Biological properties of honey from *Apis mellifera*; Efficacy on inhibition of herpes simplex virus infection and antioxidant activity

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Abstract. Herpes simplex virus (HSV) is a common cause of oral and genital lesions and remains latent in nerve ganglia. Current antiviral treatments often use antiviral drugs like acyclovir, valacyclovir, and famciclovir. However, these drugs have side effects and drug-resistant strains may emerge. Thus, natural products such as honey are being explored as an alternative agent for viral inhibition. This study aimed to investigate the antioxidant activity of longan, lychee, and polyfloral honey, and evaluate efficacy of honey against HSV-1 infection via plaque reduction assays. The HSV-1 was treated with three different types of honey before, during, and after viral attachment to human epidermal keratinocyte (HaCaT) cells. Longan honey exhibited the highest total phenolic content of 1.375 ± 0.02 mg GAE/g and antioxidant activity of 0.850 ± 0.06 mg GAE/g. All three different types of honey exhibited less than 50% inhibition when treated before and after viral attachment. However, during viral attachment, all types of honey inhibited HSV-1 infection by more than 50% at a concentration of 3.125% W/V and polyfloral honey showed the strongest inhibitory effect by 70.87 ± 3.72%. These results suggest that the antiviral mechanism of honey may involve disrupting the viral particles or interfere during attachment of the virus to the cell. Therefore, the finding supports the potential anti-HSV activity of Thai longan, lychee, and polyfloral honey.

1. Introduction

Herpes simplex virus is classified within alpha subfamily of human herpesviruses. The viral structure is large, about 125–130 nm in diameter, spherically shaped with double-stranded DNA that is enclosed by an icosahedral capsid and a lipid bilayer envelope [1-3]. HSV infection is widely prevalent and typically manifests as painful blistering lesions. It often spreads by skin contact that can be treated but not completely cured and can cause recurrent infection. There are two types of herpes simplex viruses. HSV-1 is mainly spread via oral contact and causes infections around the mouth. It can also cause keratitis and in some cases cause genital herpes. HSV-2 is primarily spread via sexual and leading to genital lesions.

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However, it can also cause oral herpes in some cases [4]. Additionally, herpes simplex viral infection can lead to severe symptoms of the disease, including encephalitis, meningitis, and keratitis, which results in blindness [5]. The treatment of herpes simplex virus can be performed using antiviral drugs including acyclovir, valacyclovir, and famciclovir by inhibition of the viral replication. However, these medications cannot completely eradicate the infection and may cause side effects such as nausea, vomiting, stomach pain, frequent urination, loss of appetite, fatigue, or weakness. Due to these limitations of current antiviral treatments, natural products with antiviral potential have gained increasing attention as alternative therapeutic options for HSV infections. [6].

Honey is a natural substance that exists as a supersaturated solution or a semi-solid substance. It is synthesized by worker bees after they collect floral nectar, plant secretions, and the honeydew secretion [7]. The bees convert these substances by secreting enzymes in their salivary glands to break down glucose and fructose into invert sugar and exude honey before storing it in the honeycomb [8]. Honey consists of 70–80 % sugar as the main component. It contains approximately 70% monosaccharides and approximately 10% trisaccharides. The minor components include minerals, vitamins, flavonoids, organic acids, and various proteins and amino acids. The composition of honey depends on the type of bee, the source of the flower, environmental variables, honey production processes, and the various ratios of these elements that result in the aroma, color, viscosity, taste and different therapy activities [9-11]. In addition, honey also contains biologically active substances such as phenolics, flavonoids, polyphenols for antioxidant, antimicrobial, and immune stimulating properties. It also provides nutritional benefits and medicinal properties, including treating gastrointestinal disorders, burns, inflamed wounds, ulcers, and abscesses. It has anti-inflammatory and anticancer properties [7, 12]. Furthermore, honey has demonstrated anti-viral activities against various types of viruses, including influenza, human immunodeficiency virus, rubella virus, rabies virus and SARS-CoV-2 virus [13]. Previous study also demonstrated that honey and royal jelly exhibit inhibitory effects against herpes simplex virus type 1, with efficacy comparable to that of acyclovir. The study also demonstrated that Manuka honey was as effective as acyclovir to HSV-1 inhibition [14]. These findings indicate that honey may serve as an alternative natural product for the treatment of herpes lesions. Therefore, the efficacy of Thai longan, lychee, and polyfloral honey on antioxidant activity and the mechanism of herpes simplex viral inhibition were investigated in this study.

2. Methods

2.1 Determination of antioxidant activity of honey by DPPH radical scavenging assay

The antioxidant potential of honey was analyzed using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. DPPH solution was prepared at 20 mg/ml by dissolving 2 mg of DPPH in 100 ml of methanol. Then, 0.75 ml of the honey sample at various concentrations was mixed with 1.5 ml of the DPPH reagent. Incubation in the dark for 15 minutes at 25°C and measured at 517 nm using a spectrophotometer. Gallic acid at different concentrations was used as a standard solution, and the percentage of antioxidant activity was calculated [15].

2.2 Determination of total phenolic compounds in honey

The total phenolic compound was measured using a modified method from Isla et al., 2011 [15]. 0.5 ml of honey was combined with 2.5 ml of Folin-Ciocalteau reagent and incubated for 5 minutes. Then, 2 ml of 5% Na_2CO_3 (75 g/l) was added to the honey sample solution, and it was further incubated in the dark at 25°C for 2 hours. After incubation, the absorbance

of the solution was measured at a wavelength of 765 nm. A calibration curve was generated using gallic acid, and the results was reported as mg gallic acid/g honey.

2.3 Cytotoxicity test of honey on human epidermal keratinocyte (HaCaT) cell line by MTT assay

HaCaT cells (CLS cell lines service, 300493) are the immortalized human keratinocytes. The cells were cultured in a 96-well plate to grow into a monolayer. Honey was diluted with DMEM medium in various concentrations and added into each well. The cells were maintained at 37°C in a 5% CO_2 incubator for 72 hours. Then, the MTT reagant was applied into each well and further incubated under the same conditions for 4 hours. The formazan crystals were dissolved with DMSO solution, and absorbance was recorded at 540 and 630 nm. The obtained values were used to calculate cell viability. The antiviral activity against herpes simplex virus was further tested using the honey at non-toxic concentration [16].

2.4 Inhibition of herpes simplex virus type 1 with honey before viral attachment to HaCaT cells

HaCaT cells were seeded with a density of 1.5×10^5 cells in 24-well plates and incubated for 24 hours. The culture medium was removed and honey at non-toxic concentrations was added to the wells. The plates were incubated on a rocking platform for 1 hour at room temperature. After incubation, herpes simplex virus was treated to the wells of the culture plate and incubated at room temperature for 1 hour on the rocking platform. Then, overlay media were added to all wells and the plates were incubated at 37°C in a 5% CO_2 incubator for 3 days. Subsequently, the infected cells were stained with 0.1% crystal violet staining. The number of viral plaques was counted and compared with the infected cell control to calculate the percentage of virus inhibition [16].

2.5 Inhibition of herpes simplex virus type 1 with honey during viral attachment to HaCaT cells

HaCaT cells was plated in 24-well plates at a density of 1.5×10^5 cell and cultured for 24 hours. The culture medium was removed, and herpes simplex virus was applied to the wells. Then, honey at non-toxic concentrations was added into each well and incubated at room temperature for 1 hour on the rocking platform. After incubation, overlay media were added to all wells and incubated at 37°C in a 5% CO_2 incubator for 3 days. Subsequently, the infected cells were stained with 0.1% crystal violet staining. The number of viral plaques was counted and compared with the infected cell control to calculate the percentage of virus inhibition [16].

2.6 Inhibition of herpes simplex virus type 1 with honey after viral attachment to HaCaT cells

HaCaT cells was seeded with a density of 1.5×10^5 cells in 24-well plates and incubated for 24 hours. The culture medium was removed, and the herpes simplex virus was added to the wells and incubated at room temperature for 1 hour on a rocking platform. Then, honey at a non-toxic concentration was added to the wells, followed by the addition of overlay medium. The plates were incubated at 37°C in a 5% CO_2 incubator for 3 days. Subsequently, the infected cells were stained with 0.1% crystal violet staining. The number of viral plaques was counted and compared with the infected cell control to calculate the percentage of virus inhibition [16].

2.7 Statistical analysis

All data were analyzed using IBM SPSS Statistics version 26 and GraphPad Prism version 10.1.2. Differences among groups were evaluated using one-way analysis of variance

(ANOVA), followed by Tukey's HSD multiple comparison test. Statistical significance was defined at $p < 0.05$.

3. Result and Discussion

3.1 Total phenolic content and antioxidant activity

The total phenolic content and antioxidant activity of the honey samples were determined in this present study. The result is presented in **Table 1.**

Table 1. Total phenolic content and antioxidant activity of honey samples.

Honey	Total phenolic content (mg GAE/g)	Antioxidant activity (mg GAE/g)
Longan	1.375 ± 0.02^{a}	0.850 ± 0.06^{a}
Lychee	0.568 ± 0.02^{c}	0.337 ± 0.06^{c}
Polyfloral	1.079 ± 0.07^{b}	0.595 ± 0.05^{b}

All values are mean ± SEM of triplicate samples. Different superscript letters denote significant differences (ANOVA, $p < 0.05$).

Polyphenols are a major group of bioactive compounds in honey and are important contributors to its physicochemical characteristics. In the present study, the total phenolic content (TPC) of the honey samples ranged from 0.568 ± 0.02 to 1.375 ± 0.02 mg GAE/g. A significant difference in TPC was observed among the different honey types. Longan honey showed the highest TPC of 1.375 ± 0.02 mgGAE/g, followed by TPC of polyfloral honey (1.079 ± 0.07 mg GAE/g), while Lychee honey showed the lowest TPC of 0.568 ± 0.02 mg GAE/g. The TPC values of longan and polyfloral honey analyzed in this study were higher than those reports of similar honey from Vietnam (0.8930 to 1.110 mgGAE/g) [17], as well as honey from Malaysia (563.55 mg/kg) [18], and Bangladesh honey (152.4-688.5 mg/kg) [19]. Hence, the variation in TPC was varied among honey from different geographical regions, floral source, and climate conditions, which are known to influence the concentration and profile of phenolic compounds in honey [18].

The antioxidant activities of the three honey varieties in this study were evaluated using the DPPH radical scavenging assay. The results showed that the radical inhibition capacity of Longan honey exhibited the highest antioxidant capacity compared to the other types of honey. This finding is consistent with reported antioxidant properties from longan, lychee, and polyflora honey collected in Thailand between February and April 2012 [20], as well as those from Indian honey [21] and Malaysian honey, including Tualang, Gelam, and Borneo tropical honey [22]. Furthermore, the antioxidant capacity of the honey was correlated with their total phenolic content [23].

3.2 Cytotoxicity of honey

The cytotoxicity of the three honey types was performed on HaCaT cells. The honey solution was prepared at a concentration of 50% W/V and the honey was diluted by two-fold serial dilution with Dulbecco's Modified Eagle Medium (DMEM). After 72 hours, Longan honey showed the highest cytotoxicity, with a 50% cytotoxic concentration (CC_{50}) value of 3.097 ± 0.01% w/v, followed by polyfloral honey at 3.125 ± 0.11%, and lychee honey at 3.314 ± 0.02% w/v **(Fig. 1)**.

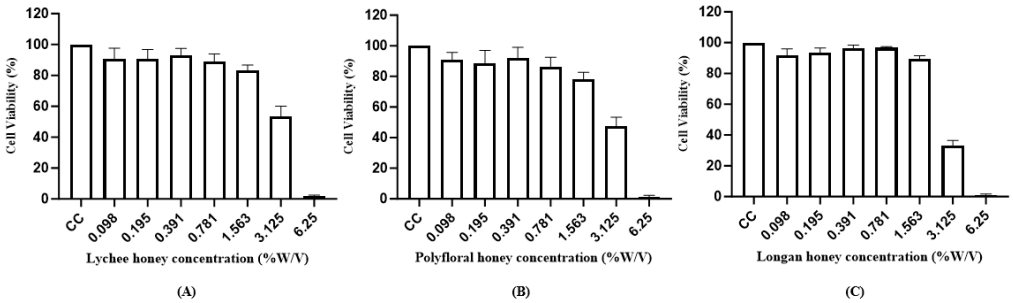

Fig. 1. Cytotoxicity effects of three types of honey on HaCaT cell. The result shows percentage cell viability at various concentrations of (A) Lychee honey, (B) Polyfloral honey, and (C) Longan honey. Data are reported as mean ± standard deviation from three independent experiments.

The cytotoxicity test of the honey samples on HaCaT cells depended on the type of honey and the cell line. These findings were consistent with previous studies that evaluated the cytotoxicity of various honeys on the murine fibroblast cell line (L929). They reported IC_{50} values of 1.56 ± 0.14% for heather honey, 2.00 ± 0.24% for buckwheat honey, 3.55 ± 0.61% for linden honey, and 3.78 ± 0.57% for rapeseed honey [24]. Similarly, cytotoxicity of Malaysian *kelulut* honey was investigated on HGF-1 cells and they found that honey (3.125 to 200 mg/ml) showed the cell viability higher than 70%, suggesting low toxicity of the honey [25]. Furthermore, previous studies have reported that honey tends to be more toxic toward tumors or cancer cells while having lower toxicity to normal cells [26]. For example, Tualang honey exhibited cytotoxic effects on human breast cancer cell lines (MCF-7 and MDA-MB-231) but had no effect on normal breast epithelial cells MCF-10A [27]. Additionally, Gelam honey showed IC_{50} value of 25% against HepG2 cells, whereas normal human hepatocytes (WRL-68) exhibited IC_{50} value of 70% [28]

3.3 Inhibition of herpes simplex virus type 1 with honey before viral attachment to HaCaT cells

The antiviral activity of three different types of honey against herpes simplex virus type 1 (HSV-1) infection was evaluated by plaque reduction assay. Different concentrations of honey at a maximal concentration of 3.125% w/v could inhibit HSV-1 with percentages of inhibition less than 50%. Lychee, longan and polyfloral honey showed percentages of HSV-1inhibition by 29.64 ± 2.12%, 32.63 ± 2.12% and 35.05 ± 4.37%, respectively **(Fig. 2.)**

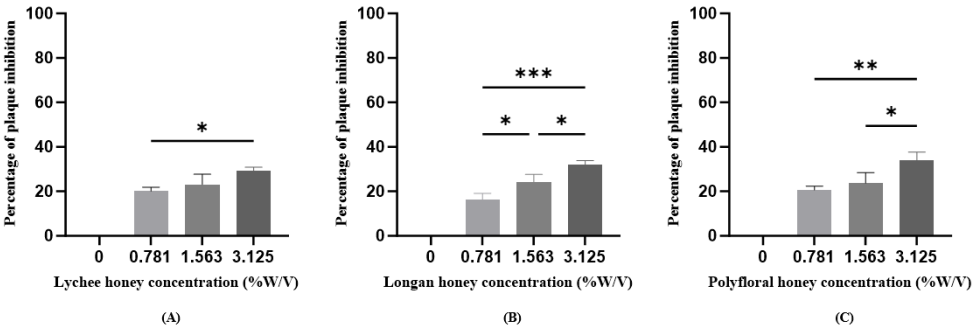

Fig. 2. Inhibition of HSV-1 activity with (A) Lychee honey, (B) Polyfloral honey, and (C) Longan honey before viral attachment on HaCaT cell. Results are presented as mean ± standard deviation from three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

These findings demonstrated that three types of honey samples showed low efficacy in preventing viral infection at this stage, suggesting that honey may not effectively interact or block host cell surface receptors. Furthermore, the relatively low concentration of honey used in this study may have contributed to its limited inhibitory effect against HSV-1. This result aligns with the finding that a higher concentration of honey is necessary for viral inhibition, as a previous study demonstrated that honey at a concentration of 5% completely inhibited HSV infection before viral attachment to cell culture [29].

In addtion, other study reported that propolis extract obtained from bee hives strongly interacted with the surface of Vero cells, resulting in preventing viral entry into the cells [30]. Additionally, propolis samples from various geographical origins and different phytochemical compositions showed different levels of antiviral activity against the influenza virus [31]. Therefore, the botanical origin and geographical source of honey and propolis may influence the content and composition of bioactive compounds responsible for antiviral activity.

3.4 Inhibition of herpes simplex virus type 1 with honey during viral attachment to HaCaT cells

The efficacy of honey against HSV-1 was evaluated during the viral attachment to cell culture. Non-cytotoxic concentrations of honey were mixed with the HSV-1 to the cell and compared with the virus control group. The results indicated that lychee, longan and polyfloral honey at a concentration of 3.125% W/V inhibited HSV-1 infection by 50.20 ± 4.32%, 56.07 ± 6.88%, and 70.87 ± 3.72%, respectively, relative to the control group **(Fig. 3).**

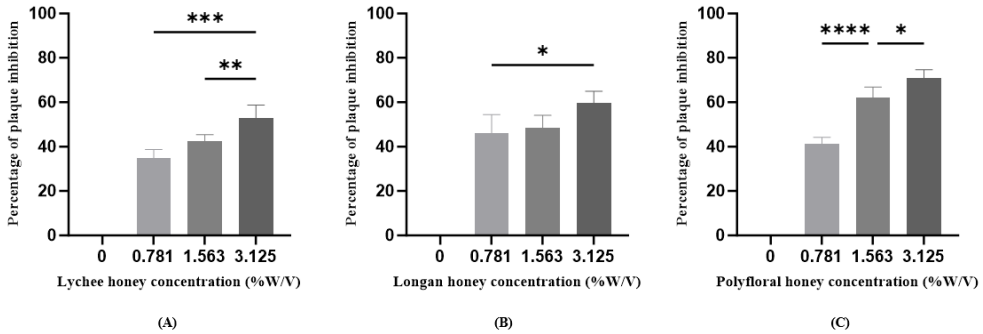

Fig. 3. Inhibition of HSV-1 activity with (A) Lychee honey, (B) Polyfloral honey, and (C) Longan honey during viral attachment on HaCaT cell. Results are presented as mean ± standard deviation from three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

The inhibitory effect of honey against HSV-1 during the cell attachment exhibited that the honey may interfere with viral proteins involved in host cell binding and entry. This finding aligned with the recent study that investigated the antiviral properties of natural extracts, honey, and propolis against norovirus. Their results demonstrated that several samples of honey and propolis were able to inhibit the interaction between Norovirus GII.4 and GII.10 virus-like particles (VLPs) with histo-blood group antigens (HBGAs) on the surface of cell culture [32]. Moreover, the honey samples examined in this study may contain bioactive compounds with potential antiviral properties. For example, Korean chestnut honey (KCH) was reported to inhibit viral infection by interfering with viral attachment, possibly through compounds other than kynurenic acid (KYNA), which is well known for anti-influenza activity [33]. Flavonoids can bind to viral surface proteins, thereby preventing the virus from entering host cells [34]. In addition, the antiviral efficacy is influenced by the

bioactive compounds in honey, as the diverse floral sources of polyfloral honey contribute to a variety of these compounds compared to monofloral honey [35]. Consequently, despite longan honey exhibiting higher antioxidant activity, its antiviral activity was lower than that of polyfloral honey. These findings suggest that viral inhibition may depend on the presence of antiviral compounds in honey and may not correlate with antioxidant potential.

3.5 Inhibition of herpes simplex virus with honey after viral attachment to HaCaT cells

The antiviral efficacy of honey against herpes simplex virus type 1 (HSV-1) was evaluated by adding honey after the virus had attached to the cell culture. The results showed that all three types of honey exhibited less than 50% inhibition against HSV-1 **(Fig. 4.)**

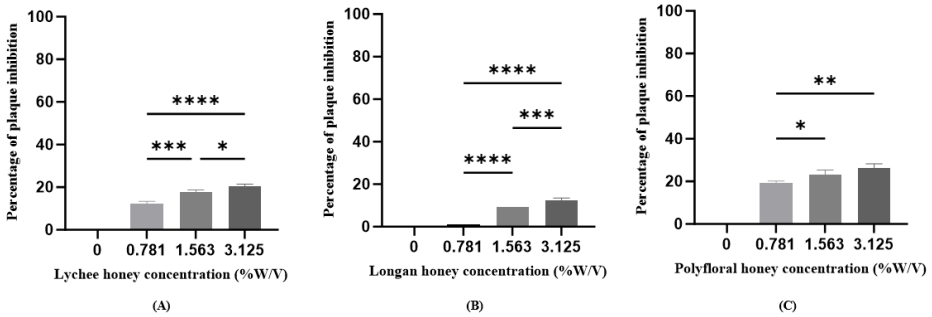

Fig. 4. Inhibition of HSV-1 activity with (A) Lychee honey, (B) Polyfloral honey, and (C) Longan honey after viral attachment on HaCaT cell. Results are presented as mean ± standard deviation from three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

This result indicated that honey did not effectively inhibit the virus after attachment, implying that honey may not interfere with the viral DNA replication or the translation and assembly of viral proteins. In particular, the chemical nature of flavonoids could contribute to the limited antiviral activity. The flavonoid components of honey are typically present as flavonoid glycosides, which are often derived from the floral sources of honey. These glycosylated forms are more polar and have lower lipid solubility than flavonoid aglycones, making it difficult to penetrate the lipid bilayer of cell membranes. As a result, these compounds may be unable to inhibit viral replication when the virus has entered host cells [36-38]. Moreover, the differences in honey types can lead to variations in bioactive constituents. For example, Manuka honey has been reported to inhibit other viral replication at the translational level such as respiratory syncytial virus (RSV). The inhibition is likely due to its high methylglyoxal content, that is known to have antibacterial and antiviral properties [39-40] Similarly, Tualang honey contains luteolin, which inhibits the replication of Japanese encephalitis virus (JEV) [41], while apigenin, a flavonoid with a 5,7-dihydroxyflavone structure, has been shown to inhibit Chikungunya virus (CHIKV) replication rather than viral entry [42].

4. Conclusion

The analysis of total phenolic content and antioxidant activity of honey determined by DPPH assay showed that longan honey exhibited the highest values. Lychee honey exhibited the lowest cytotoxicity on HaCaT cells compared to longan and polyfloral honey. Moreover, the efficacy of honey against HSV-1 infection showed low efficacy inhibition when treatment

before and after viral attachment to HaCaT cell. However, all samples of honey significantly inhibited HSV-1 more than 50 % during viral attachment at a concentration of 3.125% W/V. Polyfloral honey showed the highest inhibition by 70.87 ± 3.72%. Notably, polyfloral honey showed the strongest antiviral effect despite longan honey having higher antioxidant capacity, indicating that antiviral efficacy may not be directly correlated with antioxidant potential. Thus, the antiviral mechanism of the honey may involve disrupting the viral particles or interfere during attachment of the virus to the cell.

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