



RESEARCH ARTICLE

In vitro Evaluation of Anti-Chikungunya Virus Activities of Tualang Honey

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ABSTRACT

Chikungunya is an infection caused by chikungunya virus (CHIKV). Although chikungunya has affected many countries in recent times, specific treatment or licensed vaccine are non-existent. In this study the potential antiviral properties of Tualang honey against *in vitro* CHIKV infection was evaluated. Cytotoxic test was performed using the XTT Cell Viability assay to determine maximum non-toxic dose (MNTD) in Vero cells. Using plaque assay, the potential antiviral activities of Tualang honey at various non-toxic concentrations and treatment regimens were evaluated. Tualang honey demonstrated virucidal effect with maximum inhibition CHIKV observed was 99.71% ($p < 0.05$). Tualang honey also had a prophylactic property by conferring protection to Vero cells during pre-treatment assay, resulting in up to 98.22% reduction of CHIKV replication under certain treatment regimen. Furthermore, Tualang honey exhibited anti-viral activities, with as much as 94.87% inhibition following post-treatment assay of Tualang honey in CHIKV-infected Vero cells. Additionally, Tualang honey also affected viral entry up to 82.21% after 48 hours of infection. These results suggest that Tualang honey has wide anti-CHIKV activities in Vero cells and exerts its effect through different mechanisms although these need to be further validated in other cells or model of CHIKV infection.

Keywords: Chikungunya, Tualang honey, antiviral, virucidal.

INTRODUCTION

Chikungunya is an infection caused by chikungunya virus (CHIKV) which is transmitted mainly by two species of *Aedes* mosquitoes; *Aedes aegypti* and *Aedes albopictus* (Morrone, 2007; La Beaud, 2008). CHIKV was first identified in Tanzania in 1952 and previous outbreaks have been confined to Africa and Asia (Perera-Lecoin *et al.*, 2016; Sanyaolu *et al.*, 2016). However, a global CHIKV epidemics that started in Kenya and spread to La Reunion Island in 2005-2006 followed by other countries was a major event that saw the spread of CHIKV to countries including India, Europe, the Caribbean, Latin America and South East Asia (Schuffenecker *et al.*, 2006; Morrison, 2014). Chikungunya may induce similar clinical manifestations to dengue and Zika (Heang *et al.*, 2012; Deeba *et al.*, 2016) including joint swelling and pain, fever, rashes, headache and muscle pain (Naresh Kumar *et al.*, 2007).

Despite its health threat, there is no exclusive antiviral agent or vaccine to treat or prevent CHIKV infection (Devasvaran & Yong, 2016). Alternative therapy for chikungunya using drugs that are used to treat other infections such as chloroquine, arbidol, ribavirin have been investigated, although there are issues regarding their effectiveness. Chloroquine has prophylaxis properties (Bernard *et al.*, 2010) but it is

ineffective in the late stage of infection when tested *in vivo* and during clinical trial (De Lamballerie *et al.*, 2008; Khan *et al.*, 2010). Ribavirin exhibits antiviral activity against CHIKV *in vitro* particularly when combined with doxycycline (Rothan *et al.*, 2015). However, ribavirin is only effective during the early stage of CHIKV life cycle (Mishra *et al.*, 2016). The antiviral properties of medicinal plants and their derivatives against CHIKV infection, albeit at *in vitro* level, have also been investigated by many. These include studies on *Vernonia amygdalina* (Chan *et al.*, 2016), curcumin from *Curcuma longa* (Von Rhein *et al.*, 2016), Epigallocatechin-3-gallate (EGCG) derived from green tea (Steinmann *et al.*, 2013), and silymarin extract from *Silybum marianum*'s fruit (Lani *et al.*, 2015). These compounds exert their effects against CHIKV through various mechanisms including direct inactivation of virus, blocking of CHIKV entry into cells and interruption of protein synthesis although the *in vivo* potential use of these compounds is unknown yet.

Tualang honey is a local Malaysian honey which is gathered from the honey combs of giant honey bee, *Apis dorsata* (Ahmed & Othman, 2013). The bees build their hives on Tualang tree (*Kompassia excels*) which is commonly found in Malaysian tropical forests (Bashkaran *et al.*, 2011). Similar to other honeys, Tualang honey also exhibits antibacterial

effects against a wide range of bacteria including *Salmonella typhi*, *Escherichia coli* and *Shigella sonnie* (Ahmed & Othman, 2013). Unfortunately, there is a dearth of evidence on the antiviral properties of Tualang honey which warrants further investigation. This current study was a preliminary study designed to examine the effect of Tualang honey on *in vitro* CHIKV replication.

MATERIALS AND METHODS

Honey

Tualang Honey was a kind gift from Prof. Siti Amrah Sulaiman. Five gram of fresh honey was diluted in 10 mL Dulbecco's Modified Eagle Media (DMEM) and filtered using 0.22 µm syringe filter. Further dilution was prepared as required during subsequent experiments.

Cell Lines

Vero cells were maintained in DMEM, containing 5% of foetal bovine serum (FBS), 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and penicillin/streptomycin and subsequently grown at 37°C with 5% CO₂.

CHIKV

CHIKV used in this study was originally isolated from a patient and was a kind gift from Prof. Shamala Devi (Universiti Malaya). CHIKV was propagated in Vero cells and viral titres were determined by plaque assay.

Cytotoxicity assay

Cytotoxicity test was performed to determine the maximum non-toxic dose (MNTD) of Tualang honey. Vero cells at 2x10⁴ cells per well were seeded overnight in 96-well plates. After incubation, following washing with PBS, the cells were treated with different Tualang honey concentrations in triplicates. Healthy cells without honey were used as a negative control, whereas cells treated with 5% DMSO in DMEM were used as a treated positive control as DMSO induces cell death in cells, followed by incubation for 48 hours at 37°C with 5% CO₂. Then, the XTT Cell Viability Assay was added to the cells for 3 hours at 37°C with 5% CO₂. The absorbance was read using ELISA plate reader at 475 nm. Cell viability was calculated as described in Mohamat *et al.* (2018).

Virucidal activity of Tualang honey

Virucidal activity is when particular compound has the ability to inactivate or kill free virus directly outside of infected host. Different amounts of CHIKV; 2 x 10⁵, 2 x 10⁴ and 2 x 10³ pfu/mL, were directly treated with different concentrations of honey; 5 mg/mL, 10 mg/mL, 15 mg/mL and 20 mg/mL in triplicates for 2 hours at 37°C with 5% CO₂. Following incubation, the inhibitory effect was determined by plaque assay. The experiments were done in duplicates or triplicates and repeated three times.

Pre-treatment assay

Vero cells were seeded (1 x 10⁵ cells per well) in a 24-well plate and incubated overnight. The cells were then washed with PBS before pre-treated with different concentrations of honey; 5 mg/mL, 10 mg/mL, 15 mg/mL and 20 mg/mL in triplicates for 2, 4, 6, 12 or 24 hours. Following this, the cells were washed with PBS and infection of CHIKV was done at a multiplicity of infection (MOI) of 0.05 prior to incubation at 37°C for 1.5 hours. The cells were washed with PBS and were then supplemented with DMEM medium containing 1% FBS and were further incubated at 37°C with 5% CO₂. The

supernatants from CHIKV-infected Vero cells were subsequently harvested 48 hours post infection (hpi) to determine viral titre. The experiments were done in duplicates or triplicates and repeated three times.

Post-treatment assay

Vero cells were seeded in a 24-well plate (1 x 10⁵ cells per well) incubated overnight at 37°C with 5% CO₂. The cells were infected with CHIKV at MOI of 0.05 for 1.5 hour. The infected cells were washed twice with PBS to remove the un-absorbed virus and incubated with the different concentrations of honey; 5 mg/mL, 10 mg/mL, 15 mg/mL and 20 mg/mL in triplicates for 0, 2, 4, 6 or 8 hours at 37°C with 5% CO₂. The supernatants were harvested after 48 hpi CHIKV infection. The experiments were performed in duplicates or triplicates and repeated three times.

Anti-adsorption assay

1 x 10⁵ cells per well were seeded in 24-well plates overnight. The cells were infected with CHIKV at 0.05 MOI, and added with honey before being incubated at 4°C for 1 hour. Following this, the treated Vero cells were washed three times with PBS to remove the non-adsorbed virus, and subsequently supplemented with DMEM containing 2% FBS and incubated at 37°C with 5% CO₂. Following this, supernatants were collected 48 hpi to determine viral titre. The experiments were performed in duplicates or triplicates and repeated three times.

Anti-entry assay

Confluent Vero cells in 24-well plates were infected with CHIKV (MOI 0.05) for 1 hour at 4°C. The infected cells were washed twice with PBS to remove the non-adsorbed virus, and then treated with different concentrations of Tualang honey, 5 mg/mL, 10 mg/mL, 15 mg/mL and 20 mg/mL, in triplicates for 2 hours at 37°C with 5% CO₂. The cells were washed again with PBS before were added with citrate buffer (pH 3) to inactivate the non-internalized viruses. The cells were washed again three times with PBS before supplemented with DMEM containing 2% FBS and incubated for 48 hours at 37°C with 5% CO₂. Viral titres were determined from the CHIKV-infected Vero cells' supernatants. The experiments were performed in duplicates or triplicates and repeated three times.

Plaque assay

Plaque assay was performed to determine CHIKV titres in supernatants collected from virus-infected and honey-treated samples. The assay was performed by seeding Vero cells in a 24-well plate. Then, the supernatants were diluted 10-fold in DMEM media supplemented with 1% FCS and 100 µl was added onto Vero cells prior to incubation for 3 hours. Subsequently, Vero cells were overlaid with 500 µl of 4% carboxymethylcellulose in DMEM. The cells were incubated for 3 days at 37°C with 5% CO₂. The cells were stained with methylene blue in 10% formaldehyde and plaques were counted. Viral titres were expressed as plaque forming unit per millilitre (pfu/mL).

Statistical analysis

Statistical analysis was performed using Statistical Package of Social Sciences (SPSS) software, version 25. All assays were performed in three independent experiments (biological replicates) and the values were presented as mean ± standard error (SE). Data from all the experiments except virucidal assays had normal distribution and thus, were analysed by using one-way ANOVA with Tukey's post-hoc test.

Data with non-normal distribution were analysed by Kruskal-wallis test. *P* value 0.05 was considered to be statistically significant.

RESULTS

Cytotoxic of Tualang honey

Prior to determining the potential anti-CHIKV activity of Tualang honey, its cytotoxic effect on Vero cells was investigated. Appropriate dose of honey that would not cause toxicity to Vero cells must be established such that cell changes during antiviral studies were affected solely by CHIKV. In this study, the viability of cells was inversely proportional to honey concentration and $\geq 90\%$ of Vero cells were viable when exposed to ≤ 20 mg/mL Tualang honey (Figure 1). As such, nontoxic dose of Tualang honey at 5 mg/mL, 10 mg/mL, 15 mg/mL and 20 mg/mL were selected for further antiviral experiments as described below.

Virucidal activity of Tualang honey

The ability of Tualang honey to kill or inactivate CHIKV directly was investigated by incubating 5, 10, 15 or 20 mg/mL of Tualang honey directly with different amounts of CHIKV; 2×10^5 , 2×10^4 , and 2×10^3 pfu. As shown in Figure 2, Tualang honey possess a moderate killing activity when 2×10^5 pfu/mL of CHIKV was exposed to 10 mg/mL and 15 mg/mL Tualang honey with inhibitory effect of 67.64% and 77.61%, respectively. The exposure of lower amount of CHIKV, 2×10^4 pfu and 2×10^3 pfu, to various concentration of Tualang honey resulted in a dose-dependent virucidal effect. At 2×10^4 pfu of CHIKV, Tualang honey at 15 mg/mL and 20 mg/mL caused CHIKV titres to reduce from \log_{10} 5.71 to 4.71 and 4.35 pfu/ml, respectively (90% and 95.63% reduction). Tualang honey had the greatest virucidal activity when it was exposed to 2×10^3 pfu CHIKV, the lowest amount of virus tested in this study. The viral titres reduced from \log_{10} 5.82 to 4.25, 3.83, 3.57 and 3.27 pfu/mL (97.30%, 98.97%, 99.43% and 99.71% reduction, $p < 0.05$) upon incubation with 5 mg/mL, 10 mg/mL, 15 mg/mL and 20 mg/mL, respectively (Figure 2).

Effect of pre-treatment assay

The ability of Tualang honey to confer protection to Vero cells prior to CHIKV infection was evaluated by pre-treatment assay. To examine the effect of concentration and incubation period of honey, Vero cells were pre-incubated with 5 mg/mL, 10 mg/mL, 15 mg/mL or 20 mg/mL for 2, 4, 6, 12 or 24 hours followed by CHIKV infection. In general, Tualang honey pre-treatment on Vero cells affected CHIKV replication although it was more influenced by the pre-treatment time rather than honey concentration (Figure 3). At 12-hour pre-treatment, Tualang honey at various concentrations exerted similar levels of inhibition (reduced from \log_{10} 7.25 pfu/ml to \log_{10} 6.80 pfu/ml and below). The highest inhibitory effect of Tualang honey was observed when Vero cells were exposed for 24 hours prior to CHIKV challenge (Figure 3). Statistically significant reduction at this time point was demonstrated following pre-treatment with 5 mg/mL and 10mg/mL Tualang honey. Viral replication decreased from \log_{10} 7.31 pfu/mL to 5.65 and 5.56 pfu/mL, respectively (97.81% and 98.22% reduction, $p < 0.05$) (Figure 3).

Effect of post-treatment assay

Post-treatment assay was conducted to determine the antiviral activities of Tualang honey against CHIKV. Vero cells were infected with CHIKV and various concentrations of honey were added at 2, 4, 6 or 8 hpi. Based on the plaque assay results obtained at 48 hpi, post-incubation of CHIKV-infected Vero cells with Tualang honey at 4 hpi resulted in a viral reduction. When exposed to 5 mg/mL and 10 mg/mL honey, viral titres significantly decreased from \log_{10} 7.06 pfu/mL to 6.20 and 6.12 pfu/mL, respectively (86.19% and 88.51% reduction, $p < 0.05$) (Figure 4). Similarly, CHIKV titres declined from \log_{10} 6.85 pfu/mL to 6.21, 5.96 and 6.04 pfu/mL (77.09%, 87.11% and 84.51% reduction) when 5, 10, and 15 mg/mL honey was added at 6 hpi, respectively (Figure 4). Interestingly, the most significant inhibitory effect was observed when between 5 to 15 mg/mL honey were added 8 hours after CHIKV infection. Viral titres diminished from \log_{10} 6.65 pfu/mL to

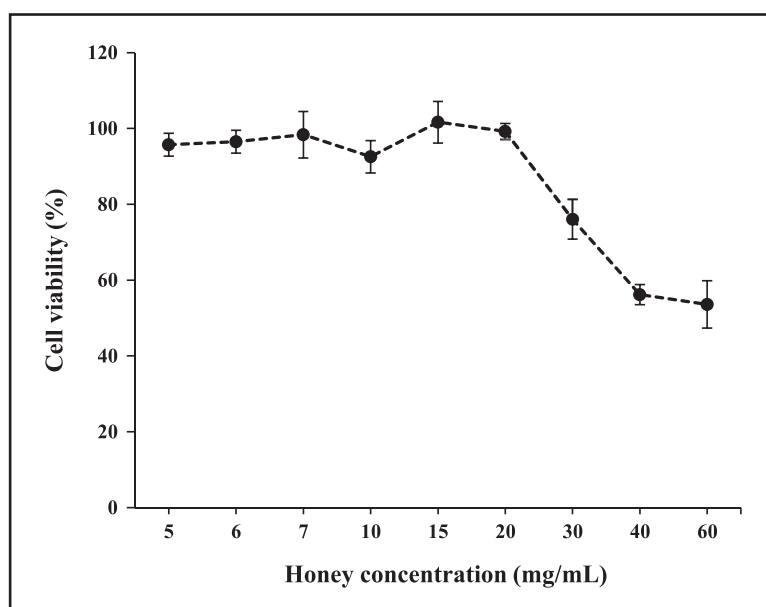


Figure 1. Cytotoxicity analysis of Tualang honey on Vero cells. The assay was performed after 48 hours of treatment with various concentrations of Tualang honey. The results are presented as percentage of cell viability in triplicates.

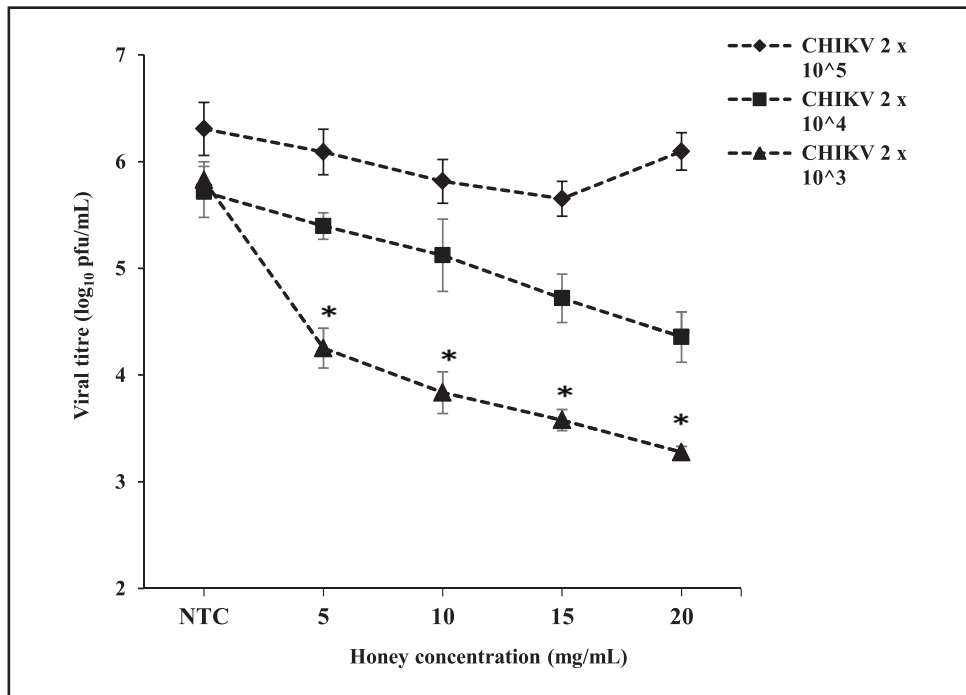


Figure 2. Virucidal activity of Tualang honey against different amounts of CHIKV. NTC refers to non-treated control, which was CHIKV-infected Vero cells without Tualang honey treatment. Values were expressed as mean \pm SE and $p < 0.05$ was considered statistically significant (*).

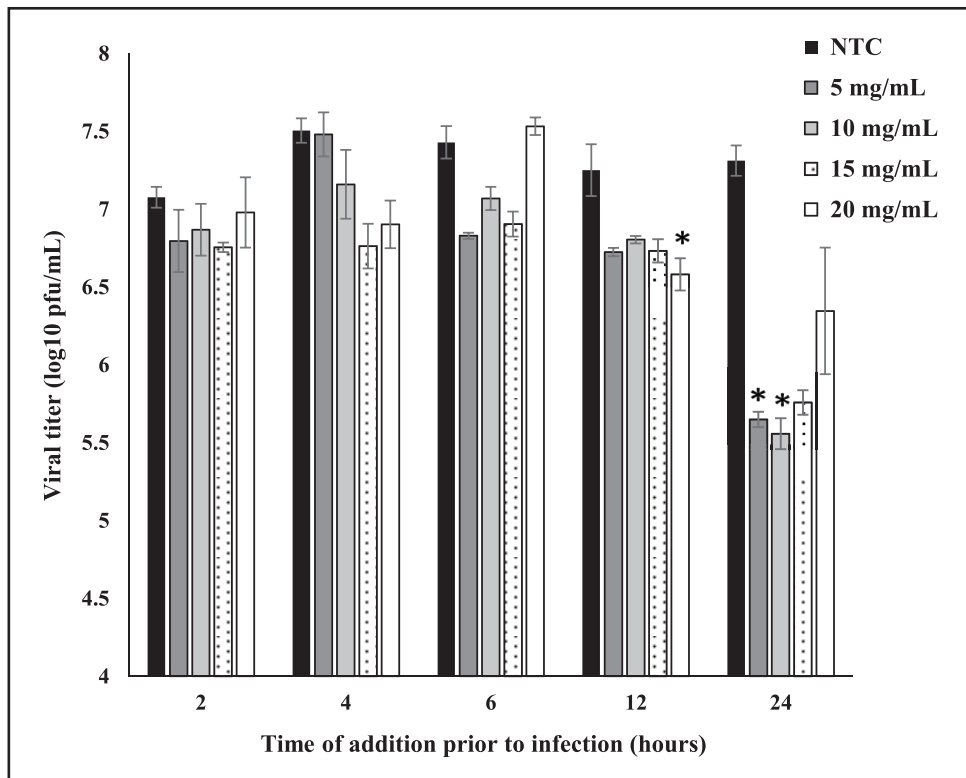


Figure 3. The effect of Tualang honey pre-treatment at different concentrations and hours on CHIKV replication. Vero cells were pre-incubated with Tualang honey at 2, 4, 6, 12 and 24 hours. Viral titres were monitored 48 hpi. NTC refers to non-treated control, which was CHIKV-infected Vero cells without Tualang honey treatment. Values were expressed as mean \pm SE and $p < 0.05$ was considered statistically significant (*).

\log_{10} 5.75, 5.61 and 5.36 pfu/mL, respectively (87.41%, 90.87% and 94.87% inhibition, $p < 0.05$) (Figure 4).

Anti-adsorption and anti-entry assays

As shown in Figure 5a, Tualang honey did not exert inhibitory effect during anti-adsorption assay as CHIKV replication was similar between CHIKV-infected Vero cells without Tualang honey pre-treatment (NTC) (\log_{10} 7.42) and CHIKV-infected Vero cells treated with different concentrations of honey (between \log_{10} 7.24 to 7.36 pfu/mL). In contrast, during anti-entry assay, CHIKV titres had reduced from \log_{10} 7.25 pfu/mL to \log_{10} 6.69, 6.56, 6.50 and 6.54 pfu/mL (72.45%, 79.58%, 82.21%, and 80.50% reduction) upon treatment with 5, 10, 15 and 20 mg/mL of Tualang honey, respectively (Figure 5b), suggesting a moderate anti-entry activity of Tualang honey.

DISCUSSION

Tualang honey possesses numerous biological activities including antimicrobial, anticancer, antioxidant and wound healing (Ghashm *et al.*, 2010; Khoo *et al.*, 2010; Moniruzzaman *et al.*, 2013). The composition, physicochemical properties, and pharmacokinetic of Tualang honey have been previously reported (Khalil *et al.*, 2011; Jaafar *et al.*, 2012). Tualang honey has various phenolic acids including gallic, syringic, benzoic, trans-cinnamic, p-coumaric, caffeic acid and flavonoid compounds such as catechin, kaempferol, naringenin, luteolin and apigenin (Khalil *et al.*, 2011). However, the actual composition of this honey varies depending on geographical region, floral and entomological sources and storage time (Jaafar *et al.*, 2012). Tualang honey has a dark colour intensity, indicating a higher levels of phenolic and flavonoid compounds compared with Manuka honey and other Malaysian honeys (Kishore *et al.*, 2011). Manuka honey

is a monofloral honey obtained from the manuka tree (*Leptospermum scoparium*) which naturally grows in New Zealand and Australia (Alvarez-Suarez *et al.*, 2014). Unlike Tualang honey, Manuka honey has been extensively studied for its various antimicrobial activities against many pathogens.

To the best of our knowledge, data pertaining to the antiviral activities of Tualang honey is still lacking. Hence, in this preliminary study, the potential effects of Tualang honey towards CHIKV were investigated through various assays as Tualang honey could directly inactivate or interfere with various CHIKV replication stages such as attachment, viral entry, and RNA viral replication. In this study, Tualang honey was demonstrated to have viral dose and honey concentration dependent virucidal activities against CHIKV. Tualang honey may exert its virucidal activity by inactivating the free virus particles which eventually may affect CHIKV infection rate in Vero cells. Similar to our finding, Manuka honey was shown to possess direct killing activity against influenza and varicella zoster virus (Watanabe *et al.*, 2014). Meanwhile, hepatitis C virus inactivation occurred directly following exposure to natural compound chebulagic acid (CHLA) and punicalagin (PUG) which protected the cell from subsequent infection (Tai *et al.*, 2015). In the current study, although reduced viral replication was demonstrated, Tualang honey did not exert significant virucidal activity when exposed to the highest dose of CHIKV. This could be because the concentration of honey may be insufficient in comparison to the number of CHIKV particles present. Hence, a higher number of CHIKV particles may not be inhibited leading to insignificant viral reduction. Perhaps, Tualang honey virucidal activity can be bolstered if the concentration is increased proportionately to the viral concentration/number of particles.

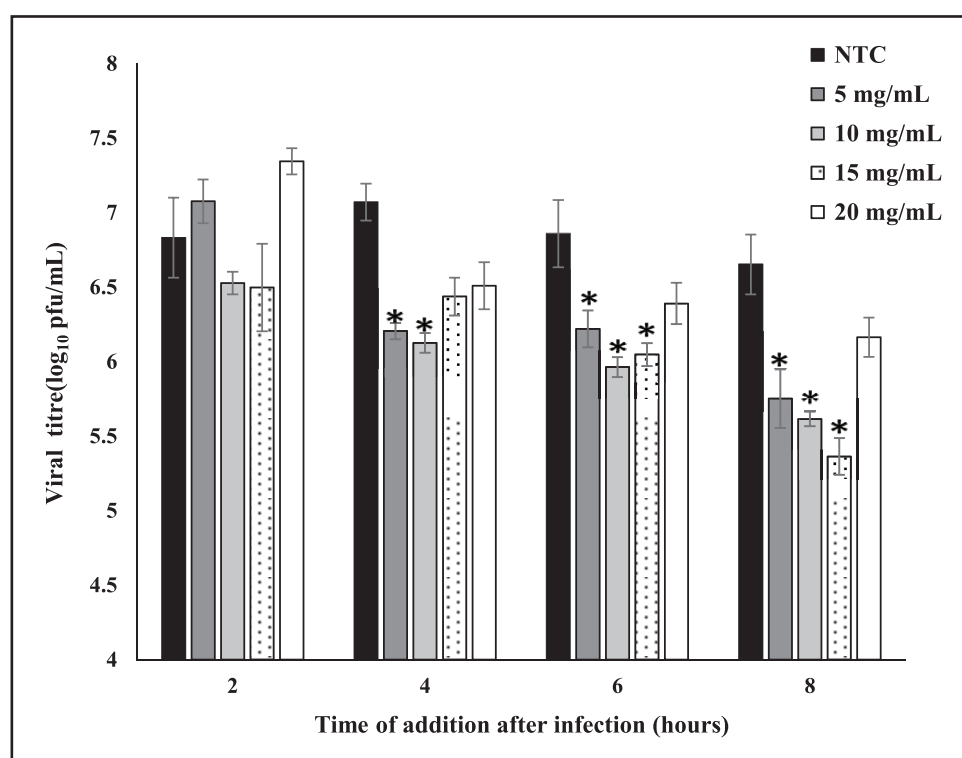


Figure 4. The effect of Tualang honey post-treatment at different concentrations and hours on CHIKV replication. NTC refers to non-treated control, which was CHIKV-infected Vero cells without Tualang honey treatment. Values were expressed as mean \pm SE and $p < 0.05$ was considered statistically significant (*).

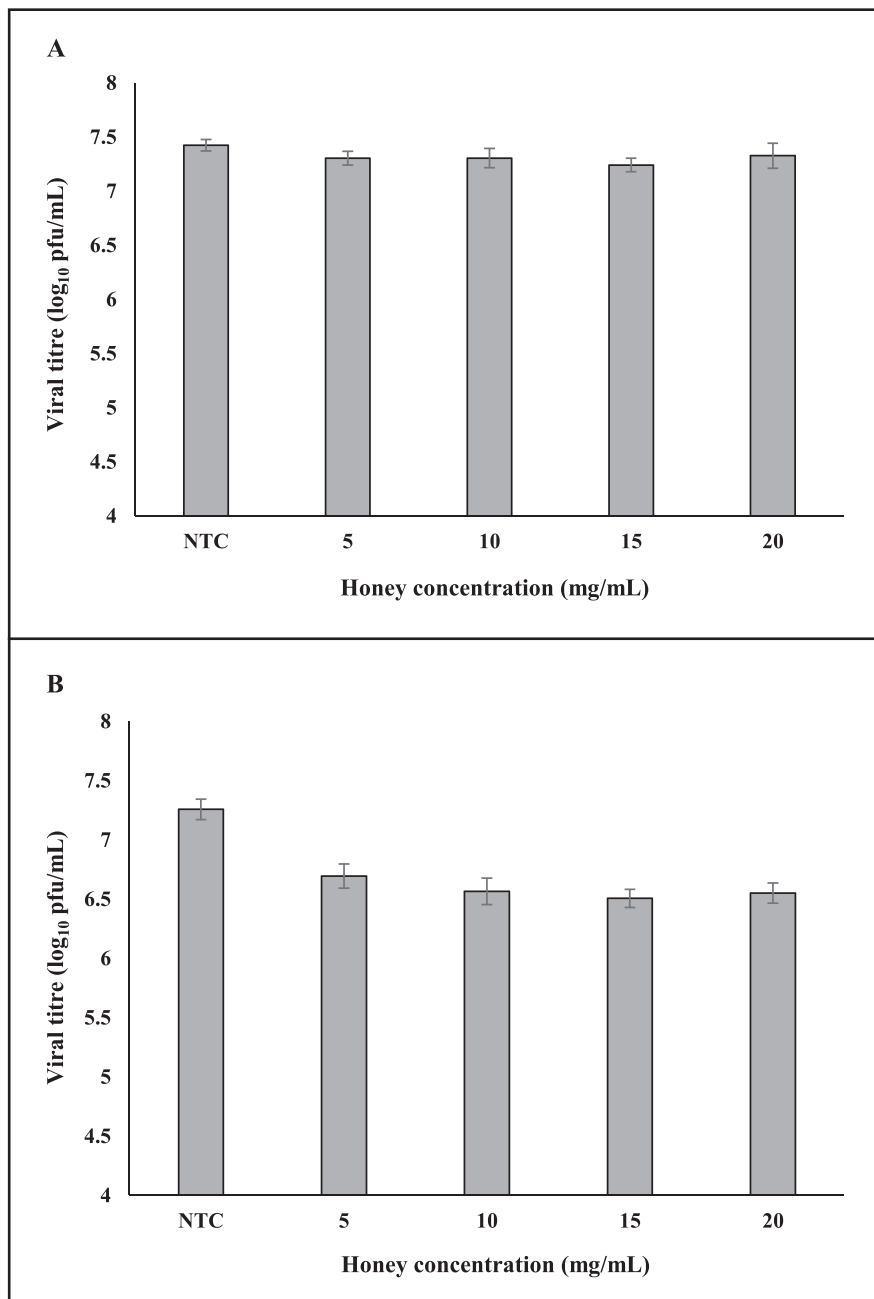


Figure 5. The effect of Tualang honey on CHIKV (a) adsorption and (b) entry. NTC refers to non-treated control, which was CHIKV-infected Vero cells without Tualang honey treatment. Values were expressed as mean \pm SE and $p < 0.05$ was considered statistically significant (*).

Tualang honey was demonstrated in this study to possess good prophylactic activities against CHIKV. The 24-hour pre-treatment conferred the most protection to the cells from CHIKV infection, resulting in as much as 98.22% viral inhibition. Perhaps this is because prolonged exposure to Tualang honey enabled cells to be protected sufficiently and prevented CHIKV from interacting with its target receptor on Vero cells and eventually from initiating replication. As such, Tualang honey has therapeutic potential at least at *in vitro* level if appropriate amount of honey is used at the right time. In contrast, it was demonstrated that Manuka honey could not confer cell protection against influenza virus following pre-treatment with 6.25 mg/mL or 25 mg/mL of honey (Watanabe *et al.*, 2014). A separate study had shown that Manuka honey was also unable to protect cell from

respiratory syncytial virus infection (RSV) (Zareie, 2011). Future study should be performed to shed further light on the different prophylactic activities of Tualang and Manuka honeys.

The post-treatment assay suggests that Tualang honey also affects the intracellular activities of CHIKV such as replication of viral RNA or translation/assembly of viral protein in infected Vero cells. As much as 94.87% inhibition of CHIKV replication was observed following treatment at 8 hours after CHIKV infection. Given that Tualang honey did not directly affect CHIKV binding and moderately disrupt CHIKV entry, as demonstrated by the anti-adsorption and anti-entry assays, respectively, this suggests that Tualang honey confers its strongest anti-CHIKV effect after viral entry, although it is not known currently at which CHIKV replication

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Conflict of Interests

The author declare that they have no conflict of interests.

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- stage is affected. Post-treatment of Manuka honey was shown to affect RSV replication by inhibiting the virus at the translation and transcription level as a reduction of viral mRNA was observed (Zareie, 2011). Methylglyoxal which is a major antibacterial compound in honey was shown to also possess great antiviral activity as it suppressed RSV activity. Methylglyoxal in some Iranian monofloral honeys has been shown to affect the late stage of HIV infection by blocking the assembly of new virion (Behbahani, 2014). Meanwhile, flavonoid apigenin extract was reported to interrupt viral RNA that is associated with host trans-acting factors during enterovirus 71 infection (Zhang et al., 2014). Thus, it is possible that Tualang honey could suppress CHIKV replication through the action of certain bioactive compounds, although further study is needed to verify this.
- The *in vitro* anti-CHIKV activity of Tualang honey also could be contributed by its other flavonoid and phenolic contents. Luteolin in Tualang honey could be another compound of interest. Luteolin has potent anti-Japanese encephalitis virus (JEV) activity as it exerts extracellular virucidal activity and inhibits JEV replication after viral entry stage (Fan et al., 2016). As mentioned earlier, apigenin is a flavonoid compound that is also present in Tualang honey and previous study reported that apigenin suppressed CHIKV replication by having 5,7-dihydroxyflavone structure that targets CHIKV replication rather than entry (Pohjala et al., 2011). It will be of interest to elucidate the role of these compounds in Tualang honey during CHIKV infection.
- It should be noted here that higher Tualang honey concentration does not correlate with better anti-CHIKV activity (Figure 3 and 4). In general, Tualang honey at 20 mg/mL did not have superior antiviral activity than the lower Tualang honey concentrations used during pre-treatment and post-treatment assays. In fact, this particular honey concentration not only caused the least reduction of viral titres, it sometimes also resulted in enhancement of CHIKV replication. This finding agrees with Muller and co-workers (2011) who observed an augmentation of yellow fever virus and dengue virus replication following exposure to crude venoms and isolated toxin from a South American rattlesnake. Similarly, flavone was also shown to enhance dengue virus replication when added during and after virus adsorption (Zandi et al., 2012). It is possible that at higher Tualang honey concentration, significant amount of particular component may present that could promote either viral binding/entry or intracellular activities associated with viral replication. As such, future study should include determining effective dose for Tualang honey as inappropriate dosage might have adverse effect on CHIKV-infected host.
- Taken together, this study provides further insights on the antiviral properties of Tualang honey and demonstrated that Tualang honey exerts *in vitro* anti-CHIKV activities through different mechanisms, by direct inactivation of CHIKV, protecting cells from CHIKV infection or affecting the intracellular activities during CHIKV replication. However, these results should be interpreted with cautious due to several limitations in the study. Firstly, the study was conducted using Vero cells which is of non-human origin, therefore it should not be presumed that similar effects would be observed in other cells particularly human cells or *in vivo* until further validation. As previous study has shown that certain medicinal plants have different antiviral effects depending on the host cells used (Husin et al., 2015), further testing in appropriate cells or animal models is needed. Lastly, the current study did not incorporate known antiviral agent such as ribavirin and therefore the efficacy of Tualang honey to inhibit CHIKV replication compare to a reference compound is unknown.
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