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Virucidal activity of proanthocyanidin against Mayaro virus

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ABSTRACT

Mayaro virus (MAYV) is a sublethal arbovirus transmitted by mosquitoes with possible installation of an urban cycle in the Americas. Its infection causes disabling arthralgia, and still, there is no vaccine or treatment to it. We recently investigated nearly 600 compounds by molecular docking and identified epicatechin as a potent antiviral against MAYV. The root extract of *Maytenus imbricata* showed anti-MAYV activity and two isolated compounds from this plant were also evaluated *in vitro*. Proanthocyanidin (PAC), a dimer containing epicatechin, showed an effective concentration for 50% of the cells infected by MAYV (EC_{50}) of 37.9 ± 2.4 μM and a selectivity index (SI) above 40. PAC showed significant virucidal activity, inhibiting 100% of the virus proliferation (7 log units), and caused moderate effect during adsorption and virus internalization stage. However, PAC was unable to block the infection when only the cells were pretreated. It was observed a reduction in virus yields when adding PAC at different moments after infection. The set of results indicates that PAC binds to viral and non-cellular elements and may inactivate the MAYV. The inactivation occurs before infection or when the virus reaches the extracellular environment from the 2nd cycle of infection that could block its progression cell-to-cell or to tissues not yet infected.

In the last decades, the emerging and reemerging viral diseases had gained attention to public health in an attempt to minimize future epidemics (Devaux, 2012; Acosta-Ampudia et al., 2018). In this context, arboviruses (viruses transmitted by arthropods, such as *Haemagogus* spp. mosquitoes) stand out as being globally distributed and responsible for the cause of different human diseases (Figueiredo, 2015).

Belonging to the *Togaviridae* family, *Mayaro virus* (MAYV) (*Alphavirus* genus), is kept in wild cycles involving vertebrates, non-human primates and hematophagous arthropods (Weaver and Reisen, 2010; ICTV, 2019). Since its isolation from blood samples of febrile rural workers in Trinidad and Tobago (Anderson et al., 1957), sporadic cases and small epidemics of Mayaro virus Disease (MVD) have been described in South and Central America countries and other tropical regions (Munõz and Navarro, 2012; Mota et al., 2015). The discovery of its potential transmission by the urban vectors *Aedes aegypti* and *Aedes albopictus* contributed for the classification of MAYV as an emergent virus, with the potential to installing an urban cycle, as recently happened with the Chikungunya virus (Long et al., 2011; Figueiredo and Figueiredo, 2014; Wiggins et al., 2018). For non-endemic areas, a recent study reported an 8-year-old boy infected by MAYV in Haiti in a context of Dengue virus co-infection. Since in Haiti non-human primates are not native, this episode suggested that the virus possibly found a new reservoir or a human-human transmission by *Aedes* spp. mosquitoes (Lednicky et al., 2016; Hotez and Murray, 2017).

As other arboviruses, MAYV is highly neglected, and its clinical manifestations are often confused with Dengue, Zika, and Chikungunya symptoms. The most characteristic symptoms observed in MVD are fever, polyarthralgia, and persistent arthritis for months or even years,

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which cause incapacitating deficiency (Munõz and Navarro, 2012). Moreover, licensed vaccines and antiviral specific agents against MAYV are unavailable, and the treatment of MVD is just symptomatic (Mota et al., 2015).

Some studies describe the properties of plant extracts and isolated compounds against viruses that affect both animals and humans (Cecílio et al., 2012; Ganjhu et al., 2015). As examples, fractions of *Salacia crassifolia* (Celastraceae), *Silybum marianum* (Compositae) and *Cassia australis* (Leguminosae) extracts rich in flavonoids and tannins, showed anti- MAYV activity (Spindola et al., 2014; Camini et al., 2018; Ferreira et al., 2018). Thus, we evaluated the antiviral activity of two flavonoids isolated from the methanol extract of *Maytenus imbricata* (Celastraceae) roots against the MAYV.

The MAYV BeAr20290 strain (GenBank accession no. KY618127) used in this study was originally isolated from a pool of *Haemagogus* spp. in Belém (Pará, Brazil) in 1960, propagated in Vero cells and later titrated by Dulbecco's plaque formation assay (Dulbecco, 1952). Vero cells (ATCC CCL-81) were cultured in Dulbecco's modified minimal essential medium (DMEM) (Cultilab, BRA), supplemented with 5% fetal bovine serum (FBS) (Cultilab, BRA), 100U/mL of penicillin, 100 µg/mL of streptomycin and 2.5 µg/mL of amphotericin B (Sigma-Aldrich, USA), and maintained in a humidified incubator at 37 °C with 5% CO_2. The stock solutions of compounds were prepared in 20% (v/v) dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA).

The crude root fractions of *M. imbricata* are rich in flavonoids and tannins, and presented a promising antiviral activity against the MAYV (data not shown). From these fractions, we isolated the flavonoids *4′-O*-methylepigallocatechin (MEP) (Fig. 1A) and proanthocyanidin [(−)-epicatechin-(4β→8)-(−)-4′-methylepigallocatechin] (PAC, a MEP dimerized with the epicatechin) (Fig. 1B) for testing their anti-MAYV activity.

Cytotoxicity of MEP and PAC was evaluated against Vero cells by the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich) colorimetric assay (Mosmann, 1983). Even at the highest concentration used in the assays (1640 µM), all cells remained viable, which means that MEP and PAC were unable to alter cells basal functions after 48 h of treatment. Thus, the 50% cytotoxic concentration (CC_{50}) values were considered higher than 1640 µM for MEP and PAC (Table 1). No cytotoxic effect was observed in control cells treated with 0.2% (v/v) DMSO (Sigma-Aldrich).

The global antiviral activity for different concentrations of compounds was tested on MAYV-infected Vero cells during 48 h, using moi 0.1, 1, 5 and 10. Cells and the viral inoculum were pretreated with the compounds for 30 min at 37 °C. This pretreatment was performed to include all stages of the viral cycle in which the compound could act, *i.e.*, in adsorption, penetration, viral replication, or virucidal effect. For the moi 0.1, 82.2 µM PAC was able to protect approximately 100% of the MAYV-infected cells (Fig. 2E) when compared to the infected and untreated cells (Fig. 2B). In the MEP's assay conditions (Fig. 2D), it was not observed activity against MAYV, even at the highest concentration tested (1000 µM). However, potent inhibition of MAYV infection was detected with the effective concentration for 50% of the cells infected (EC_{50}) equals to 37.9 ± 2.4 µM to PAC. Such higher antiviral activity in dimeric forms of flavonoids has been reported for Herpes simplex virus type 1 and Hepatitis C virus (Takeshita et al., 2009; Gescher et al., 2011). In a previous study of our group, we obtained a similar result, wherein the epicatechin monomer showed activity against the MAYV with EC_{50} 247 µM (moi 0.1) (Ferreira et al., 2018), which corresponds to an effective concentration 6.5 times greater than that obtained with PAC. As expected, the DMSO control (vehicle) (Fig. 2C) did not induce any protective activity. Ribavirin (Sigma-Aldrich, positive control), showed an effective antiviral concentration (EC_{50}) 486.4 ± 8.1 µM. Therefore, less effective when compared to the protective effect promoted by PAC (Fig. 2F, Table 1). We also determined the selective index (SI) as the ratio of CC_{50}/EC_{50}, and the relative potency (RP), expressed as the ratio of ribavirin EC_{50} (reference antiviral) to EC_{50} of the tested compound. PAC showed SI higher than 43 and RP 12.8. Compounds with SI greater than 10 are considered safe and with the possible absence of toxicity due to the significant window between the pharmacological and toxical doses (Aguiar et al., 2018).

Ribavirin and PAC showed a dose-dependence effect, since the reduction of the concentration decreases the protection of cells against viral infection. Cell viability was measured by the MTT colorimetric assay after infection (Fig. 3A). We also performed a plaque assay to evaluate the viral load after PAC infection treatment. This assay was performed with the supernatant of MAYV infected cells (moi 0.1) and

Table 1
Cytotoxicity and anti-Mayaro virus activity of MEP, PAC, and ribavirin (positive control).

Substance	CC_{50} (µM)[a]	EC_{50} (µM)[b]	SI[c]	RP[d]
MEP	> 1640.0	Nd	Nd	Nd
PAC	> 1640.0	37.9 ± 2.4	> 43	12.8
Ribavirin	2142.1 ± 174.0	486.4 ± 8.1	4.4	Nd

PAC: proanthocyanidin (−)-epicatechin-(4β→ 8)-(−)-4′-methylepigallocatechin.
MEP: 4′-O-methylepigallocatechin.
nd: not detected.
All data were obtained from three independent experiments with three replicates each.
[a] 50% cytotoxic concentration.
[b] 50% effective concentration of viral replication.
[c] Selectivity Index: the ratio between substance's CC_{50} and EC_{50}.
[d] Relative Potency: the ratio between ribavirin EC_{50} and PAC EC_{50}.

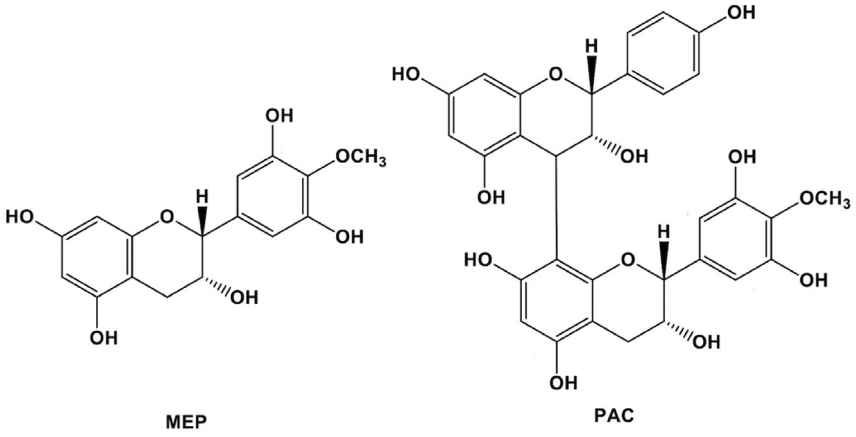

Fig. 1. Chemical structure of flavonoids 4′-*O*-methylepigallocatechin (MEP) and proanthocyanidin (−)-epicatechin-(4β→8)-(−)-4′-methylepigallocatechin (PAC).

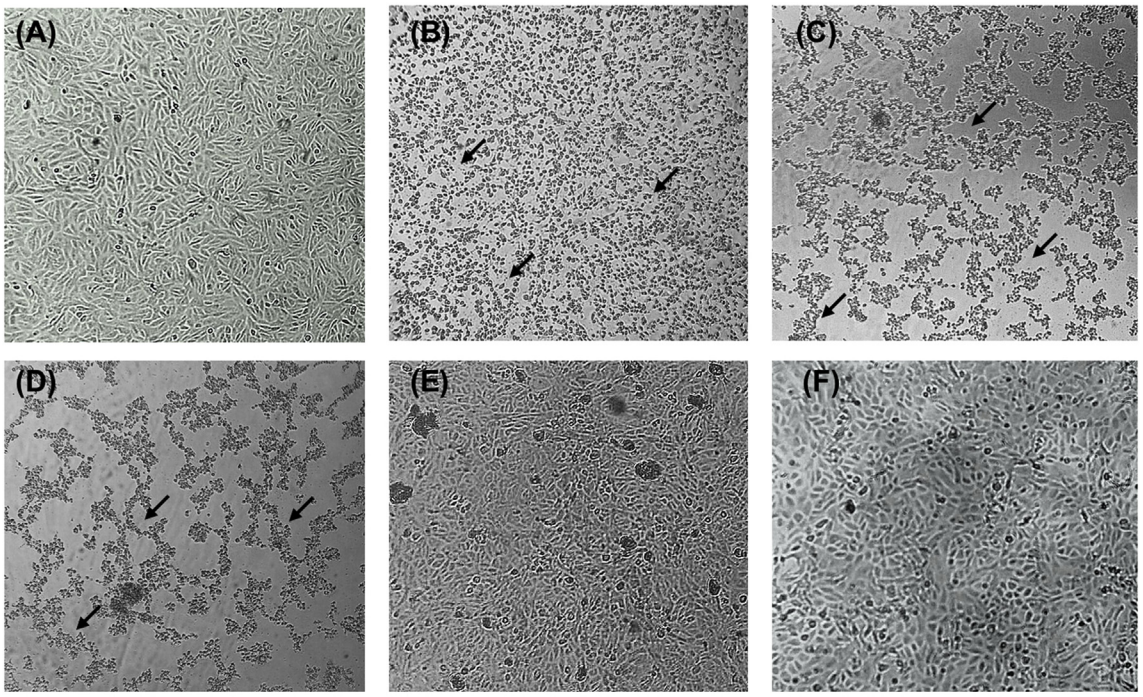

Fig. 2. Viral cytopathic effect (CPE) in Vero cells treated with MEP, PAC, and Ribavirin 2 days after the MAYV infection. (A) Cellular control: untreated and uninfected cells. (B) Virus control: infected and untreated cells. (C)Vehicle control: infected and treated cells with 0.2% (v/v) DMSO. (D) MEP treated (500 μM) and infected cells. (E) PAC treated (82 μM) and infected cells. (F) Positive control: Ribavirin treated (2000 μM) and infected cells; The CPE reduction was observed only in PAC and Ribavirin when compared to viral control. Black arrows indicate some clusters of cells from the cytopathic effect of the MAYV. Magnification, 100×.

treated with PAC in different concentrations (164 - 41 μM). At concentrations of 164 and 82 μM, no viral particles were detected (Fig. 3B), the concentration of 41 μM PAC resulted in 45.5% inhibition of the lysis plates, very close to the calculated EC_{50} of 37.9 μM. Thus, the cells infected with MAYV and treated with PAC from 82 μM, did not affect cell viability, and no virus was detected 48hpi and treatment.

A higher PAC concentration was required to inhibit a higher viral load. Results showed that the EC_{50} was 57.9 ± 3.9 μM, 98.9 ± 3.2 μM and 146.8 ± 7.9 μM for moi 1, 5 and 10, respectively (Table 2). However, the increase was not proportional, while the viral titer was increased by 100x, PAC concentration increased only 4x. PAC's viral inhibition using larger moi followed the same dose-dependence behavior presented by moi 0.1. Also, PAC's concentration of 164 μM induced more than 80% protection of the infected cells independent of the moi.

The MAYV replication kinetics were performed to complement the analysis of the antiviral activity of PAC. Such assay was done both in the absence and presence of PAC treatment at a concentration of 164 μM and a moi 5. From the kinetic curves (Fig. 4), we notice a minimum of 3 log units decrease in the virus yield in PAC treated cells. In the initial phase (0–12 h), MAYV was not detected in the treated sample, reaching a maximum reduction of almost 7 log units when compared to the control. Wu et al. (2017) obtained a reduction of only 1 log in the replication kinetics of Hepatitis E virus (HEV) in the presence of sofosbuvir, an antiviral used in the treatment of chronic hepatitis C. Even so, the decrease in the PAC effect after 12 h is still unclear.

In assessing the PAC mechanism of action, the virucidal activity against MAYV was first analyzed aiming to elucidate if this compound would have a direct effect on the viral particles (Pujol et al., 2012). Thus, after pretreatment of MAYV with PAC (82 μM) for 1 h, no remaining viral activity was detected, inhibiting 100% infection (~7 logs). To verify the virucidal activity obtained, 10^4 PFU of MAYV was treated with PAC at 82 μM for 15, 30, 60 and 120 min at 37 °C. After incubation, virus + compound and viral control were placed on a dialysis membrane (Spectra/Por® Dialysis Membrane - MWCO: 12–14000 Da), which would allow PAC (610 Da) to pass and the MAYV (~70 nm) would be retained. The dialysis in PBS 1x (1 L) at 4 °C was replaced by fresh PBS 1x (1 L) after 4 h and incubated overnight at 4 °C. Thus, even if it had remnant PAC, it was diluted 1,000,000 times after dialysis, reaching a concentration of 0.82 nM, much lower than inhibitory concentration. Dialyzed samples were quantitated by plaque reduction assay, and MAYV was inactivated (Table 3), while in the viral control it was quantified around 5×10^2 PFU/mL. Therefore, we can affirm that the PAC treatment irreversibly blocks the MAYV, even with only 15 min. Tsukuda et al. (2017) described the virucidal action of two PAC analogs; they observed a significant reduction in Hepatitis B and D virus infectivity in 30 min of pre-treated cells.

In cellular pretreatment, wherein only the cells were treated with PAC (1 h at 37 °C) and washed before viral adsorption, no inhibitory effects and no disruption on the replication cycle were observed. A similar result was observed by Tsukuda et al. (2017), in which cells pretreated for 24 h with PAC analogs failed to inhibit HBV infection, showing that they also did not act on cell receptors to inhibit viral infection. In the adsorption stage, compound along with the virus was added on time of viral adsorption at 4 °C for 1 h; then, cells were washed and added to a compound-free medium. In the internalization of the viral particle, cells were infected in the compound-free medium and after 1h from the adsorption stage at 4 °C, cells were washed and incubated at 37 °C during 1 h in the presence of PAC. After that, cells were treated with citrate buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl, pH 3) for 1 min to inactivate the adsorbed but not internalized viruses. In both stages, PAC treatment resulted in an inhibition of about 99,9% of the infection, which was statistically different from the viral control and also supported by the results obtained by Tsukuda et al. (2017). It is possible that some of the virions are only adsorbed or partially detached in the cell surface, therefore still susceptible to antiviral binding and blockade. However, it will be possible to confirm this hypothesis only using electron microscopy or immunofluorescence methods. PAC treatment at different times after viral internalization (1, 3, 6, 12, 24 and 36 hpi) reduced on average 93%, or 15 times, of virus

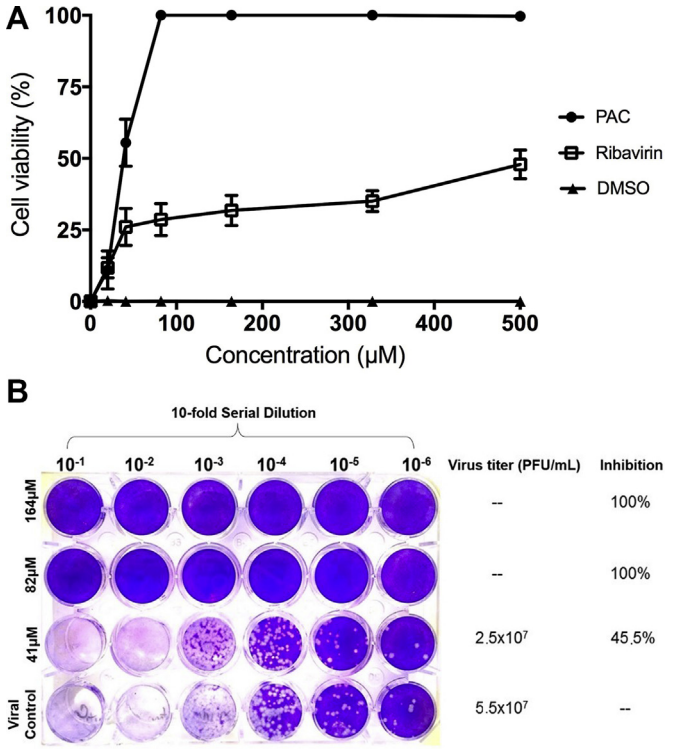

Fig. 3. The anti-MAYV activity of PAC. Vero cells and MAYV (moi 0.1) were pretreated with the compounds prior infection for 30 min at 37 °C. They remained incubated for 48 h in 96-well microplates. The untreated virus (viral control) also remained 30 min at 37 °C. After 48 hpi, the supernatant was collected for viral quantification by plaque assay and the metabolic activity of the cells was measured indirectly by the MTT colorimetric method at 492 nm. (A) The inhibitory activity of PAC showed a dose-dependent behavior, inhibiting 100% of the infection by the MAYV in low concentrations when compared to Ribavirin (positive control). (B) Viral quantification of the supernatant of the infected cells and treated with different PAC concentrations, where MAYV was not detected after treatment at concentrations above 82 μM. PAC results were statistically significant ($p < 0.001$) when analyzed using one-way ANOVA and Tukey's post-test.

Table 2
Effective Concentration values for 50% of cells (EC50) of PAC comparing different moi of MAYV. The MAYV infection inhibition by PAC followed the same behavior independent of moi. With a higher amount of virus per cell, higher concentrations of PAC are needed to inhibit infection by the MAYV, but not proportionally.

moi	EC_{50} (μM)
0.1	37.9 ± 2.4
1	57.9 ± 3.9
5	98.9 ± 3.2
10	146.8 ± 7.9

All data were obtained from three independent experiments with three replicates each.

yield (Fig. 5), and there was no significant difference regarding the time that the cells were treated post-infection. Tsukuda et al. (2017) reported a similar result.

Some studies highlight the potential of the proanthocyanidin classes to induce activity against Hepatitis C virus (Ishida et al., 2014), Hepatitis B and D virus (Tsukuda et al., 2017), Herpes simplex virus type 1 (Gescher et al., 2011) and type 2 (Terlizzi et al., 2016), Respiratory

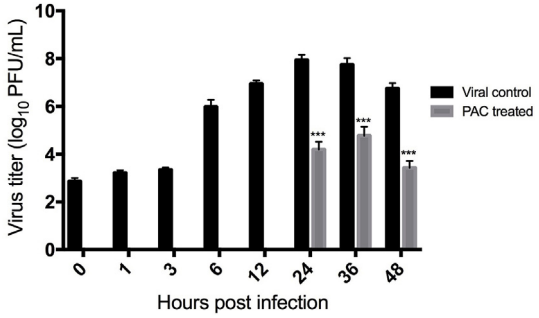

Fig. 4. Kinetics of MAYV multiplication (moi 5) in the absence and presence of PAC's treatment (164 μM). Cells and virus were pretreated for 30 min before infection. After infection and one hour of viral adsorption, the inoculum was removed, the cells washed with PBS 1x and fresh medium containing PAC in the concentration of 164 μM was added. There was no supplementation of PAC after the beginning of the experiment. Incubation was continued for 48 h; aliquots were withdrawn at times 0, 1, 3, 6, 12, 24 and 36 hpi. The supernatant was titrated by the plate-forming method. The same procedure was performed for infection without any treatment of the cells and inoculum, in order to evaluate the kinetic behavior of the MAYV in the absence of the antiviral. All data were obtained from three independent experiments with three replicates each and were plotted and analyzed using *t*-test nonparametric, with ***$p < 0.001$.

Table 3
MAYV viral quantification after PAC virucidal activity and dialysis. MAYV virus (10^4 PFU) was treated with PAC (82 μM) at different times followed by overnight dialysis at 4 °C in Spectra/Por® Dialysis Membrane - MWCO: 12–14000 Da, which allowed passage of the PAC (610 Da) but not virus. Even after dialysis, MAYV was not detected in any of the treatment conditions, confirming the potent virucidal activity of the PAC.

Treatment time (min)	Viral control (PFU/mL)	Treated virus (PFU/mL)
15	$4{,}3 \times 10^2$	Nd
30	$6{,}4 \times 10^2$	Nd
60	$7{,}0 \times 10^2$	Nd
120	$6{,}8 \times 10^2$	Nd

Nd: not detected

syncytial virus (Lee et al., 2017), Canine distemper virus (Gallina et al., 2011), and Rotavirus (Lipson et al., 2017). Reports have shown that oligomeric forms of proanthocyanidins inhibit adsorption and viral penetration (Gescher et al., 2011). Other studies describe that dimeric proanthocyanidins target viral envelope glycoproteins, resulting in loss of infectivity of viral particles (Terlizzi et al., 2016) and inhibition of entry into host cells (Tsukuda et al., 2017). In addition, it decreases the synthesis of viral RNA by inhibiting the viral replication complex (Gallina et al., 2011). Also, the property of proanthocyanidins in chelating with calcium ions is known, and this action is not discarded in the virus-cell system (Epasinghe et al., 2017). In this way, the data set of the present work indicates that PAC inhibits the multiplication cycle of the MAYV by direct action in the viral particle and not in the host cell. Furthermore, we do not rule out the possibility of PAC inhibiting other arboviruses, since in a preliminary assay it was also active against ZIKV (Data not shown). Details of how PAC inactivates virion infectivity, possible synergistic interactions with other antivirals, and *in vivo* assay are part of an upcoming study.

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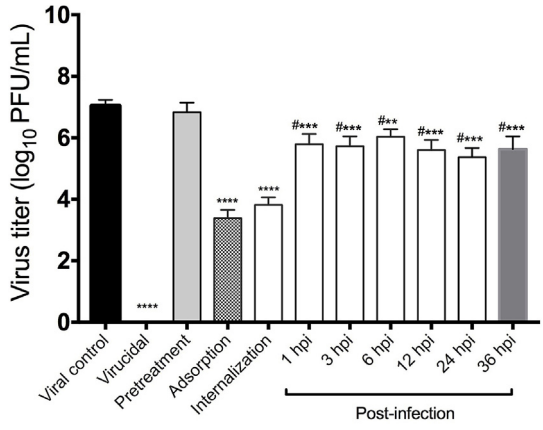

Fig. 5. Characterization of the global mechanism of action of PAC (82 μM) against the MAYV. PAC treatment was performed at different times of the viral multiplication cycle, in order to evaluate which stage the antiviral was interrupting. Vero cells were used in all assays with moi 0.1 and 48 h of incubation at 37 °C after infection. Virucidal: only the inoculum was pretreated before the infection for 1 h at 37 °C in order to evaluate if PAC would have a direct action on the viral particles. Pretreatment: only cells were pretreated with PAC for 1 h at 37 °C; then, the cells were washed to remove the compound, and viral inoculum was added; after viral adsorption, we added PAC-free medium. Adsorption: PAC was added along with virus during the adsorption period at 4 °C for 1 h, after that, virus remaining and compound were washed, and PAC-free medium added. Internalization: cells were infected in compound-free, and after 1h adsorption at 4 °C cells were washed and incubated at 37 °C during 1 h in the presence of PAC. After that, cells were treated with citrate buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl, pH 3) for 1 min to inactivate adsorbed but not internalized viruses. Post-infection: cells were infected with MAYV at 37 °C for 1 h; then, the cells were washed, and PAC-free medium added. After 1, 3, 6, 12, 24 or 36 h of infection, PAC was added to the medium. All data were obtained from three independent experiments with three replicates each and were plotted and analyzed using one-way ANOVA and Tukey's post-test, with # indicating that there was no statistically significant difference ($p > 0.05$) between post-infection treatment times, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$.

Conflicts of interest

The authors declare that there is no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.05.008.

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