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α-Mangostin Effectively Inhibits Chikungunya Virus Replication in HepG2 Cells

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ABSTRACT

Chikungunya virus (CHIKV) is an arthropod-transmitted *Alphavirus* endemic to countries in Africa and Asia, including Indonesia, which causes debilitating arthralgia which can last several years. The rapid spread of CHIKV to new areas makes the discovery of antiviral agents a high priority. α-mangostin is a xanthone from mangosteen (*Garcinia mangostana*) pericarp and has antiviral activity against Hepatitis C and Dengue viruses. We investigated the antiviral activity of α-mangostin against CHIKV in HepG2 cells in pre-, post- and combination treatments compared to the common antiviral medicine ribavirin, as well their cytotoxicity. Our results show dose-responsive reductions in viral titer in all treatment regimes, with post- and combination treatments being more effective than pre-treatment only (IC_{50} = 7.79, 5.99 and 6.39 µM, respectively), but with poor specificity (SI = 1.39, 1.81 and 1.70, respectively) compared to ribavirin. Neither compound showed a direct virucidal effect. These results suggest α-mangostin effectively inhibits CHIKV replication in this cell line.

1. Introduction

Chikungunya virus (CHIKV) is a positive single-strand RNA virus from the *Alphavirus* genus transmitted through the bite of *Aedes mosquitoes*, which now circulates in more than 60 countries in Asia, Africa, Europe and the Americas, including Indonesia (Silva and Dermody 2017; Vu *et al.* 2017; Harapan *et al.* 2019). Infection causes chikungunya fever (CHIKF), which may result in significant liver damage with associated diabetes mellitus, as well as debilitating Guillain-Barré syndrome requiring respiratory support, along with a mortality risk, especially in old age (Economopoulou *et al.* 2009; Lebrun *et al.* 2009; Ganesan *et al.* 2017). Recent increases of fatal cases of CHIKF are largely attributed to better reporting and naivete of populations rather than an increased risk, but repeated and independent occurrences of mutations that increase CHIKV replication in *Aedes albopictus* suggest that the virus will continue to spread to newer populations, as this species is well adapted for global spread driven by human activity (Schuffenecker *et al.* 2006; Benedict *et al.* 2007; Tsetsarkin *et al.* 2007, 2009, 2014; Vazeille *et al.* 2007; de Lamballerie *et al.* 2008; Higgs and Ziegler 2010). As there is no specific drug or approved vaccine against CHIKF, treatment is limited to symptom alleviation using analgesic-antipyretic drugs, with ribavirin being the only FDA-licensed drug that has been tested in humans and has shown good results in patients, though it is only effective early in the CHIKV replication cycle (Galán-Huerta *et al.* 2015; Cunha and Trinta 2017; Silva and Dermody 2017; Vu *et al.* 2017). Given the need for new antiviral agents, the xanthone α-mangostin from the pericarp of the mangosteen (Garcinia mangostana Linn.) fruit shows promise, successfully inhibiting replication of against Hepatitis C virus (HCV) (Choi *et al.* 2014) and dengue virus (DENV) (Subudhi *et al.* 2018; Sugiyanto *et al.* 2019; Panda *et al.* 2021), with *in silico* analyses suggesting a potential action on SARS-CoV-2 (Hidayat *et al.* 2021). Here we report our findings on the antiviral activity of α-mangostin against CHIKV in HepG2 cells. α-mangostin and ribavirin action were tested in post-, pre-, and full treatment scenarios in

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order to better identify their mechanism of antiviral action. Effective antiviral concentrations were compared to their relative cytotoxicity in these cells to give a standardized measure of safety and efficacy.

2. Materials and Methods

HepG2 and BHK-21 cells were obtained from ATCC and maintained in RPMI-1640 (Gibco 11875093). CHIKV strain JMB-192 (isolated from a patient in Jambi, Indonesia in 2015 (Sasmono *et al.* 2017) was propagated using Vero CCL-81 cells. The cytotoxicity of α-mangostin (Sigma-Aldrich M3824), ribavirin (Sigma-Aldrich R9644) and DMSO (Applichem A3672) in HepG2 was assessed using the Vybrant MTT Cell Proliferation kit (Thermo-Scientific V13154). The virucidal and antiviral activity of α-mangostin and ribavirin in pre-, post- and full treatment regimens were assessed using CHIKV plaque assay at multiplicity of infection (MOI) of 1 as previously described (Hayati *et al.* 2021). Statistical analysis was done using GraphPad Prism v.9, with two-way and one-way ANOVAs for antiviral and virucidal effects, respectively, with Dunnet's-adjusted $p<0.05$ being considered significant.

3. Results

3.1. Cytotoxicity assay of α-mangostin

To assess the cytotoxic activity of α-mangostin in HepG2 cells, we treated the cells with various concentrations of α-mangostin. Our assay reveals a dose-responsive pattern of α-mangostin, with concentrations ≤7 µM being non-toxic, with cell viability remaining above 80% compared to control after 48 hours (Figure 1A). Above 10 µM, the compound proved significantly toxic, with cell viability reaching 0% at ≥20 µM, while DMSO had no major cytotoxic impacts at the tested concentrations (0.4%, data not shown). When plotted in a logistic curve (R^2 = 0.965, Figure 1A), we extrapolate a 50% cytotoxic concentration (CC_{50}) of 10.84 µM. Similarly, ribavirin showed a dose-responsive toxicity, with significant cell death at ≥20 µg/ml and a CC_{50} of 88.07 µg/ml (R^2 = 0.958, Figure 1B), although the falloff in cell viability occurred slower with increasing concentrations compared to α-mangostin.

3.2. Antiviral activity of α-mangostin

The inhibition of CHIKV growth by α-mangostin was assessed using CHIKV plaque assay. Upon

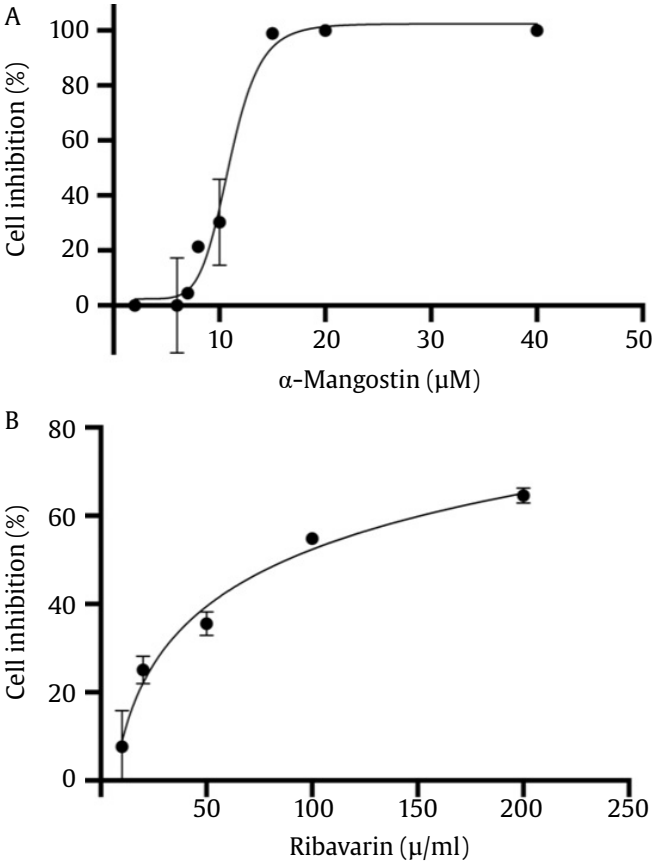

Figure 1. Cytotoxicity of compounds in HepG2 cells. Mean cell viability values after 18 hours, errors bars represent SD. Data are representative of two independent experiments

treatment with α-mangostin, the growth of CHIKV was significantly affected by both treatment type (pre-, post-, and full treatment) and the concentration of the compound (two-way ANOVA $p = 0.046$ and <0.001, respectively). The addition of α-mangostin caused a significant reduction in the CHIKV titer in all three treatments at 12.5 µM, with no effect at only 3.125 µM (Figure 2A). At lower concentrations, pre-treatment was less effective, with full treatment showing the greatest inhibition of CHIKV at the higher concentrations, as reflected by extrapolated 50% inhibitory concentration (IC50) values of 7.79 µM, 5.99 µM, and 6.39 µM for pre-, full and post treatments, respectively (Table 1). Unlike α-mangostin, ribavirin inhibitory action was more prominent in full- and post-treatments assays with both 10 µg/ml and 20 µg/ml significantly inhibited the CHIKV growth. The ribavirin pre-treatment assay did not have any effect on viral growth (Figure 2B) and requiring a far higher IC_{50} (Table 1).

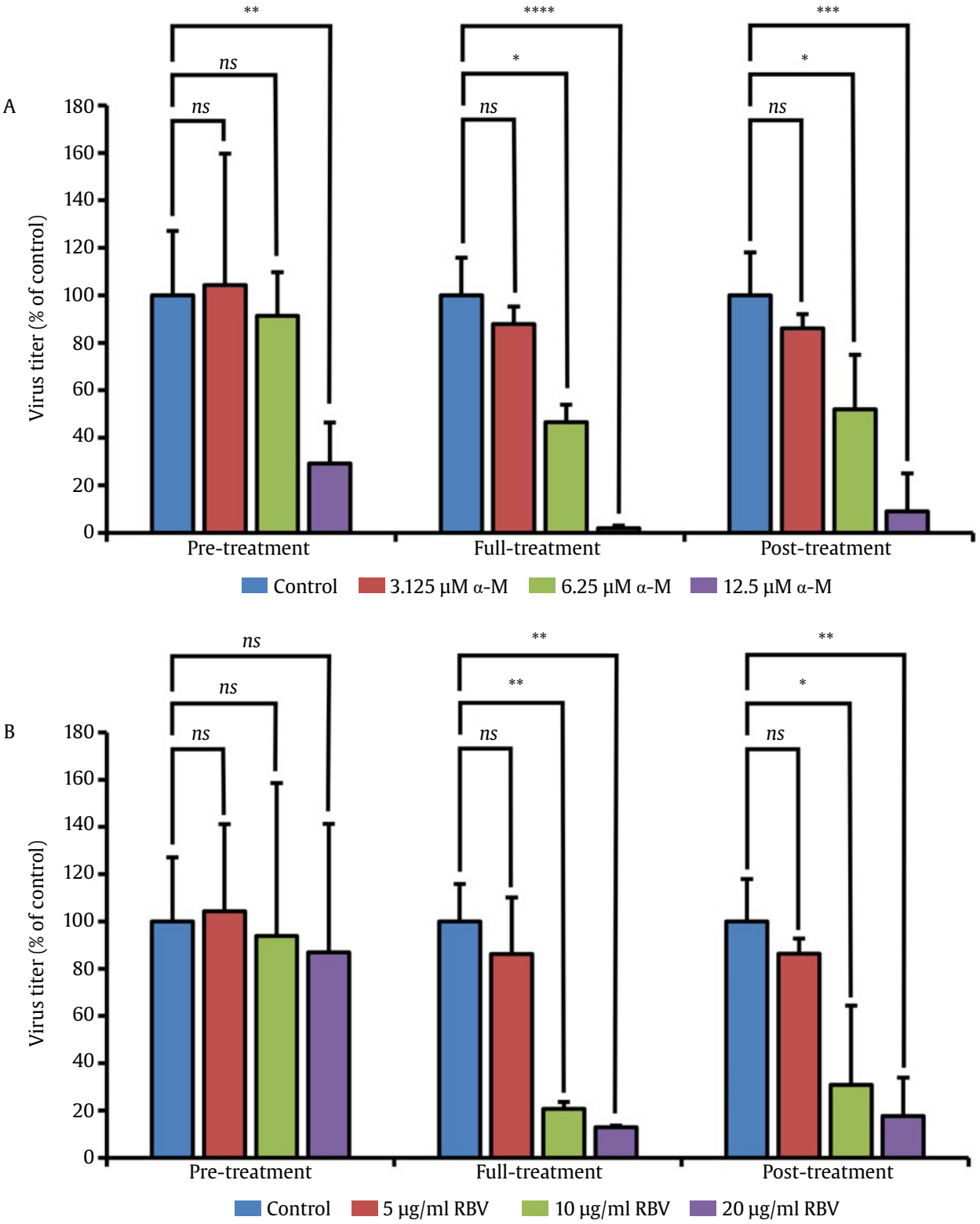

Figure 2. α-mangostin and ribavirin antiviral assay results. Mean CHIKV titer reduction induced by addition of either α-mangostin (α-M) (A) or ribavirin (RBV) (B). Error bars represent SD. *ns* = not significant, * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001. All p-values are Dunnett's corrected

Table 1. IC_{50} and SI value of α-mangostin and ribavirin

	α-mangostin		Ribavirin	
	IC_{50} (μM)	SI value	IC_{50} (μg/ml)	SI value
Pre-treatment	7.79	1.39	55.62	1.58
Full treatment	5.99	1.81	7.08	12.44
Post-treatment	6.39	1.70	7.63	11.54

4. Discussion

In this study, we assessed the potential antiviral activity of α-mangostin against CHIKV *in vitro*. In terms of cell death, the patterns of cytotoxicity of α-mangostin in our study are generally in accordance with the existing literature, as previous studies have shown that α-mangostin at a concentration of 5-10 μM induces cell death in HepG2 cells (Wudtiwai *et al.* 2018). This cell death is also affected by the incubation time, as 24- and 48-hour incubations resulted in CC50 measurements of 5.5 μM and 20.44 μM (8.39 μg/ml), respectively (Fazry *et al.* 2018; Wudtiwai *et al.* 2018).

Other studies have also shown that α-mangostin can induce significant cell death with a concentration of ≥10 μM in A549 cells (Phan *et al.* 2018) and ≥20 μM in HeLa cells (Lee *et al.* 2017), which are also human cancer cell lines. The anticancer effects of α-mangostin have been observed extensively both in vitro in a variety of human cancer cell lines (Chao *et al.* 2011; Wang *et al.* 2011; Shan *et al.* 2014; Won *et al.* 2014; Lee *et al.* 2017; Mohamed *et al.* 2017; Fazry *et al.* 2018; Phan *et al.* 2018; Wudtiwai *et al.* 2018; Markowicz *et al.* 2019), as well as *in vivo* in xenograft models (Johnson *et al.* 2012; Hsieh *et al.* 2013; Lee *et al.* 2016). In HepG2 cells, α-mangostin induces apoptosis and necrosis, possibly mediated by the caspase-dependent pathway (extrinsically and intrinsically) through caspase activation (Mohamed *et al.* 2017). Administration of α-mangostin has been observed to activate caspase-3, -8 and -9, and block the ERK1/2 and AKT signaling pathways in these cells (Fazry *et al.* 2018; Wudtiwai *et al.* 2018). Additionally, α-mangostin has been found to be a potent agonist of human stimulator of interferon genes (STING) receptor, and stimulates conversion of macrophages into pro-inflammatory versions, both of which likely contribute to its anticancer and antiviral action (Zhang *et al.* 2018).

Previous studies have also noted the dose-dependent cytotoxicity of ribavirin, a known potent CHIKV antiviral, which is traced to its inhibition of cell proliferation, namely through G1 arrest following 16-48 hours of exposure (Liu *et al.* 2012). In this study, we assessed the cytotoxicity of ribavirin in HepG2 cells. Out data show that ribavirin exerts its cytotoxic effect in HepG2 cells at CC_{50} of 88.07 μg/ml. Previous measurements of CC_{50} values of ribavirin following a three-day incubation were 65.01 μg/ml and 50.21 μg/ml in Vero and A549 cell lines, respectively (Franco *et al.* 2018), which puts our findings roughly in line with the literature. Ribavirin toxicity varies between cell lines, as different cell types have variability in intracellular metabolic rates and host cell kinases, causing variations in intracellular concentrations (Liu *et al.* 2012).

In terms of antiviral activity, our results show a strong inhibition of CHIKV replication at higher concentrations of α-mangostin. The reported IC_{50} values obtained in this study are in line with results against other viruses, as previous α-mangostin studies demonstrated its ability to inhibit DENV production in HepG2 cells in a post treatment scenario (Tarasuk *et al.* 2017) and inhibit DENV replication in PBMC cells with an IC_{50} of 5.77 μM after a 48-hour incubation (Sugiyanto *et al.* 2019). α-mangostin has also been found to inhibit replication of HCV, with a 50% effective concentration (EC_{50}) of 6.3 μM (Choi *et al.* 2014). Another study (Shaneyfelt *et al.* 2006) showed that α-mangostin can inhibit rotavirus infectivity in pre-treatment and inhibit virus replication in post-treatment, possibly by stimulating inflammatory and antiviral cellular pathways, namely through NF-κB activation and stimulation of IL-8 secretion (Shaneyfelt *et al.* 2006).

A previous evaluation of α-mangostin against CHIKV (Patil *et al.* 2021) did not report an IC_{50} value, although it showed ≥89% reduction of viral titer at 8 μM in all treatment, which is a stronger effect than reported here. These differences are likely to originate from differences in cell lines (Vero E6 vs. HepG2) and CHIKV strains, as well as the use of a different purity of α-mangostin (83% vs ≥98% in this study). This study shows a similar pattern when comparing treatment types, with the full combined treatment producing the strongest effect. Their molecular docking analysis suggested that α-mangostin binds strongly to CHIKV replication complexes, including its ns4p RNA-dependent RNA polymerase (RdRp), further suggesting the antiviral effects of the compound are due to its inhibition of CHIKV replication. While molecular docking also

suggested that α-mangostin may inhibit CHIKV entry into cells by targets on the E1 and E2 domains, as it does in rotaviruses (Shaneyfelt *et al.* 2006), our findings do not support this suggestion.

Our results also show ribavirin to be a potent inhibitor of CHIKV replication, with a significantly reduced effect if compound exposure occurs before infection (Figure 2B). This is in line with previous findings, although some variability may be expected due to the use of different cells, MOIs, virus strains and incubation times between studies (Pabbaraju *et al.* 2019). Similar to our results here, studies using the post treatment condition with three-day incubation reported an IC_{50} values of 2.58 µg/ml in Huh-7 cells (Franco *et al.* 2018), and 3.79 µg/ml in Vero cells (Rothan *et al.* 2015). Just as in our study, pretreatment with ribavirin has been observed to produce a much weaker response, with the IC_{50} value in Vero cells reported at 83.3 µg/ml following 18 hours incubation (Briolant *et al.* 2004). These results support observations that ribavirin exerts its antiviral action by inhibiting viral genome replication by the CHIKV RdRp (Crotty *et al.* 2001, 2002; Graci and Cameron 2002).

To compare the relative effective use and safety of each compound, we calculated the ratio of IC_{50} to CC_{50}, also known as the selectivity index (SI). High SI values are always desirable to proceed from in vitro to *in vivo* studies, as SI values greater than 1 indicate more benefits than risks (Subudhi *et al.* 2018). The SI values of α-mangostin and ribavirin at full treatment were greater than that of pre-treatment and post-treatment (Table 1), indicating that this treatment produces the highest effective benefits and safety. The SI value of α-mangostin was also lower in all cases than that of ribavirin, indicating that ribavirin demonstrates better efficacy and safety than α-mangostin. When comparing the antiviral activity of α-mangostin to its toxicity using the selectivity index, it becomes clear that this compound provides a rather narrow therapeutic window. While there is no fixed requirements to consider a compound sufficiently safe, other antiviral compounds already approved for human use have shown higher SI values (reviewed in (Hucke and Bugert 2020)). However, previous in vivo evaluations of α-mangostin did not remark on toxicity in mice at doses of up to 138 mg/kg (Parkhe *et al.* 2020; Rana *et al.* 2020; Patil *et al.* 2021), suggesting that this may not be a major concern for future research.

The results obtained from the virucidal assay showed that neither α-mangostin nor ribavirin had a direct virucidal effect on CHIKV (ANOVA $p = 0.12$ and 0.99 for α-M and RBV, respectively), with no significant differences in titer compared to control in any case (not shown). This is in accordance with previous research (Shaneyfelt *et al.* 2006; Choi *et al.* 2014; Tarasuk *et al.* 2017), which showed that the antiviral activity of α-mangostin occurs by inhibiting viral replication. This is reflected in our results, as antiviral activity occurs when the test compound is added to cells that have already been infected with the virus. These results are not, however, sufficient to determine at which exact point in the replication cycle this inhibition occurs, as more direct assays would be necessary to do this, such as has been done with other antiviral compound screening of alphaviruses (Varghese *et al.* 2022). While molecular docking of α-mangostin with CHIKV has identified some potential binding sites, as mentioned above (Patil *et al.* 2021), these would need confirmation in an in vitro setting in future studies.

This study has demonstrated that α-mangostin effectively inhibits CHIKV replication in HepG2 cells in a concentration-dependent manner. We have shown that the 'full treatment' condition of incubating the virus with the compound before infection and adding the compound after infection to be the most effective. Our results also show that ribavirin consistently demonstrates a better efficacy and safety profile than α-mangostin in the tested treatments. We also showed that neither α-mangostin nor ribavirin have any direct virucidal effect against CHIKV, supporting the theory that these compounds inhibit viral replication in the cell.

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