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RESEARCH ARTICLE

Antiviral potential of Tualang honey in Chikungunya virus-infected human synoviocytes

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ABSTRACT

Chikungunya is a mosquito-borne viral disease caused by chikungunya virus (CHIKV), characterised by fever, polyarthralgia, myalgia, rash, and headache, with chronic arthralgia persisting for months or years. Although vaccine is recently available, it is not widely used, and treatment focuses on symptom relief. Many natural products or their active compounds have been investigated for their anti-CHIKV activities on Vero cells which is irrelevant in CHIKV pathogenesis in human. Tualang Honey has also been shown to exert anti-CHIKV in Vero cells. This study advances the evaluation of Tualang honey's antiviral activity against CHIKV by utilising human fibroblast-like synoviocyte (HFLS) cells, a model more reflective of joint pathology in chronic infection. The maximum non-toxic dose (MNTD) of honey was determined using XTT assay. The effects of different concentrations and incubation times of honey were explored using pre-treatment and post-treatment assays, while anti-adsorption and anti-entry assays were used to investigate its antiviral activities. The MNTD of Tualang honey on HFLS cells was determined to be 50 mg/mL. Tualang honey at concentrations $\leq$ 20 mg/mL exhibited variable prophylactic activity in the pre-treatment assay, reducing viral titres by 29.21% to 94.87%. Significant CHIKV inhibition ($p < 0.05$) was observed with 10 and 15 mg/mL pre-treatment for 6 and 12 hours before infection, respectively. Notably, its anti-CHIKV effects were stronger during pre-treatment than post-treatment assay. Post-treatment with honey caused minimal to strong CHIKV inhibition, lowering viral titres by 6.67%–72.46%, depending on the concentration and incubation time, although it was not statistically significant. Tualang honey showed stronger anti-entry than anti-adsorption effects, decreasing viral titres by up to 90.45% and 66.89%, respectively. This study highlights Tualang honey's anti-CHIKV activity through various mechanisms, although further research is needed to confirm its clinical relevance. Importantly, the effects of concentration and incubation time are essential factors in determining antiviral efficacy.

Keywords: antiviral; Tualang honey; CHIKV; HFLS.

INTRODUCTION

CHIKV is a re-emerging alphavirus transmitted by mosquitoes, first detected in Tanzania in 1952. Over the next five decades, the virus spread among vertebrate hosts and *Aedes (Ae.) aegypti* mosquitoes, triggering multiple outbreaks across Asia and Africa (Schmidt & Schnierle, 2022). A significant turning point occurred during the 2006 outbreak in La Réunion, where genetic analysis revealed that 90% of CHIKV isolates carried a mutation in the E1 glycoprotein (E1-A226V), replacing alanine with valine at position 226. While this mutation does not impact viral replication in *Ae. aegypti*, it notably enhances viral fitness in *Ae. albopictus*, increasing the virus's ability to spread via this mosquito species (Vairo *et al*., 2019; Schmidt & Schnierle, 2022). As a result, CHIKV has rapidly expanded its geographical range and is now documented in over 100 countries (Schmidt & Schnierle, 2022).

CHIKV infection typically manifests as a mild, self-limiting illness, characterised by fever, skin rash, myalgia, and arthralgia, which can persist for weeks or even months. Symptoms generally appear 4–7 days after viral exposure (Khongwichit *et al*., 2021). While most individuals recover from the acute phase within 5–14 days, over 40% experience prolonged arthritic complications lasting more than three months (Amaral *et al*., 2020). In chronic cases, severe joint pain, sometimes accompanied by arthritis can persist for months or even years, significantly reducing quality of life (Khongwichit *et al*., 2021). Currently, no specific antiviral treatment exists for CHIKV infection, and treatment primarily focuses on symptom relief through nonsteroidal anti-inflammatory drugs (Patil *et al*., 2021). Although the chikungunya vaccine IXCHIQ has been approved in the United States for adults aged 18 and older, its availability and use remain limited (CDC, 2024).

During infection, CHIKV can infect various cell types, including monocyte-derived macrophages, human epithelial and endothelial cells, primary dermal fibroblasts, and synovial fibroblasts (Sukkaew *et al*., 2018). Synovial tissue is a predominant target of chronic CHIKV infection, often associated with severe joint pain or arthritis (Sukkaew *et al*., 2018). HFLS which are the dominant cell type in synovial tissue, are among the target cells of CHIKV and are known to play a pivotal role in the pathogenesis of CHIKV-induced arthritis (Sukkaew *et al*., 2020). Infection of HFLS by CHIKV triggers the production of pro-inflammatory cytokines and chemokines, contributing to the recruitment of immune cells and the amplification of joint inflammation (Sukkaew *et al*., 2018).

Given the lack of effective antiviral therapies, researchers are exploring natural compounds as potential drug candidates for chikungunya fever. *In vitro* studies have examined the anti-CHIKV properties of various compounds, including myricetin (Muñoz *et al*., 2023), *Boswellia serrata* (Von Rhein *et al*., 2016), *Andrographis paniculata* (Wintachai *et al*., 2015), silvestrol (Henss *et al*., 2018), and *Oroxylum indicum* (Mohamat *et al*., 2018). These natural compounds demonstrated diverse anti-CHIKV properties, offering protection through multiple mechanisms, including virucidal activity, inhibition of viral replication, and blockage of viral entry (Mohamat *et al*., 2020). However, many of these *in vitro* studies utilised cell lines that do not directly contribute to CHIKV pathogenesis, particularly in severe and chronic infections. For instance, although chloroquine effectively inhibited CHIKV infection in Vero cells (a kidney cell line derived from the African green monkey) by targeting the early stages of the viral life cycle (Khan *et al*., 2010), clinical trials showed that it was ineffective in treating CHIKV infections in humans. Moreover, prolonged use of chloroquine may have adverse residual effects (Roques *et al*., 2018).

Tualang honey is a local Malaysian wild honey and it has been associated with valuable medicinal properties (Azman *et al*., 2021). Tualang honey is primarily composed of monosaccharides, including glucose (47.13%) and fructose (41.73%), as well as disaccharides such as maltose (4.49%) and sucrose (1.02%) (Azman *et al*., 2021). Apart from its carbohydrate composition, honey consists of approximately 20% water and a variety of minor but significant components, including proteins, vitamins, lipids, organic acids, enzymes, amino acids, volatile compounds, flavonoids, phenolic acids, carotenoid-like substances, and minerals (Moniruzzaman *et al*., 2013). Recent studies suggest that Tualang honey exhibits a range of therapeutic properties, including antibacterial (Al-Kafaween *et al*., 2020), anti-inflammatory (Ahmad *et al*., 2012), antioxidant (Mohamed *et al*., 2010), anticancer (Ghashm *et al*., 2010), and antidiabetic effects (Erejuwa *et al*., 2010), along with its ability to promote wound healing (Mat Lazim *et al*., 2013). Many of these benefits are comparable to Manuka honey, a well-documented honey from New Zealand and Australia (Ahmed & Othman, 2013; Kamal *et al*., 2021). Furthermore, *in vitro* studies have demonstrated that Tualang honey possesses antiviral activity against CHIKV infection in Vero cells (Barkhadle *et al*., 2021). Specifically, it achieved up to 99.71% inhibition in virucidal assays, demonstrated prophylactic effects with up to 98.22% reduction in viral replication during pre-treatment, and exhibited post-infection antiviral activity with 94.87% inhibition (Barkhadle *et al*., 2021). This present study aimed to investigate the effect of Tualang honey on CHIKV infection in HFLS cells, which are involved in the pathogenesis of CHIKV.

MATERIALS AND METHODS

Honey

Tualang honey was obtained from the Federal Agricultural Marketing Authority (FAMA) in Kuala Nerang, Kedah, Malaysia. A concentration of 0.5 g/mL honey was prepared by diluting the honey in 10 mL of ready-to-use synoviocyte growth medium without fetal bovine serum (FBS), followed by filtration using a 0.22 µm syringe filter. The stock solution was stored at 4°C and diluted further as required for subsequent experiments.

Cell lines

Primary HFLS, the main cells used in this study, were cultured in a ready-to-use synoviocyte growth medium. Vero C1008 cells were used for virus propagation, while Vero 76 cells were used for plaque assay to quantify viral titres. Both Vero cell lines were maintained in DMEM supplemented with 10% FBS. All cells, including HFLS and Vero cells, were maintained at 37°C with 5% CO_2.

CHIKV

The CHIKV strain used in this study, MY019 IMR/06/BP (GenBank accession number EU703761), belongs to the Asian genotype and was generously provided by Assoc. Prof. Dr. Wang Seok Mui from Universiti Teknologi MARA (UiTM). The virus was propagated in Vero C1008 cells, and the viral titres were measured using a plaque assay.

Cytotoxicity assay

A cytotoxicity test was conducted to determine the maximum non-toxic dose (MNTD) of Tualang honey. 100 µL of HFLS cells at a density of 2 x 10^4 cells/well were plated in a 96-well plate overnight. Following incubation, the cells were washed with 100 µL of hank's balanced salt solution (HBSS) and treated with 100 µL of ten different concentrations of Tualang honey (5–80 mg/mL) in triplicates. Untreated healthy cells were used as negative control, while cells treated with 5% DMSO were used as positive control. After 48 hours of incubation at 37°C with 5% CO_2, 25 µL of XTT Cell Viability Assay was added to the cells for 4 hours at 37°C with 5% CO_2. The absorbance was measured at 475 nm, and cell viability was calculated as a percentage using the following formula:

$$\text{Percentage cell viability} = \left(\frac{\text{average absorbance of treated calls}}{\text{average absorbance of negative control}}\right) \times 100$$

Pre-treatment assay

100 µL of HFLS cells were seeded at 2 x 10^4 cells/well in a 96-well plate and incubated overnight. The cells were washed with 100 µL of HBSS and then treated with 100 µL of various honey concentrations (5, 10, 15, and 20 mg/mL) in synoviocyte growth medium for 0, 6, 12, and 24 hours before infection. After washing, the cells were infected with 100 µL of CHIKV at a multiplicity of infection (MOI) of 0.5 diluted in 2% synoviocyte growth medium for 1.5 hours, then washed again and maintained in 260 µL of 2% synoviocyte growth medium at 37°C with 5% CO_2. The cells incubated with only media were used as negative control, while the cells incubated with only virus were used as positive control. The supernatant was collected 48 hours post-infection (hpi) to determine viral titres.

Post-treatment assay

100 µL of HFLS cells at 2 x 10^4 cells/well were plated in a 96-well plate and incubated overnight. After washing with 100 µL of HBSS, cells were infected with 100 µL of CHIKV (MOI 0.5) in 2% synoviocyte growth medium for 1.5 hours at 37°C with 5% CO_2. The cells were then washed again and treated with 260 µL of different honey concentrations (5, 10, 15, and 20 mg/mL) in synoviocyte growth medium at 0, 2, 4, and 8 hpi in triplicates. Cells infected with CHIKV without honey treatment served as the positive control, while cells maintained in media alone were used as the negative control. Supernatants were collected after 48 hpi and stored at -80°C for viral titre analysis.

Plaque assay

Plaque assay was used to quantify CHIKV viral titre. 500 µL of Vero 76 cells were seeded in a 24-well plate at a density of 1.5 x 10^5 cells/well. Then, serial 10-fold dilutions of virus-infected supernatant

were prepared in 2% DMEM. A volume of 100 µL from each virus dilution was added to the corresponding wells in duplicate. Cells without infection were used as negative controls. After 3 hours of incubation at 37°C with 5% CO_2, 500 µL of 4% CMC was added, and the plate was incubated for 72 hours. After staining with methylene blue in 10% formaldehyde overnight at room temperature, plaques were counted and expressed as pfu/mL.

Anti-adsorption assay

A total of 100 µL of HFLS cells at a density of 2×10^4 cells/well was seeded into a 96-well plate and incubated overnight. The cells were then washed with 100 µL of HBSS and exposed to 100 µL of CHIKV (MOI 0.5) in 2% synoviocyte growth medium. This was followed by treatment with 100 µL of Tualang honey at concentrations of 5, 10, 15, and 20 mg/mL. After a 1-hour incubation at 4°C, the cells were washed twice with 100 µL of HBSS and maintained in 260 µL of synoviocyte growth medium. After 48 hours of incubation at 37°C with 5% CO_2, the supernatant was collected and stored at -80°C for viral titre determination using a plaque assay.

Anti-entry assay

100 µL of HFLS cells at a density of 2×10^4 cells/well were cultured overnight in a 96-well plate. After washing with 100 µL of HBSS, cells were incubated with 100 µL of CHIKV (MOI 0.5) in 2% synoviocyte growth medium at 4°C for 1 hour. Following another washing step, 100 µL of Tualang honey (5, 10, 15, and 20 mg/mL) was added and incubated for 2 hours at 37°C with 5% CO_2. The cells were subsequently washed with 100 µL of HBSS and treated with 50 µL of citrate buffer (pH 3) to inactivate non-internalised viruses. The cells were then washed twice with 100 µL of HBSS, followed by the addition of 260 µL of synoviocyte growth medium per well. The plate was incubated at 37°C with 5% CO_2 for 48 hours, after which the supernatant was collected from CHIKV-infected HFLS cells for measurement of viral titres.

Statistical analysis

Statistical analysis was performed using SPSS software (version 28.0). Data were analysed using one-way ANOVA followed by Tukey's post-hoc test, considering a p-value < 0.05 statistically significant.

RESULTS

Determination of maximum non-toxic dose (MNTD) of Tualang honey on HFLS cells

Before evaluating Tualang honey's antiviral activity against CHIKV in HFLS cells, its cytotoxicity was assessed to ensure a safe, non-toxic concentration, confirming that any anti-CHIKV effects are due to its antiviral properties, not toxicity. In general, a concentration-dependent effect was evident, with higher concentrations of Tualang honey causing a progressive decline in HFLS cell viability. As shown in Figure 1, HFLS cells exposed to Tualang honey at 5, 10, 15 and 20 mg/mL maintained over 90% viability with 103.87% ± 2.14, 101.83% ± 5.20, 95.72% ± 1.53 and 92.76% ± 4.28, respectively. Exposure to Tualang honey at concentrations of 30, 40, and 50 mg/mL led to a reduction in cell viability, ranging from 82.67% ± 2.96 to 84.20% ± 7.54. However, when HFLS cells were treated with concentrations exceeding 50 mg/mL, cell viability further declined, ranging from 77.06% ± 3.87 to 50.66% ± 0.71. As defined by the ISO 10993-5:2009 standard (ISO 10993-5:2009, 2009), a compound is considered non-toxic if treated cells maintain a viability of 80% or higher. As shown in Figure 1, 50 mg/mL of Tualang honey was identified as the MNTD for HFLS cells. However, to ensure reliable results, concentrations maintaining over 90% viability (5, 10, 15, and 20 mg/mL) were chosen for subsequent assays.

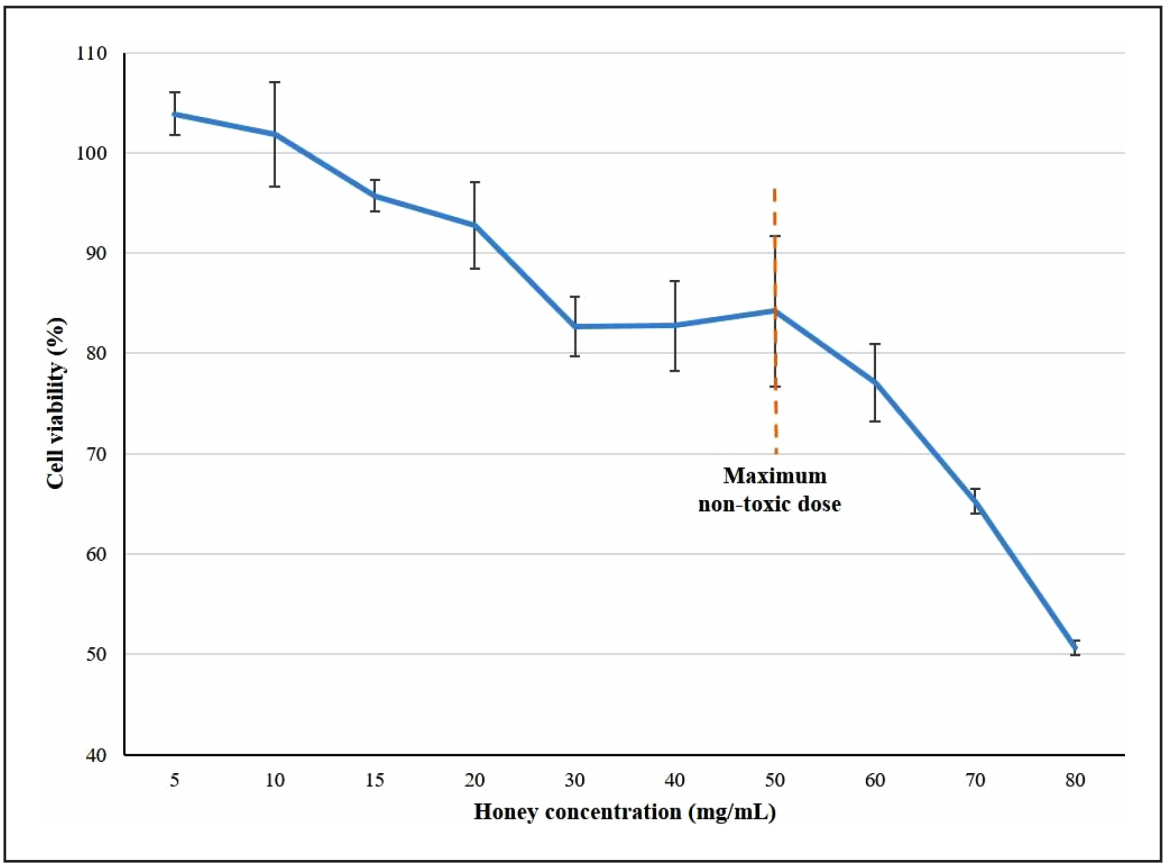

Figure 1. Determination of MNTD of Tualang honey on HFLS cells. HFLS cells were exposed to 10 different Tualang honey concentrations and cells viability was measured after 48 hours.

Effect of Tualang honey during pre-treatment assay

A pre-treatment experiment was performed to determine whether Tualang honey could protect HFLS cells from CHIKV infection. As shown in Figure 2, CHIKV replication was inhibited in a dose-dependent manner with up to 15 mg/mL of Tualang honey during 12 hours of pre-treatment prior to infection, with this time point demonstrating the best antiviral effects of Tualang honey. CHIKV titres declined from $\log_{10}$ 5.41 ± 0.21 pfu/mL to $\log_{10}$ 4.91 ± 0.24 pfu/mL (68.38% reduction), $\log_{10}$ 4.85 ± 0.15 pfu/mL (72.46% reduction), and $\log_{10}$ 4.12 ± 0.24 pfu/mL (94.87% reduction) at concentrations of 5, 10, and 15 mg/mL, respectively. Significant inhibition was also observed following pre-incubation with 10 mg/mL of Tualang honey for 6 hours prior to infection, achieving a 90.45% reduction in CHIKV replication ($p < 0.05$).

Effect of Tualang honey during post-treatment assay

The post-treatment assay evaluated the ability of Tualang honey to inhibit viral replication after CHIKV infection had already been established. As shown in Figure 3, although various concentrations of Tualang honey (5–20 mg/mL) exhibited inhibitory effects at different time points, no statistically significant reduction in viral titres was observed across all conditions. However, at 4 hpi, a dose-dependent trend was evident between 5 and 15 mg/mL of Tualang honey. Specifically, at 4 hpi, concentrations of 5, 10, and 15 mg/mL inhibited CHIKV replication in HFLS cells by 6.67%, 71.16%, and 72.46%, respectively, reducing viral titres from $\log_{10}$ 3.86 ± 0.13 pfu/mL to $\log_{10}$ 3.83 ± 0.07, $\log_{10}$ 3.32 ± 0.11, and $\log_{10}$ 3.30 ± 0.28.

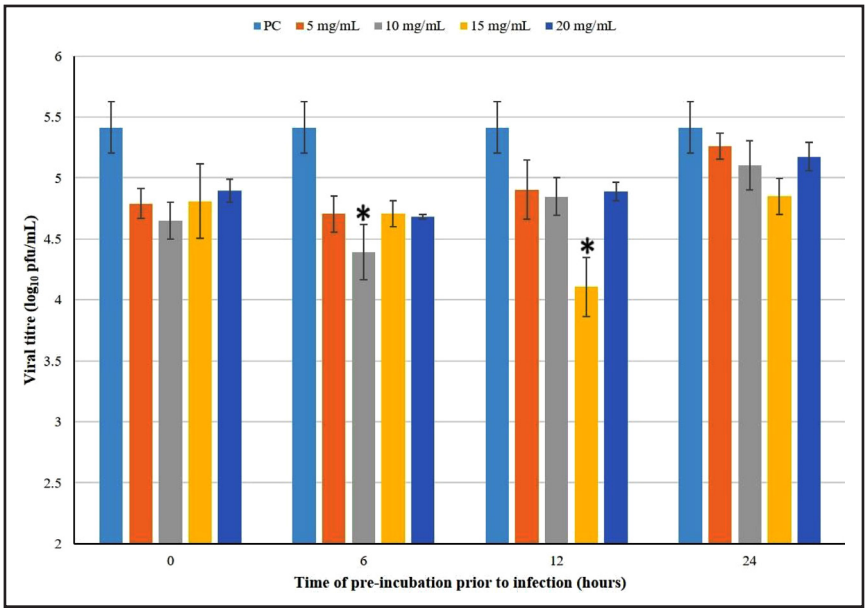

Figure 2. Effect of Tualang honey pre-treatment at different concentrations and hours on CHIKV viral titre in HFLS cells. HFLS cells were pre-incubated with Tualang honey for 0, 6, 12, and 24 hours, and viral titres were measured at 48 hpi. *There is a statistically significant difference ($p < 0.05$) between the viral titres of honey-treated virus-infected cells and those of non-treated virus-infected cells (PC).

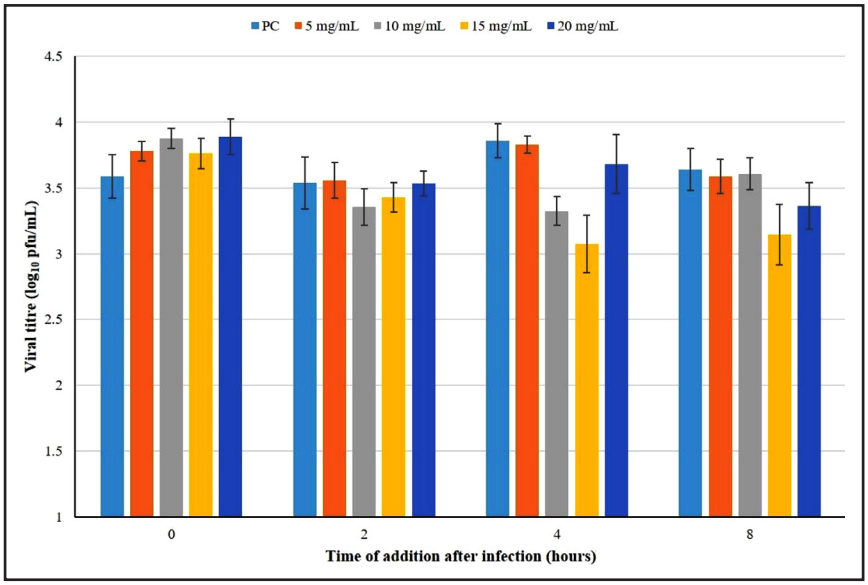

Figure 3. Effect of Tualang honey post-treatment at different concentrations and hours on CHIKV viral titres in HFLS cells. Values were expressed as the means ± S.E. from three replicates. Inhibition of viral replication in CHIKV-infected HFLS by Tualang honey was observed when the treatment was added at 2, 4 and 8 hpi, although it was not statistically significant ($p > 0.05$) compared to the untreated infected cells (PC).

Anti-adsorption effect of Tualang honey against CHIKV

The results suggested that Tualang honey might not interfere with CHIKV adsorption to HFLS cells. Although reductions in viral titres were observed, they were not statistically significant, and no consistent dose-dependent trend was evident across the concentrations tested. As shown in Figure 4, treatment of CHIKV-infected HFLS cells with Tualang honey at 10 and 20 mg/mL resulted in decreases in viral titres from $\log_{10}$ 3.87 ± 0.07 pfu/mL to $\log_{10}$ 3.39 ± 0.13 (66.89% reduction) and $\log_{10}$ 3.45 ± 0.13 pfu/mL (61.98% reduction), respectively, compared to the positive control.

Anti-entry effect of Tualang honey against CHIKV

As shown in Figure 5, the results demonstrated that Tualang honey inhibited CHIKV entry into HFLS cells in a dose-dependent manner, although statistically significant inhibition observed only at the two highest concentrations. Treatment with 5 and 10 mg/mL of Tualang honey reduced CHIKV titres from $\log_{10}$ 3.89 ± 0.10 pfu/mL to $\log_{10}$ 3.72 ± 0.13 pfu/mL (32.39% reduction) and $\log_{10}$ 3.49 ± 0.19 pfu/mL (60.19% reduction), respectively. The most substantial inhibition was observed with 15 and 20 mg/mL of Tualang honey ($p < 0.05$), with titres decreasing to $\log_{10}$ 3.08 ± 0.13 pfu/mL (84.51% reduction) and $\log_{10}$ 2.87 ± 0.11 pfu/mL (90.45% reduction), respectively.

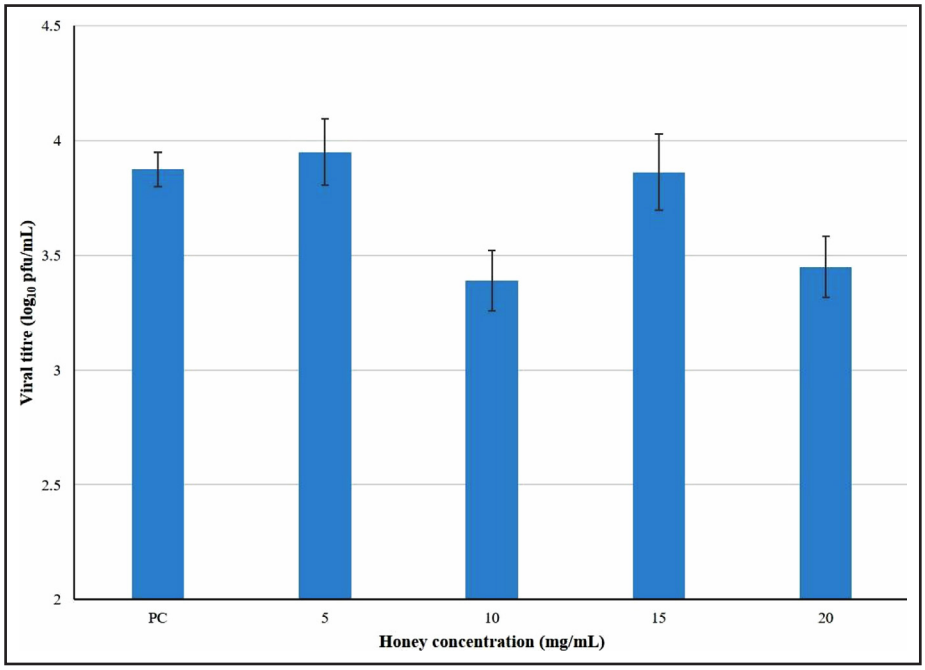

Figure 4. The effect of Tualang honey on CHIKV adsorption. Values were displayed as the means ± S.E. for three separate assays. Tualang honey inhibited viral adsorption at 10 and 20 mg/mL, although the effect was not statistically significant ($p > 0.05$) compared to the untreated control (PC).

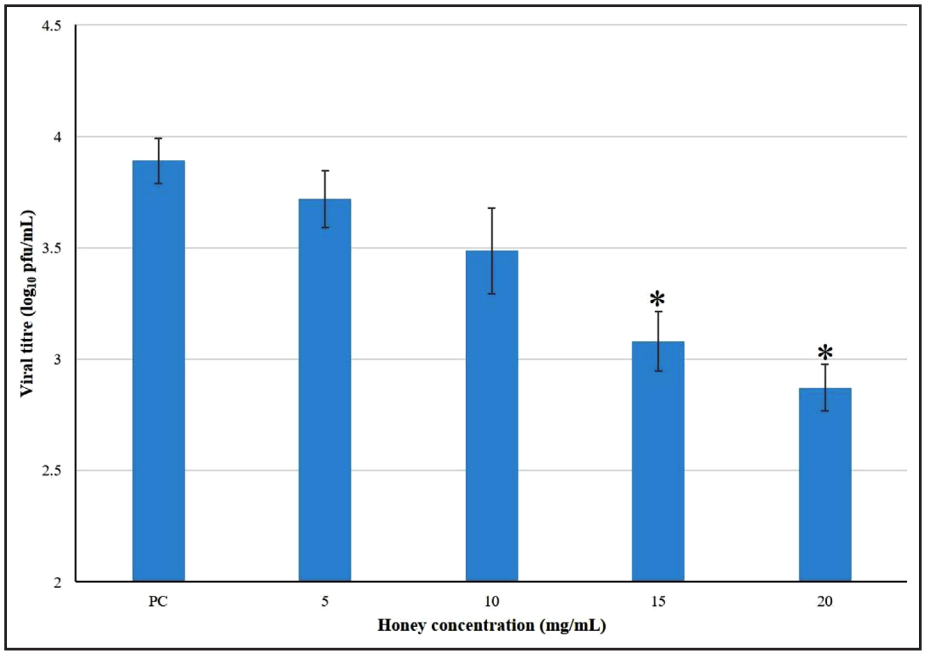

Figure 5. The effect of Tualang honey on CHIKV entry. Values were displayed as the means ± S.E. for three separate assays. Tualang honey dose-dependently inhibited CHIKV production in HFLS cells *Viral titres were significantly reduced ($p < 0.05$) in honey-treated infected cells compared to the untreated control (PC).

DISCUSSION

Tualang honey has notable medicinal properties, including antioxidant, anticancer, antimicrobial, and anti-inflammatory effects, attributed to its high phenolic acids and flavonoids (Ahmed & Othman, 2013; Kamal *et al*., 2021). Its *in vitro* antiviral activity against CHIKV in Vero cells has been demonstrated (Barkhadle *et al*., 2021), however as these are non-human cells, further research using more relevant cell models involved in chronic chikungunya is necessary. Persistent joint pain and stiffness are key features of the chronic stage of chikungunya (Abdelnabi *et al*., 2016), emphasising the importance of finding treatments to relieve these long-term symptoms. Tualang honey, recognised for its antiviral and anti-inflammatory properties (Azman *et al*., 2024), holds potential as a therapeutic option for managing chronic chikungunya. To validate these findings in a relevant human cell line, the antiviral potential of Tualang honey was investigated in HFLS cells, key targets during CHIKV chronic infection (Sukkaew *et al*., 2020), and its underlying anti-CHIKV activities were further explored.

The MNTD of Tualang honey on HFLS cells has not been investigated in previous studies. The cytotoxicity of Tualang honey in Vero cells has been examined (Barkhadle *et al*., 2021), however different cell lines may respond differently to the same compounds. Thus, determining the MNTD for HFLS cells is essential. In the present study, the MNTD of Tualang honey in HFLS cells, defined as maintaining cell viability above 80%, was determined to be 50 mg/mL. Factors like acidity, viscosity, osmolality, and hydrogen peroxide content within the honey may contribute to its potential toxicity (Brudzynski & Lannigan, 2012; Tan *et al*., 2014; Mohamad *et al*., 2018). As HFLS cells are sensitive primary cells (Phuklia *et al*., 2013; Skardal, 2015), Tualang honey concentrations of 5–20 mg/mL, maintaining >90% cell viability, were chosen for further experiments. Similar cytotoxicity levels were observed in Vero cells, where 5–20 mg/mL of Tualang honey also resulted in ≥90% viability (Barkhadle *et al*., 2021). Studies on Tualang honey's cytotoxicity across cell types show variability, often focusing on its anticancer properties and reporting the half-maximal inhibitory concentration (IC_{50}). While this differs from the MNTD used in the present study, both parameters offer valuable but distinct insights into cytotoxicity. For example, IC_{50} values for Tualang honey on human lung adenocarcinoma cell lines (H23 and A549) have been reported as 3.6 ± 0.6% and 3.1 ± 0.1% at 24 hours, decreasing to 2.7 ± 0.2% and 2.2 ± 0.0% at 48 hours, indicating increased cytotoxicity over time (Amran *et al*., 2020).

This study is the first to explore the antiviral activity of Tualang honey in HFLS cells, as prior research on these cells is unavailable. Primary cells are rarely used in antiviral studies due to their high cost, limited lifespan, and low proliferation (Eckerle *et al*., 2014). This study evaluated the anti-CHIKV activity of Tualang honey by assessing its ability to protect HFLS cells from infection, protect cells after infection has been established, and prevent CHIKV attachment and entry. Tualang honey may act as a prophylactic by preventing CHIKV infection or as a therapeutic by disrupting viral intracellular activities. Honey has demonstrated antiviral effects against various viruses, including human immunodeficiency virus (HIV), varicella-zoster virus (VZV), influenza virus, herpes simplex virus (HSV), and respiratory syncytial virus (RSV) (Al-Hatamleh *et al*., 2020; Abedi *et al*., 2021). Interestingly, methylglyoxal in Iranian honey has been found to inhibit HIV by blocking virion assembly (Behbahani, 2014), while flavonoids in honey, such as chrysin, quercetin, and kaempferol, may help combat coronaviruses by blocking viral entry and replication (Abedi *et al*., 2021).

In this study, Tualang honey exhibited minimal to excellent prophylactic activity against CHIKV infection in HFLS cells, as indicated by a reduction in CHIKV viral titres ranging from 29.21% to 94.87%. The findings suggest that Tualang honey shows strong prophylactic potential against CHIKV when applied at specific concentrations and incubation time, with 10 mg/mL and 15 mg/mL demonstrating the most effective inhibition. Pre-exposure for 6 hours with 10 mg/mL resulted in a 90.45% reduction in viral titres, while 12 hours with 15 mg/mL led to a 94.87% reduction. Significant prophylactic effects of Tualang honey have been observed, with up to a 98.22% reduction in CHIKV replication in Vero cells (Barkhadle *et al*., 2021). However, while their study showed the most protection with a 24-hour pre-treatment, this study found the least protection at the same time point. This difference is likely due to the use of different cell lines, which may respond differently to the antiviral treatment, leading to variations in the observed effects. Additionally, other studies have examined the prophylactic effects of various compounds against CHIKV. For instance, 8 µM α-mangostin reduced viral titres by 95% in Vero cells when added 4 hours before infection ($p < 0.001$), likely targeting early stages of CHIKV by disrupting receptor-mediated endocytosis (Patil *et al*., 2021). Similarly, 50 µM stearylamine reduced CHIKV FFU titres by 99.67% in Vero cells when added 4 hours before infection (Jeengar *et al*., 2021).

Beyond its prophylactic effects, Tualang honey exhibited minimal to strong therapeutic activity against CHIKV, reducing viral titres by 6.67% to 72.46%. Notably, Tualang honey exhibited stronger anti-CHIKV effects in prophylactic treatment compared to therapeutic treatment. Based on the findings, post-treatment with 15 mg/mL of Tualang honey achieved the greatest reduction in viral titres, especially at 4 and 8 hpi. This suggests that the antiviral activity of Tualang honey may be influenced by the concentration and relative composition of its bioactive constituents. Although the chemical composition of the Tualang honey used in this study was not analysed, previous study by Azman *et al*. (2021) reported that Tualang honey contains several types of flavonoids (catechin, hesperetin, quercetin, xanthohumol, chrysin, kaempferol, fisetin, naringenin, luteolin, vitexin, apigenin, isoorientin, pinobanksin-3-o-butyrate, pinobanksin-3-o-propionate, and 3,7,42 -trihydroxyflavone) and phenolic acids (gallic acid, salicylic acid, syringic acid, ferulic acid, benzoic acid, chlorogenic acid, trans-cinnamic acid, p-coumaric acid, and caffeic acid). Several compounds commonly found in honey, including methylglyoxal (MGO), copper, ascorbic acid, flavonoids, nitric oxide, hydrogen peroxide, and their derivatives have demonstrated potential antiviral activity by inhibiting viral replication and/or exerting virucidal effects (Watanabe *et al*., 2014). Moreover, Wan Yusuf *et al*. (2019) reported that specific bioactive compounds in Tualang honey, such as caffeic acid and quercetin which are strong antioxidants, exhibit potential anti-HIV 1 activity. These compounds could contribute to the observed anti-CHIKV activity of Tualang honey, although further studies are needed to confirm the exact mechanisms and active constituents involved. Significant therapeutic effects ($p < 0.05$) of Tualang honey was observed in Vero cells, with up to 94.87% viral inhibition achieved with concentrations between 5–15 mg/mL (Barkhadle *et al*., 2021). Several studies have also investigated the therapeutic potential of different compounds against CHIKV through post-treatment assays. For example, a 89% and 97% reduction in viral load in Vero cells was observed after 24 hours of treatment with 50 µg/mL and 100 µg/mL of *Uncaria tomentosa* extract, respectively (de Lima *et al*., 2024). Similarly, a 93.7% reduction in infectious virus titre was reported when tomatidine was added to Huh7 cells at 2 hpi (Troost *et al*., 2020).

It is worth noting that the highest concentration of Tualang honey (20 mg/mL) did not exhibit the anticipated antiviral effectiveness in either pre-treatment or post-treatment assays. This may be due to differences in the percentage or availability of particular chemical components between lower and higher concentrations of Tualang honey, which could influence its antiviral activity. One possible explanation is that at higher concentrations, the high sugar content, acidic pH and low water activity create an environment that is unfavourable to most pathogens (Ogwu & Izah, 2025) but may also be detrimental to host cells, potentially affecting cellular functions, virus-host interactions, and consequently, the antiviral

activity. Furthermore, it is also possible that the active components in Tualang honey reach saturation at lower doses, beyond which further increases in concentration do not result in enhanced antiviral activity. In fact, the 20 mg/mL concentration frequently exhibited little to no reduction in viral titres, which is consistent with findings reporting that the highest tested concentration of Tualang honey had minimal or no effect on CHIKV viral titres in Vero cells (Barkhadle *et al*., 2021). In contrast, andrographolide concentrations from 1 to 100 µM showed a dose-dependent reduction in CHIKV-infected cells, with up to 80% reduction at 100 µM (Wintachai *et al*., 2015).

Tualang honey demonstrated anti-entry effects against CHIKV infection in HFLS cells achieving up to a 90.45% reduction in viral titres. Its anti-entry effect likely involves Tualang honey interacting with viral or host factors, thereby blocking the virus from entering host cells (Abdul Ahmad *et al*., 2017).

Taken together, Tualang honey shows stronger prophylactic than therapeutic effects against CHIKV in HFLS cells, primarily by inhibiting early stages of infection, such as viral attachment and entry. It may also modulate cytokine production to enhance immune response, though further confirmation is needed. In conclusion, this study demonstrated that Tualang honey possesses anti-CHIKV properties and works through various mechanisms. However, these results should be interpreted with caution due to several study limitations including the lack of comparison with standard antivirals, variations in cell-type responses, and the focus on *in vitro* results, which may not fully reflect *in vivo* complexity. This study provides preliminary evidence of the potential antiviral effects of Tualang honey against CHIKV. However, at higher concentrations, the antiviral effects observed were inconsistent and sometimes contradictory, suggesting that the complexity of whole honey may influence its bioactivity. Therefore, future studies should focus on identifying and isolating the specific bioactive compounds responsible for the observed antiviral activity. Testing these individual components could offer greater insight into their mechanisms of action and help confirm their efficacy against CHIKV.

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Conflict of Interest Statement

The author declares that they have no conflict of interests.

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