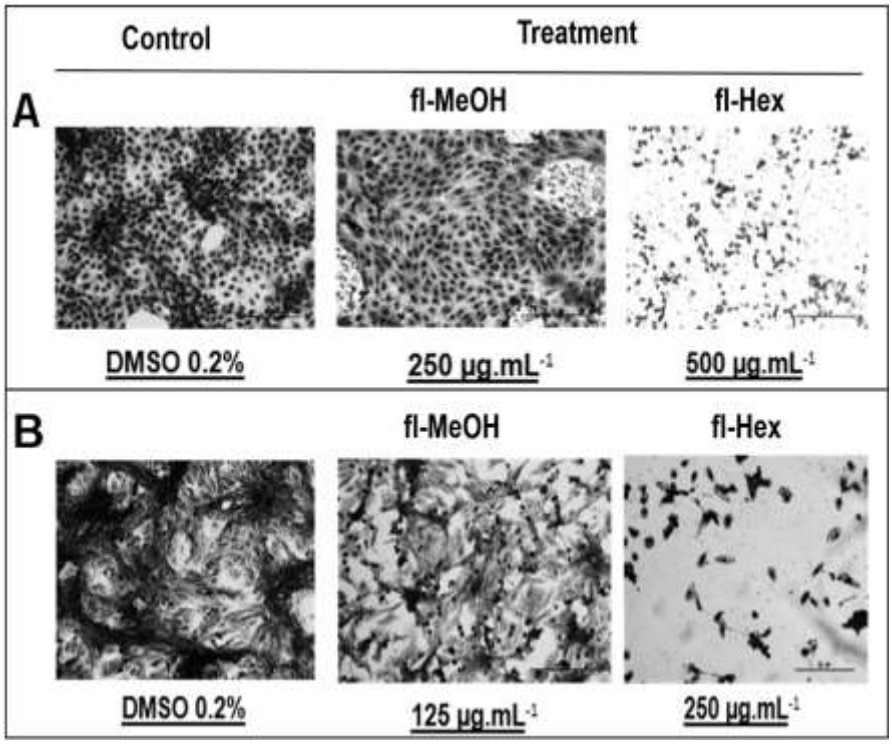

Figure 1. Morphological aspects of Vero (A) and MDBK (B) cells treated with *A. heterophyllus* flMeOH and fl-Hex at maximal non cytotoxic concentrations and high toxic concentrations. Control**,** Cells treated with dimethyl sulfoxide (DMSO) at 0.2%; fl-MeOH**,** fresh leaves methanolic extract; fl-Hex**,** fresh leaves hexane extract; Accentuated cell confluence reduction and wrinkled round cells and loss of cytoplasm in high toxic doses are visualized. 400× magnification.

Table 1. Maximal non cytotoxic concentration (MNCC) and anti-herpesvirus activity of *A. heterophyllus* fresh and dry leaves ethanolic extracts.

Extract	VERO	MDBK	EHV-1		SuHV-1		BoHV-1	
	MNCC (µg/ml)		VII	IP (%)	VII	IP (%)	VII	IP (%)
dl-MeOH	31.2	15.6	0.38	58.31	1.25	**94.38**	0.26	45.04
dl-EtOH	7.8	7.8	-0.5	-	0.38	58.31	0.75	82.22
dl-Hex	31.2	15.6	0.12	24.14	0	0	0.63	76.56
fl-MeOH	62.5	**125**	-0.13	-	0	-	2.12	**99.20**
fl-EtOH	31.2	7.8	0.62	76.01	0	-	0.26	45.04
fl-Hex	15.6	15.6	0.24	42.46	0.13	25.87	0.37	57.34

VERO, African green monkey kidney (Vero) cells; MDBK, Madin-Darby bovine kidney cells; EHV-1, equid herpesvirus 1; SuHV-1, suid herpesvirus 1; BoHV-1, bovine herpesvirus 1; VII, viral inhibition index; IP, inhibition percentage. (–), not able to calculate. dl-MeOH, dry leaves methanolic extract; dl-Hex, dry leaves hexane extract; dl-EtOH, dry leaves ethanolic extract; fl-MeOH, fresh leaves methanolic extract; fl-Hex, fresh leaves hexane extract; fl-EtOH, fresh leaves ethanolic extract.

Also, substances such as tannins, already described for jackfruit leaves (Bhattacharjee and Dutta, 2013) and for which *in vitro* cytotoxicity has been reported (Silva et al., 2014), may be exposed to hydrolysis during the extraction method and have its affinity to the solvent changed (Sieniawska and Baj, 2017).

Particular attention must be given to the methanolic extract of *A. heterophyllus* dry leaves. The *in vivo* study demonstrated that this fraction did not cause death or physiological alterations (Bhattacharjee and Dutta, 2013). However, in the present study, dl-MeOH revealed high toxicity to equine erythrocytes, which is important to consider by performing other tests, more detailed fraction partition, and a phytochemical study before proceeding to

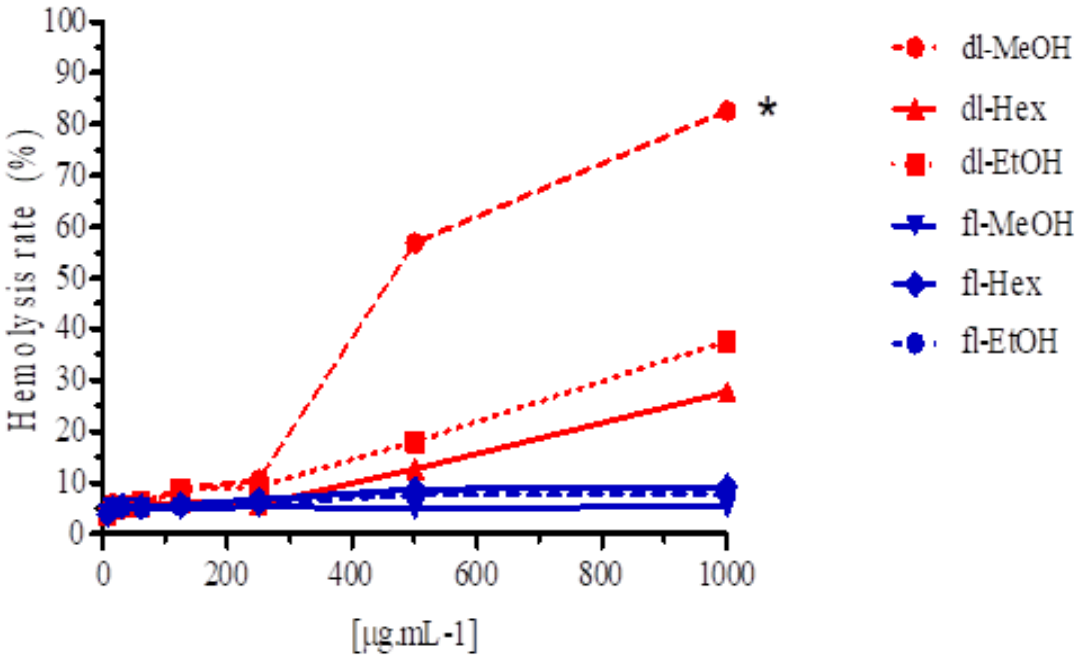

Figure 2. Hemolytic evaluation of *A. heterophyllus* leaves extracts. fl-MeOH, Fresh leaves methanolic extract; fl-Hex, fresh leaves hexane extract; fl-EtOH, fresh leaves ethanolic extract; dl-MeOH, dry leaves methanolic extract; dl-Hex, dry leaves hexane extract; dl-EtOH, dry leaves ethanolic extract. Independent T test was used to compare fresh and dried fractions phenolic compounds content (*p<0.05).

in vivo tests.

Further, dry and fresh leaf methanolic extracts may have potent antiviral substances since both extracts were highly active against two of the three viruses tested in our study, BoHV-1 (IIV=2.12; PI 99.20%) and SuHV-1 (IIV=1.25; PI 94.38%). As mentioned in the literature (Saavedra et al., 2010), in general, methanolic solvent carries phenolic molecules such as flavonoids and flavanols besides glucosinolates derivatives such as isothiocyanates and saponins. Such classes of substances are highly active biologically (Pereira and Kaplan, 2013), particularly as antiviral agents (Simões et al., 2010).

In the present study, phenolic compounds were measured in methanolic extracts from dry and fresh leaves since more relevant results were obtained with these extracts. A high significant difference (p = 0.0161 at 95%) between leaf processing was seen. Fresh leaf phenolic compounds content was 2337.52±6.09 µg/g of gallic acid equivalent (GAE) and dry leaf methanolic extract content was 514.85±1.75 µg/g GAE. The reduction by 22.02% of phenolic compounds content during dryness at low temperature (55°C) may be due to phenolic compounds degradation by enzymatic reaction, even though it is reported in the literature that controlled heating process can prevent such occurrences (Costa et al., 2005).

Although scarce, the antiviral potential of *A. heterophyllus* has been reported in the literature. The isolated substance (jacalin) showed anti-human immunodeficiency virus activity (Tamma et al., 2006) and, more recently, the dichloromethane extract from leaves of *A. heterophyllus* was highly active (selective index > 134.8) against hepatitis C virus (Hafid et al., 2017). Hence, the present study contributes to knowledge by revealing the antiviral activity of the methanolic fraction independently of leaf processing before solvent extraction against animal herpesviruses.

In conclusion, the data reported in this study show the *in vitro* anti-herpesvirus effect of *A. heterophyllus* leaf extracts at a safe level to cells. Furthermore, the use of methanolic fraction from jackfruit fresh leaves seems to be more profitable than dry leaves and further research on the mechanism of action, isolation of molecules and *in vivo* tests is required. Overall, the results add value to the jackfruit tree as a functional food.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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