**UNIVERSIDADE FEDERAL DE UBERLÂNDIA**
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# LECTINA DE LIGAÇÃO À MANOSE INIBE A INFECÇÃO PELO VÍRUS CHIKUNGUNYA

# "MANNOSE-BINDING LECTIN IMPAIRS *CHIKUNGUNYA VIRUS* INFECTION"

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Trabalho de Conclusão de Curso apresentado como requisito para obtenção do título de Bacharel em Biomedicina na Universidade Federal de Uberlândia.

Orientadora: Profa. Dra Ana Carolina Gomes Jardim

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Me. Poliana Gomes da Silva

# MANNOSE-BINDING LECTIN IMPAIRS *CHIKUNGUNYA VIRUS* INFECTION

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**ABSTRACT**

*Chikungunya virus* (CHIKV), the etiological agent of chikungunya fever disease, is mainly transmitted by *Aedes aegypty* and *A. albopictus* mosquitoes. Symptoms of the disease include fever, join pain, as well as arthralgia and polyarthalgia, which can progress to a chronic condition for months or years. There are still no specific antivirals approved to treat CHIKV infection and, therefore, it currently threats public health systems. In this context, lectins isolated from plants have demonstrated diverse biological activities, providing an interesting source of molecules with antiviral activity, due to their ability to bind to specific sugar regions on viral particles, however, there is a lack of data for the understanding of the effects of lectins against CHIKV replication. Here the anti-CHIKV activity of four lectins isolated from different plants from the northeastern region of Brazil (ConA; PPL; MaL; and VML) was evaluated. For this, all lectins were screened towards their cytotoxicity in Syrian golden hamster kidney cells (BHK-21) at concentrations of 50, 10 and 2 µg/mL using cell viability assay after 16h. The highest non-cytotoxic concentration of each lectin was selected to evaluated their effect on CHIKV replicative cycle, employing a CHIKV infectious clone carrying the nanoluciferase gene (CHIKV-*nanoluc*). All lectins were tolerated by cells at 50µg/mL, but only ConA was active against CHIKV-*nanoluc* inhibiting virus replication by 96%. The ConA inhibition profile and cytotoxicity were further evaluated performing a dose-response assay in which resulted in a selectivity index of 14.71. Therefore, our data demonstrate that ConA has a potent antiviral activity against CHIKV. Further experiments are needed to be performed to investigate the mechanism of action of ConA, and also for a better understanding of its interactions with viral proteins. Finally, the data presented here can be used as a basis for future research that seeks to develop antiviral drugs against CHIKV.

**Keywords:** Antiviral, Chikungunya Virus, Lectins, Natural compounds.

## INTRODUCTION

CHIKV is the etiological agent of chikungunya fever (Cavalcanti *et al*., 2022), a disease characterized by acute symptoms such as high fever, myalgia, nausea, headaches, and severe joint pain (Bartholomeeusen *et al*., 2023; Battisti; Urban; Langer, 2021; Kumar *et al*., 2021). Although most cases are self-limited, in more recent outbreaks, atypical manifestations of CHIKV fever have been reported including encephalitis, meningitis, and Guillain-Barré syndrome (Pereira; Franca, 2023). The acute phase of the disease can self-resolve after 7-10 days after symptoms onset, however, in approximately 40% of the patients the infection progress to a chronic phase that prolonged arthralgia and polyarthralgia can last for months to years (Vu; Jungkind; Labeaud, 2017; Marques *et al*., 2017; Amaral; Bilsborrow; Schoen, 2020).

CHIKV belongs to the genus *Alphavirus* and family *Togaviridae* (Kril *et al.*, 2021). It is a virus constituted of a positive single-stranded RNA genome, associated with an icosahedral capsid of viral protein, and evolved by the viral envelope (Haese; Poderes; Streblow, 2020). Its genome possesses approximately 12kb in length with two open reading frames (ORFs), being the first on the 5' untranslated region that encodes four non-structural proteins: nsP1 to nsP4; and the subgenomic ORF encoding six structural proteins: capisd (C), envelope proteins (E1, E2 e E3), ion channel forming protein (6K), and transframe protein (TF) (Policastro *et al.*, 2022; Santos *et al.*, 2021; Yin *et al.*, 2019;).

Genetics and phylogenetic studies classified CHIKV variants into three genotypes: West Africa (WA), East/Central/South African (ECSA), and Asian (Cavalcanti *et al.*, 2022). Its transmission occurs mainly by the bite of *Aedes aegypti* and *Aedes albopictus* mosquitoes (Constant *et al.*, 2021; Simon *et al.*, 2023), however, other species harbor the capacity of carrying the virus, such as *Aedes fluviatilis, Aedes taeniorrvinchus* and *Wyeomyia bourrouli* (Ximenes *et al*., 2020).

CHIKV was first isolated from the serum of an infected individual during an outbreak of an unknown disabling arthritic disease between 1952 and 1953 in Nawala District, Tanzania (Cavalcanti *et al*., 2022; Sharif *et al*., 2021). Since its identification, viral transmission was identified in India, Italy, France, and Southeast Asian countries (Vairo, 2019; Kril *et al*.; 2021). In the Americas, it has been representing a public health problem since 2014, when about one million cases were notified (Cavalcanti *et al*., 2022). Brazil is the country with the highest number of cases currently reported, with more than 565.000 confirmed cases and 368 deaths

from 2018 to 2023 (Ministry of Health, 2023; Pan American Health Organization, 2024). To date, the predominant strain in Brazil is ECSA of the ECSA-American sub-lineage (de Souza *et al*., 2024). Recently, an epidemiological study on CHIKV distribution in Brazil was conducted and revealed that a new lineage of the ECSA genotype was responsible for causing a new outbreak in the northeast region of the country in 2022 (de Souza *et al*., 2023).

Vector control and the use of repellents are the main alternatives to prevent and contain viral transmission, (Vairo *et al*., 2019; Cavalcanti *et al*., 2022). Furthermore, there are four vaccines in the phase three of development: VLA1553 (Roques *et al*., 2022), PXVX0317 (Raju *et al*., 2023), V184 (Rossi *et al*., 2019) and BBV87 (Cherian *et al*., 2023). VLA1533 has already received a license for application in 2022 by the FDA (Food and Drug Administration) in the United Stated of America (USA) (McMahon *et al*., 2023; Ly, 2024).

Despite the high prevalence of CHIKV on several continents, direct acting antiviral molecules to impair viral replication have not been approved yet (Kovacikova; Hemert., 2020). Therefore, the symptoms in infected patients are managed employing analgesics and non-steroidal anti-inflammatory drugs, but these therapeutically options have presented limited effectiveness (Cai *et al*., 2023; Santos *et al*., 2021). In this sense, identifying molecules that can abrogate viral replication and can be further capitalized into new chemical species is essential to combat Chikungunya Fever.

Flora is represented by a sustainable and immeasurable source of molecules for the development of novel drugs due to their anti-inflammatory, antimicrobial, and antioxidant properties that possess diverse effects on biological systems (Bakare *et al*., 2022; Borges *et al*., 2019; Hu *et al*., 2021; Kubica *et al*., 2020; Periferakis *et al*., 2022; Nieto; Rosa; Castilho, 2018). Interestingly, several molecules isolated from different plants have been extensively explored for evaluation of antiparasitic, antifungal and antimicrobial activity (Newman; Cragg, 2020, Valério *et al*., 2023), and more importantly, antiviral activities against a range of viruses, such as the *Mastadenovirus humano* (HAdV) (Bidart *et al*., 2023), *Orthoflavivirus denguei* (DENV) (Jain *et al*., 2020), CHIKV (Freitas *et al*., 2022, Martins *et al*., 2020), *Orthoflavivirus flavi* (YFV) (Wani *et al*., 2021), *Hepacivirus hominis C* (HCV) (Jardim *et al*., 2018; Shimizu *et al*., 2017; Jardim *et al*., 2015), and *Orthoflavivirus zikaense* (ZIKV) (Ramos *et al*., 2022; Silva *et al*., 2022).

Among the natural molecules, lectins are proteins that bind to specific carbohydrate structures in a reversible manner, capable of recognizing and interacting with glycoproteins present on the viral envelope, interfering with the potential to the pathogens to infect host cells (Wang *et al*., 2021; Tsaneva; Van Damme, 2020). There are several lectins from nature, specifically from plants, capable of inhibiting the infection by a variety of viruses (Mitchell; Ramessar; O'keefe, 2017; Ekowati *et al*., 2017; Vanderlinden *et al*., 2021). From those, the lectin from the banana tree *Musa acuminada* (BanLec), which has specificity for mannose, was reported with antiviral activity against *Human immunodeficiency virus 1* (HIV-1) (Swanson *et al*., 2010) and *Influenza A virus* (H1NI and H3N2) (Cóves-Datson *et al*., 2020). In other works, the lectin ConBR, derived from *Canavalia brasiliensis*, a plant found in the Brazilian flora, was capable of inhibiting *Severe Acute Respiratory Disease Coronavirus 2* (SARS-CoV-2) (Grosche *et al*., 2023) and HIV (Gondim et al., 2019), due to its specificity for mannose sugar. Additionally, the lectin isolated from *Momordica charantia* with specificity for galactose and N-acetylgalactosamine was also described to possess activity against HIV (Meng *et al*., 2012), *Influenza A* (Pongthanapisith *et al*., 2013), and SARS-COV-2 (Ogidigo *et al*., 2022; Adedayo; Famuti, 2023).

Taking into consideration the biological properties of lectins isolated from plants, and the lack of knowledge of their activity against CHIKV, here we investigated the antiviral potential of the lectins isolated from *Canavalia ensiformis* (Concanavalin A: ConA) (Araújo-Filho *et al*., 2010)*, Parkia platycephala* (*P. platycephala* lectin: PPL) (Silva *et al*., 2019), *Machaerium acutifolium* (*M. acutifolium* lectin: MAL) (Dias *et al.*, 2020)*,* and *Vatairea macrocarpa* (*V. macrocarpa* lectin: VML) (Véras *et al*., 2022), on CHIKV replication.

## MATERIAL AND METHODS

### Isolation and purification of Lectins

Seeds from the plants *Canavalia ensiformis, Parkia platycephala*, *Machaerium acutifolium,* and *Vatairea macrocarpa* located in northeastern Brazil were collected and purified as previously described (Dias *et al*., 2020; Silva *et al*., 2019; Véras *et al*., 2022). Briefly, the seeds were ground into a fine powder, aliquoted in 5 grams and further diluted in 50 mL of 150 mM NaCl and incubated at 25ºC for 4h under continuous agitation. The solution was centrifuged at 10.000× *g* at 4°C for 20 minutes to collect the proteins solubilized in the supernatant. The supernatant was submitted to affinity chromatography using

a Sephadex-G50 (Sigma, Saint Louis, MO, EUA) column (2 x 20 cm) and DEAE-Sephacel$^{TM}$ (2 x 5 cm) equilibrated with 100mM NaCl to purify the lectins. The purity of the lectins ConA, PPL, MAL and VML was confirmed in sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), as previously published by our research group (Dias *et al*., 2020; Silva *et al*., 2019; Véras *et al*., 2022). In the tests carried out, lectins were diluted in Phosphate Buffered Saline (PBS).

**Cell culture**

BHK-21 cells (fibroblasts derived from Syrian golden hamster kidney; ATCC CCL-10), were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 100U/mL penicillin (Gibco Life Technologies), 100mg/mL streptomycin (Gibco Life Technologies), 1% (v/v) non-essential amino acids (Gibco Life Technologies) and 10% (v/v) Fetal Bovine Serum (FBS; Hyclone Laboratories) in a humidified incubator at 37ºC with 5% $CO_2$ (Pohjala *et al*., 2011).

**Rescue of CHIKV carrying the *nanoluciferase* gene**

Antiviral assays were performed using the CHIKV harboring the *nanoluciferase* reporter (CHIKV-*nanoluc*). This construct is based on the CHIKV isolate LR2006OPY1 (East/Central/South African genotype), where the CHIKV-*nanoluc* cDNA was placed under the control of the CMV promoter (Matkovic *et al*., 2018). The CHIKV-*nanoluc* production was performed as previously described (de Oliveira *et al*., 2020; Freitas *et al*., 2022; Pereira *et al.*, 2021; Ruiz *et al*., 2023; Santos *et al*., 2021), where the BHK-21 cells were transfected with 1.5µg of CMV-CHIKV-*nanoluc* plasmid using Lipofectamine 2000 and Opti-Mem medium. After 48h of transfection, the supernatant was collected and stored at -80ºC. To determine the viral titer, $9x10^4$ BHK-21 cells were cultured in a 24-well plate, and after 24h cells were infected with 10-fold serial dilutions of CHIKV-*nanoluc*. Cells were incubated with virus for 1h at 37°C, then supernatant was collected, cells were washed with PBS to remove unbound virus, and fresh medium supplemented with 1% penicillin and streptomycin stock dilution, 2% FBS and carboxymethyl cellulose (CMC) at final concentration of 1%. After 48h, cells were fixed with 4% formaldehyde and stained with 0.5% crystal violet. Viral foci were counted to determine viral title, which was presented in plaque-forming units per milliliter (PFU/mL) (Santos *et al*., 2022).

**Cell viability assays**

Cell viability was measured using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay (Sigma-Aldrich), as previously described (Santos *et al*., 2022). Briefly, BHK-21 cells were seeded in a 96-well microplate at a density of $2x10^4$ cells/well and incubated for 24h in a humidified incubator at 37ºC with 5% $CO_2$. Medium containing lectins at concentrations of 2, 10, and 50µg/mL were added to the cell culture. After 16h, the media was removed and a solution containing MTT (0.5 mg/mL) was added to each well and incubated for 30 minutes at 37ºC in a humidified incubator with 5% $CO_2$. Then, solution containing MTT was removed and replaced with 300µL DMSO to solubilize the formazan crystals. Absorbance was measured by the optical density of each well at 560ηm, using a Glomax microplate reader (Promega). Viability was calculated according to the equation (T/C) x 100%, where T and C represent the average optical density of the treated group and the control group, respectively. To calculate the cytotoxic concentration of 50% ($CC_{50}$) cells were treated with a two-fold serial dilution of ConA at concentrations ranging between 0.19 to 50µg/mL for 16 h. After incubation, MTT assay was carried out as described above. The dose-response curve and $CC_{50}$ values were calculated using Graph Pad Prism 8.0 (Kamiloglu *et al*., 2020; Kumar; Nagarajan; Uchil, 2018).

**Antiviral assays**

Antiviral assays were performed as previously described (Pereira *et al*., 2021; Santos et al., 2022). BHK-21 cells were seeded at a density of $5x10^4$ cells/well in 48-well plates. After 24h, cells were infected with CHIKV-*nanoluc* at a multiplicity of infection (MOI) of 0.1 in the presence of ConA, PPL, MAL or VML at 50µg/mL (the highest non-cytotoxic concentrations defined by the cell viability assay). PBS was used as an untreated control. After 24h, cells were lysed with passive luciferase lysis buffer (Promega) and submitted to luminescence quantification employing the *Renilla luciferase* Assay System (Promega), where nanoluciferase levels are proportional to viral replication.

The active lectin ConA was further exploited to identify their effective concentration of 50% ($EC_{50}$). To this, antiviral assay was performed as descried above, while the compounds were added in a two-fold serial dilution of ConA at concentrations ranging from 0.19 to 50µg/mL. The dose-response curve and $EC_{50}$ values were calculated using Graph Pad Prism 8.0 and the selectivity index (SI) was calculated by the ratio between $CC_{50}$ and $EC_{50}$.

## Statistical Analysis

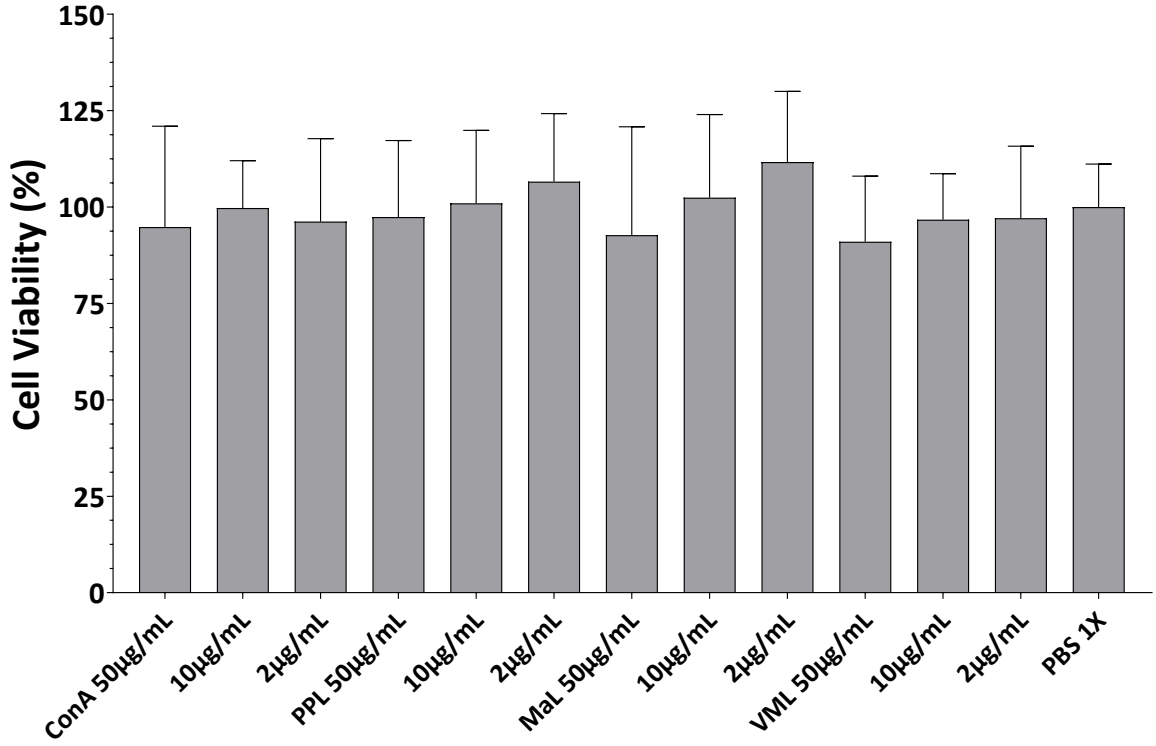
Individual experiments were performed in triplicates and all assays were performed a minimum of three times to confirm reproducibility. $EC_{50}$ and $CC_{50}$ were calculated using non-linear regression considering log(inhibitor) vs. response, with variable inclination (four parameters), and comparison was also performed using the F test ($p < 0.05$). Data were expressed as mean ± SD and subjected to normality and lognormality tests. Statistical significance was determined by one-way analysis of variance (ANOVA), complemented by Dunnett's post hoc test, performed in comparison with the untreated control (Santos *et al*., 2022). Statistical analyzes were performed using GraphPad Prism 8 software.

## RESULTS

### The mannose-binging lectin ConA inhibits CHIKV infection in vitro

To investigate the antiviral activity of the lectins ConA, PPL, MaL, and VML, first, a cell viability assay was performed by treating BHK-21 cells with the lectins at 50, 10, and 2 µg/mL. The mean values of cell viability obtained from treatment with all lectins showed no statistically significant differences when compared with the untreated control PBS ($p > 0.05$) **(Figure 1)**. Therefore, the concentrations of 50 µg/mL (the highest non-cytotoxic concentration) was used to the antiviral screen.

**Figure 1. Cell viability under the treatment of BHK-21 cells with lectins.** BHK-21 cells were treated with lectins at 50, 10, and 2 µg/mL. PBS was used as an untreated control. The graph indicates mean values from three independent experiments, each measured in triplicates, including the standard deviation. $P < 0.05$ was considered significant.




Then, to assess the antiviral activity of these natural lectins, BHK-21 cells were infected with CHIKV-*nanoluc* and treated with the lectins ConA, PPL, MaL, and VML at 50µg/mL, the highest non-cytotoxic tested concentration. Viral replication was quantified 16h post infection (h.p.i.). Cell viability assay was performed in parallel. As a result, ConA impaired viral replication in 96% ($p < 0.0001$) with no significant impact on cell viability **(Figure 2)**. On the other hand, PPL, MaL and VML did not significantly decrease CHIKV replication, although presented no cytotoxicity to the cells **(Figure 2 and Table 1)**. Therefore, from all lectins evaluated here, only ConA was selected for further analysis.

**Figure 2. Effect of ConA, PPL, MaL, and VML on CHIKV-*nanoluc* infection**. BHK-21 cells were infected with CHIKV-*nanoluc* and treated with ConA, PPL, MaL, and VML at 50µg/mL. Viral replication was measured 16 h.p.i. by quantifying nanoluciferase activity. The cell viability (MTT assay) was performed in parallel. PBS was used as an untreated control. The mean values from three independent experiments, each measured in triplicates, including the standard deviation are shown. $P < 0.05$ was considered significant. (****) $P < 0.0001$. All images were generated using GraphPad Prism 8.

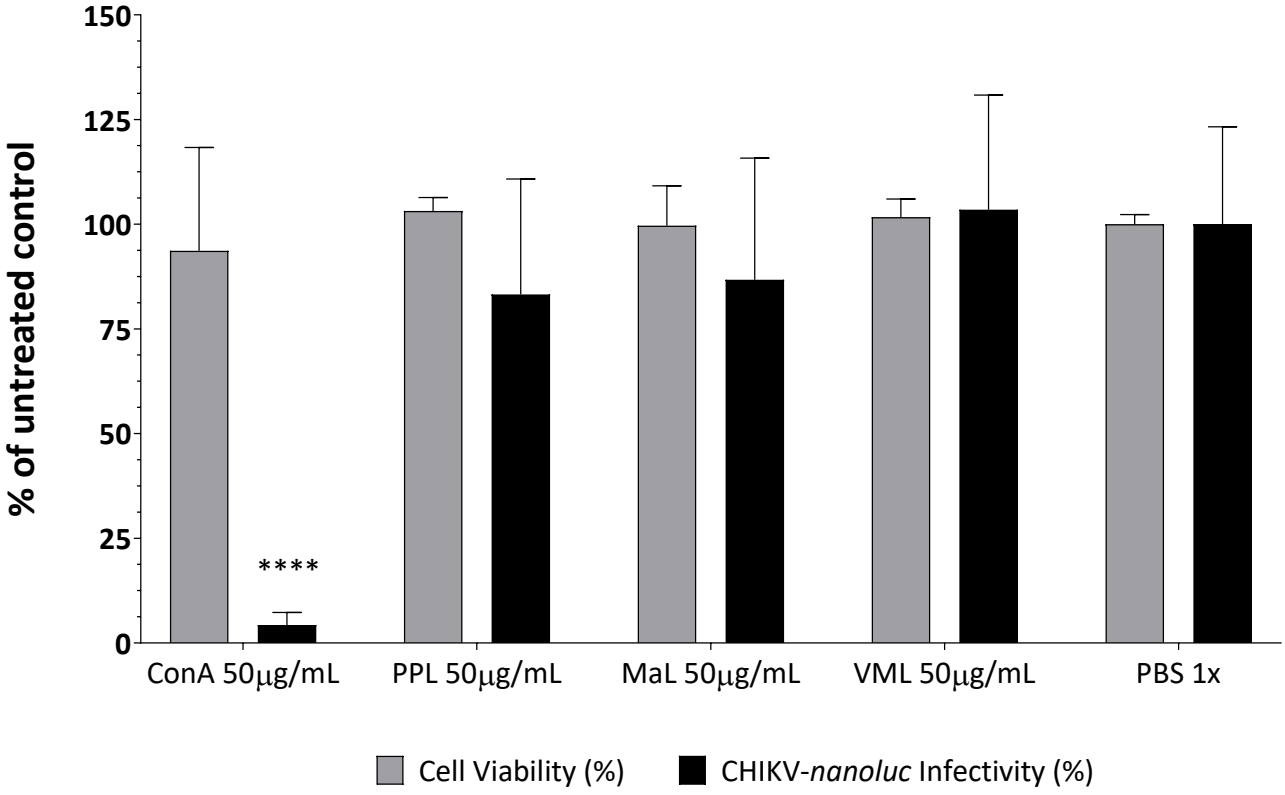



**Table 1.** Antiviral effects of lectins in CHIKV-*nanoluc*.

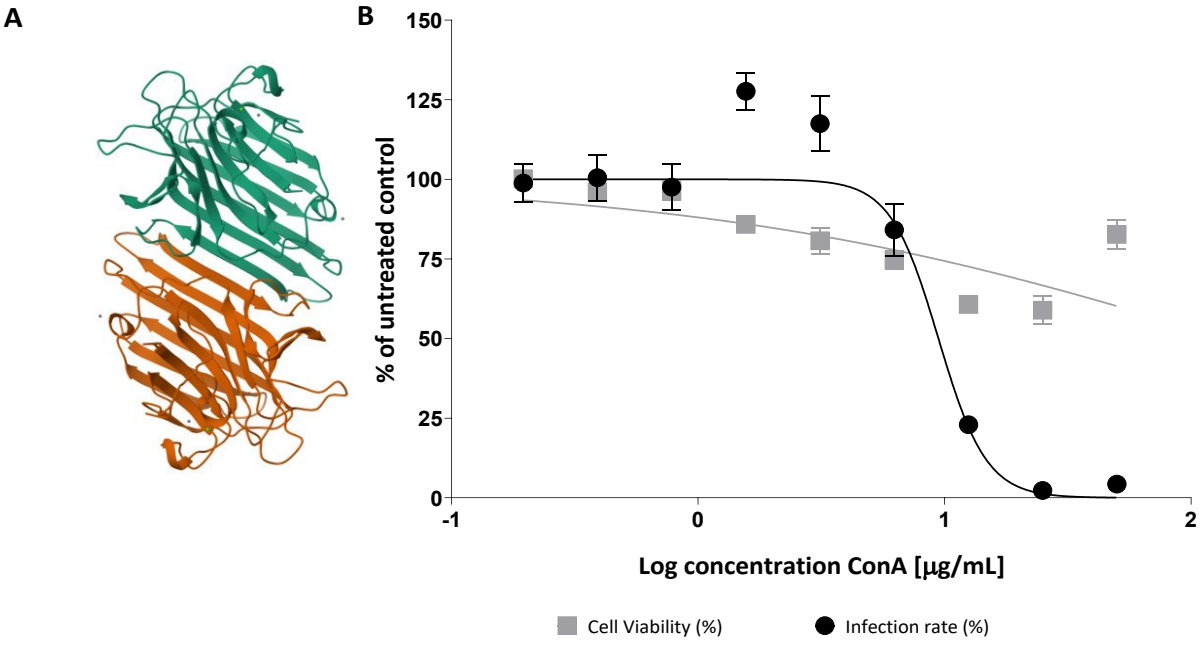
| Lectins | Inhibition (%) |
|---|---|
| **ConA** | 95.7 |
| **PPL** | 16.8 |
| **MaL** | 13.3 |
| **VML** | -3.5 |

## Lectin ConA strongly impairs CHIKV replication

In order to further investigate the antiviral activity of ConA **(Figure 3A)**, a dose response assay was performed to determine the effective concentration of 50% ($EC_{50}$) and the cytotoxicity of 50% ($CC_{50}$), and to calculate the SI ($CC_{50}/EC_{50}$). For this, BHK-21 cells were treated with ConA in a two-fold serial dilution at concentrations ranging from 0.19 to 50 µg/mL

in the presence of absence of CHIKV-*nanoluc*. As an outcome, ConA had an $EC_{50}$ of 9.44 µg/mL, $CC_{50}$ of 138.9 µg/mL, and SI of 14.71. (**Figure 3B**). Therefore, ConA represents a promising inhibitor of CHIKV infection that can be further exploited towards its antiviral activity**.**

**Figure 3. Dose-response activity of ConA against CHIKV infection and its effects on cell viability**. **(A)** Structure of the lectin ConA (PDB: 3ENR). **(B)** BHK-21 cells were infected with CHIKV-*nanoluc* at an MOI of 0.1 and treated with the ConA lectin in a two-fold serial dilution ranging from 0.19 to 50µg/mL. The $CC_{50}$ is indicated by the gray squares curve and the $EC_{50}$ is indicated by the black circles curve. The assays were performed in triplicates in a minimum of three independent experiments.




## DISCUSSION

CHIKV represents a global health threat, as it has already been identified on several continents, being capable of causing large outbreaks and, despite the recent prophylaxis vaccine licensed in a few countries, there are still no effective antivirals for the treatment of chikungunya fever (de Lima *et al*., 2024).

As an *Alphavirus*, CHIKV has glycoproteins anchored on the surface of its viral envelope, composed of mannose, galactose, and N-acetylglusosamine (NAG) (Simão *et al*., 2020), capable of recognizing and binding to the target cell receptor (Kumar *et al*., 2021). In an interesting matter, lectins bind to specific carbohydrate structures in a reversible manner, therefore are capable of recognizing and interacting with viral envelope glycoproteins, consequently, impairing viral entry into the cell host (Budhadev *et al*., 2020; Mazur-Marzec *et al*., 2021; Tsaneva; Van Damme, 2020; Wang *et al*., 2021; Naik; Kumar, 2022). In this context, lectins are attractive molecules to be further investigated towards their antiviral properties that can contribute to the development of antiviral drugs against CHIKV.

Herein, four lectins extracted from different species of plants from northeastern Brazil were screened against CHIKV infection, in which three have specific binding to mannose/glucose and one to lactose. Among them, the well-studied Concanavalin A (ConA) was the only lectin that showed to strongly impairs CHIKV replication. The other lectins, despite showing no cytotoxicity, did not significant inhibit CHIKV infection.

ConA is extracted from the seeds of *Canavalia ensiformis*. Its isolation was first reported in 1919 (Cavada *et al*., 2019) and, to date, this is the most explored lectin isolated from plants. ConA has mannose/glucose binding specificity (Barre *et al*., 2019), and has been exploited for its diverse biological activities, such as the activation of T lymphocytes, playing an important role in processes of viral infections, as well as antifungal, antimicrobial, and antitumor activities (Fonseca *et al*., 2022; Ibrahim *et al*., 2021; Jin; Lee; Hong, 2019; Kar *et al*., 2021; Kuo *et al*., 2007). Recently, ConA was described as a potential inhibitor of SARS-COV-2 infection, with $EC_{50}$ of 2.5 and 2.9 µg/mL performed using A549-ACE2 and Calu-3 cells, respectively, also presenting low cytotoxicity profile, and resulting in high SI values (>299) (Klevanski *et al*., 2024). It corroborates with data described here, since ConA inhibited CHIKV with a relatively low $EC_{50}$ of 9.44 µg/mL, comparable to the one reported against SARS-CoV-2. However, the differences in SI might be due to the cytotoxicity of the molecules on different cell lines, which might be expected (Sudeep *et al*., 2019; Yeh *et al*., 2022), and also to particularities between the tested protocols. Still, the effects described here also emphasize the broad-spectrum activity of ConA as antiviral, to date, reported against different families of virus.

What is more, ConA has been also described as an inhibitor of early stages of infection against SARS-CoV-2 and HIV through interaction with Spike (S) and gp120 glycoproteins,

respectively, resulting in virucidal activity (Akkouh *et al*., 2015, Klevanski *et al*., 2024). So far, further mechanistic target of ConA against CHIKV has not been explored in this work or even in literature yet, but it is possible to suggest that ConA might be interacting with CHIKV E1-E2 glycoprotein receptors. Further studies are necessary to understand the effects of the lectin during the early stages of CHIKV replication. As an interesting matter, a similar lectin purified from tamarind, the tamarind seed kinase-like lectin (TCLL), was described as a specific ligand for N-acetylglucosamine on CHIKV glycoprotein virion, reducing viral infectivity (Kaur *et al*., 2019). In another study, TCLL was employed for quantification and diagnosis of CHIKV using an antigen capture assay (Choudhary *et al*., 2021). In this sense, it also emphasizes the specificity that this lectin could have towards viral glycoproteins, as well as be used as scaffold for further drug development.

Plant lectins have wide applicability for the development of new therapeutics, including antiviral therapies (Maier, 2022). However, despite being explored towards their antiviral activity against several viruses, to the best of our knowledge, this is the first description of ConA as a CHIKV inhibitor. To better characterize the effects of ConA, further analyses are being conducted by our research group.

## CONCLUSION

Of the four lectins tested against CHIKV infection, the lectin from *Canavalia ensiformis* (Concanavalin A: ConA) showed a high inhibition rate. In summary, the data showed here is the first evidence of antiviral activity of the ConA lectin against CHIKV. Further assays are needed to confirm its mechanism of action, as well as characterize its interactions with CHIKV viral proteins. The data presented here can be used as scaffold for further drug development of antivirals against CHIKV and other emergent viruses such as ZIKV and DENV.

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