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ORIGINAL ARTICLE

Anti-influenza Viral Effects of Honey *In Vitro*: Potent High Activity of Manuka Honey

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Background and Aims. Influenza viruses are a serious threat to human health and cause thousands of deaths annually. Thus, there is an urgent requirement for the development of novel anti-influenza virus drugs. Therefore, the aim of this study was to evaluate the anti-influenza viral activity of honey from various sources.

Methods. Antiviral activities of honey samples were evaluated using MDCK cells. To elucidate the possible mechanism of action of honey, plaque inhibition assays were used. Synergistic effects of honey with known anti-influenza virus drugs such as zanamivir or oseltamivir were tested.

Results. Manuka honey efficiently inhibited influenza virus replication (IC_{50} = 3.6 ± 1.2 mg/mL; CC_{50} = 82.3 ± 2.2 mg/mL; selective index = 22.9), which is related to its virucidal effects. In the presence of 3.13 mg/mL manuka honey, the IC_{50} of zanamivir or oseltamivir was reduced to nearly 1/1000th of their single use.

Conclusions. Our results showed that honey, in general, and particularly manuka honey, has potent inhibitory activity against the influenza virus, demonstrating a potential medicinal value.

Key Words: Manuka honey, Anti-viral drug, Influenza virus, Synergistic effect, Virucidal activity.

Introduction

Influenza viruses are enveloped, negative-strand RNA viruses with a segmented genome and belong to the *Orthomyxoviridae* family. Two subtypes of the influenza virus A and B cause influenza in humans. Influenza A virus mutates easily, thereby often resulting in the emergence of new antigenic variants of each subtype. The threat of a human influenza pandemic has greatly increased over the past 18 years. The highly pathogenic avian influenza viruses, notably the H5N1 virus, emerged in 1997 (1). The 2009 pandemic virus (H1N1) quickly spread throughout the world (2), and more recently, human infection with avian influenza virus (H7N9) has been reported (3). These outbreaks should serve as warnings to responsible agencies to prepare for the next pandemic threat. At present, two main classes of anti-influenza drugs are available: M2 ion channel inhibitors (amantadine and rimantadine) and neuraminidase inhibitors (zanamivir, oseltamivir, laninamivir, and peramivir). The use of M2 ion channel inhibitors is limited because of the rapid emergence of drug resistance and side effects (4); moreover, resistance to neuraminidase inhibitors is also rapidly increasing in clinical isolates (5). It is noteworthy that neuraminidase inhibitor resistance without loss of virulence was observed in certain H7N9 clinical isolates (6). These factors necessitate the development of novel anti-influenza virus drugs with reduced potential for the emergence of resistance.

Natural products such as microbial metabolites and medicinal plants offer great promise as potentially effective novel antiviral drugs. To date, many anti-influenza agents isolated from these natural products have been reported.

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For example, polyketide leptomycin B from *Streptomyces* spp. (7), polyphenols pentagalloylglucose (PGG) and (−) -epigallocatechin-3-gallate (EGCG) from *Phyllanthus emblica* L. (8) and green tea (9), respectively, and alkaloid (−) -thalimonine from *Thalictrum simplex* L. (10) showed anti-influenza viral activity by inhibiting nuclear export of viral ribonucleoprotein complex (vRNP), interacting with viral hemagglutinin (HA), inhibiting viral neuraminidase (NA), and inhibiting viral protein synthesis, respectively. It was also reported that valtrate from Valerianae Radix and 1′-acetoxychavicol acetate (ACA) from the roots of *Alpinia galanga* suppress nuclear export of vRNP, thereby inhibiting influenza viral activity (11). More recently, it was found that *Alchemilla mollis* extracts suppress the growth of influenza virus because of its virucidal activity (12). However, the toxicity of these natural products is a cause for concern in their application as antiviral drugs.

Honey is a natural product obtained from flowers via bees. Although the composition of honey varies depending on the plants on which the bees feed (13,14), the main constituents of honey from any source are the sugars fructose, glucose, and sucrose, which account for ~80% of its weight and 20% water. Honey also contains amino acids, vitamins, minerals, enzymes, phenolic acids, and flavonoids. Honey exhibits anti-inflammatory (15), wound healing (16), antioxidant (17), and anti-neoplastic activities (18). In addition, honey has been used as a traditional remedy for bacterial infections, cold, and cough since ancient times, suggesting that it is effective against many infectious diseases. Natural honey shows broad spectrum activity against gram-positive and gram-negative pathogenic bacteria and fungi, including methicillin-resistant *Staphylococcus aureus* (MRSA) (19), *Shigella sonnei* (20), *Helicobacter pylori* (21), and *Candida albicans* (22). The antibacterial and antifungal activities of honey are attributable to its osmolarity, low pH, hydrogen peroxide (H_2O_2), phenolic acids, and flavonoids. Moreover, natural honey has been reported to exhibit antiviral activities against rubella virus (23) and varicella-zoster virus (VZV) (24) and is used to treat recurrent herpes simplex lesions (25). From these observations about antiviral activities, traditional use and phytochemicals in honey, it was presumed that honey is effective against influenza virus. Therefore, in the present study, the anti-influenza viral activities of honey were investigated.

Materials and Methods

Cells, Virus, and Chemicals

Madin–Darby canine kidney (MDCK) cells were grown in Eagle's minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS) at 37°C in a 5% CO_2 atmosphere. For all experiments, MEM supplemented with 100 units/mL penicillin G and 100 μg/mL streptomycin sulfate was used. Influenza virus A/WSN/33 (H1N1), a well-studied neurotropic laboratory strain (26), was propagated in MDCK cells, and culture supernatants were titrated using the 50% tissue culture infectious dose ($TCID_{50}$) assay in MDCK cells. Manuka (UMF15+; *Leptospermum scoparium*), soba (*Fagopyrum esculentum*; buckwheat), kanro (honeydew), acacia (*Robinia pseudoacacia*), and renge (*Astragalus sinicus*) honey samples were supplied by Yamada Bee Farm Co. (Tsuyama, Japan). Zanamivir and oseltamivir were purchased from GlaxoSmithKline PLC (Middlesex, United Kingdom) and F. Hoffmann-La Roche Ltd. (Basel, Switzerland), respectively.

Evaluation of Anti-influenza Viral Effects

Anti-influenza viral effects were evaluated as previously described (12) with some modifications. In brief, MDCK cells were seeded onto 48-well plates at a density of 1.2×10^5 cells per well in 200 μL of MEM containing 5% FBS, and incubated overnight. After washing with MEM, 100 μL of 2-fold serially diluted honey samples in MEM were added to the cells followed by the immediate addition of a 100 μL virus suspension (250 $TCID_{50}$/mL) in MEM supplemented with 1% 100× vitamin solution (MEM−vitamin). The culture plates were incubated at 37°C in a 5% CO_2 atmosphere for 2 days. Subsequently, the culture medium was removed, and the cells were treated with 0.5% crystal violet (CV) in 70% ethanol for 5 min. After washing with water and drying in air, absorbance was measured at 560 nm using an Infinite M200 Tecan plate reader (Wako Pure Chemical Industries, Osaka, Japan). The 50% cell toxicity concentration (CC_{50}) and 50% virus inhibitory concentration (IC_{50}) of the samples were calculated from dose–response curves using the GraphPad Prism software program (Prism v. 5.01, GraphPad Software) (27).

Plaque Inhibition Assay

Plaque inhibition assay was performed as previously described (11) with some modifications. In brief, ~300 plaque-forming units (pfu) of virus in MEM−vitamin were used for infection. The detailed procedures for each treatment are as follows. (i) Pretreatment of cells before viral infection: before plaque assays, MDCK cells were pretreated with test samples at 37°C for 1 h. After the medium was removed, cells were washed with MEM and infected by adding the virus suspension containing 300 pfu of virus in MEM−vitamin medium. (ii) Pretreatment of virus before infection: approximately 10^7 pfu/mL of virus stock was preincubated with the test samples at room temperature for 1 h. These mixtures were subsequently diluted in MEM−vitamin to obtain approximately 600 pfu/mL, and 500 μL aliquots of the diluted mixtures (300 pfu) were used for infection. (iii) Treatment of cells during virus infection: 250 μL aliquots of the test samples in MEM−vitamin were added to MDCK cells followed by 250 μL of virus suspension (300 pfu). The solution was then added and incubated

for 1 h. (iv) Treatment of cells after viral infection: after viral infection (300 pfu) for 1 h, the cells were overlaid with 3 mL of agarose solution containing the honey samples and MEM supplemented with 0.8% agarose, 0.1% BSA, and 1% 100× vitamin solution.

Evaluation of Synergistic Effects

MDCK cells were seeded onto 96-well plates at a concentration of 3×10^4 cells per well in 100 µL of MEM supplemented with 5% FBS and incubated overnight. The medium was removed, and 100 µL aliquots of MEM−vitamin containing 2-fold serially diluted test samples and 100 µL of the influenza virus A/WSN/33 suspension in MEM−vitamin (250 $TCID_{50}$/mL) were immediately added. The culture plates were incubated at 37°C in a 5% CO_2 atmosphere for 2 days. The plates were subsequently fixed and stained with CV, and optical density (OD) values were determined as described above.

Results

Honey Inhibits Influenza Virus Replication

Anti-influenza virus effects of honey samples were evaluated using MDCK cells (Figure 1).

When MDCK cells were infected with influenza virus, the cells died and detached from the plate (cytopathic effect, CPE). CPE was suppressed in the presence of honey samples, including manuka (*L. scoparium*), soba (*F. esculentum*; buckwheat), kanro (honeydew), acacia (*R. pseudoacacia*) and renge (*A. sinicus*) honey. As a result, relative CV stain values (%) increased in a dose-dependent manner. These results suggest that all tested honey samples had antiviral activity. The CC_{50}, IC_{50}, and selective index (SI) values were calculated for each honey sample (Table 1). The CC_{50} values of manuka, soba, kanro, acacia, and renge honey samples were 82.3 ± 2.2, 80.9 ± 2.0, 82.8 ± 1.5, 81.1 ± 1.4, and 80.6 ± 0.7 mg/mL, respectively. The IC_{50} values of the honey samples ranged from 3.6 ± 1.2 to 11.3 ± 3.7 mg/mL. Among the honey samples tested, manuka honey exhibited the highest activity, with the IC_{50} and SI of 3.6 ± 1.2 mg/mL and 22.9, respectively, followed by soba (IC_{50} = 6.7 ± 0.5 mg/mL; SI = 12.1), kanro (IC_{50} = 8.6 ± 1.8 mg/mL; SI = 9.6), acacia (IC_{50} = 10.3 ± 2.7 mg/mL; SI = 7.9), and renge (IC_{50} = 11.3 ± 3.7 mg/mL; SI = 7.1). The SI of zanamivir was calculated to be $>3.53 \times 10^3$ (Table 1). These data indicate that among the honey samples tested, manuka honey has the most potent viral-inhibitory activity.

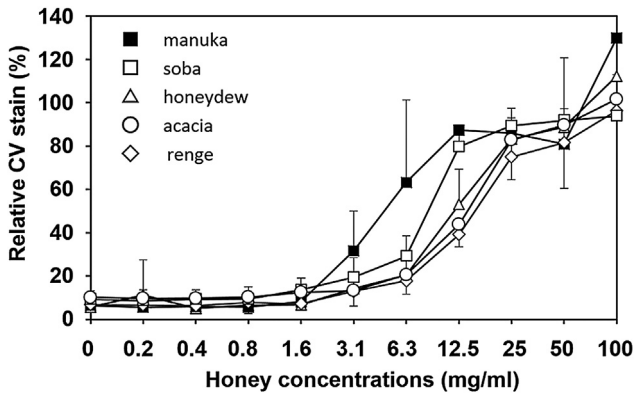

Figure 1. Antiviral activity of honey. Evaluation of anti-influenza virus effects of the honey samples was performed as described under Materials and Methods. MDCK cells were grown in 48-well plates and infected with influenza virus A/WSN/33 in the presence of 2-fold serially diluted samples (manuka, closed square; soba, open square; honeydew, open triangle; acacia, open circle; renge, open diamond). Two days after infection, the cells were fixed and stained with CV, and the absorbance was measured using a plate reader. Relative CV stain (%) is expressed as a percentage of the uninfected cells. Data are representative of three independent experiments.

Manuka Honey has Virucidal Activity

Plaque inhibition assays were performed to determine whether manuka honey affects influenza virus growth (Figure 2). For these experiments, manuka honey was either (i) added to the cells for 1 h and subsequently washed out before viral infection (‘‘pretreatment of cells’’), (ii) mixed with influenza virus suspension for 1 h before viral infection (‘‘pretreatment of virus’’), (iii) added during virus adsorption for 1 h and subsequently washed out (‘‘during infection’’), or (iv) added to the agarose gels (‘‘after infection’’). Pretreatment of cells with manuka honey had no effect on plaque numbers (6.25 mg/mL, 102.3 ± 1.3% and 25 mg/mL, 110.3 ± 6.4%; Figure 2B). In contrast, plaque numbers were significantly decreased when the virus was treated with manuka honey before infection (6.25 mg/mL, 13.7 ± 0.8% and 25 mg/mL, plaques were not observed; Figure 2B), suggesting potent virucidal activity of manuka honey. Moderate reductions in plaque numbers were also obtained on treatment of cells with honey during infection (6.25 mg/mL, 51.0 ± 2.7% and 25 mg/mL, 7.5 ± 4.2%; Figure 2B) and after infection (6.25 mg/mL,

Table 1. Summary of antiviral activities of the honey samples

Honey samples	IC_{50}[a]	CC_{50}[a]	SI[b]
Manuka (*L. scoparium*)	3.6 ± 1.2 mg/mL	82.3 ± 2.2 mg/mL	22.9
Soba (buckwheat)	6.7 ± 0.5 mg/mL	80.9 ± 2.0 mg/mL	12.1
Kanro (honeydew)	8.6 ± 1.8 mg/mL	82.8 ± 1.5 mg/mL	9.6
Acacia (*R. pseudoacacia*)	10.3 ± 2.7 mg/mL	81.1 ± 1.4 mg/mL	7.9
Renge (*A. sinicus*)	11.3 ± 3.7 mg/mL	80.6 ± 0.7 mg/mL	7.1
Zanamivir	28.3 ± 14.9 nM	>100 µM	$>3.53 \times 10^3$

[a]Mean ± standard deviation (SD) from three independent experiments.
[b]SI = CC_{50}/IC_{50}; IC_{50}: 50% virus inhibitory concentration, CC_{50}: 50% cell toxicity concentration; SI, selective index.

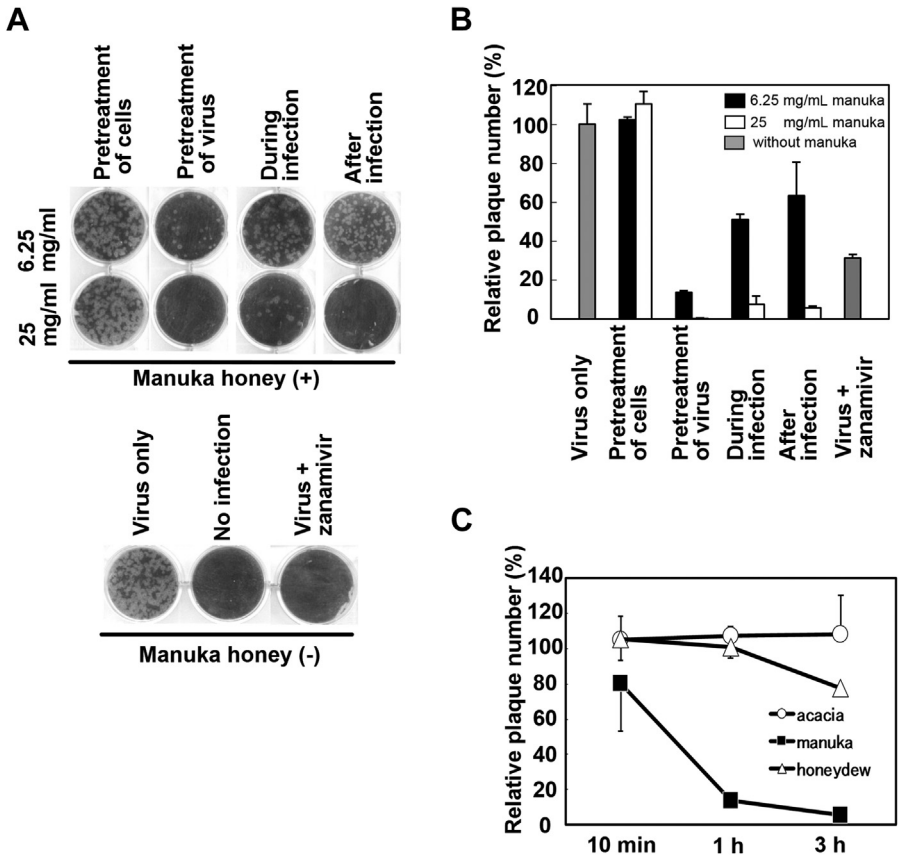

Figure 2. Virucidal activity of honey. (A) Plaque formation in the presence of manuka honey. Confluent monolayers of MDCK cells were grown in six-well plates and infected with dilutions of virus that produced ~300 plaques per well. After 1 h, the virus solution was removed, the cells were washed and overlaid with an agarose solution (0.8% agarose in MEM), and plaques were counted after 3 days. For the "pretreatment of cells" experiment, manuka honey was added to the cells 1 h before infection. For the "pretreatment of virus" experiment, virus and manuka honey were mixed at room temperature 1 h before addition to the cells. For the "during infection" experiment, manuka honey/virus solution was added at the beginning of the 1 h infection period. For the "after infection" experiment, manuka honey was mixed with the agarose solution. For the control, zanamivir (100 nM) was mixed with the agarose solution (virus + zanamivir). Representative data from three independent experiments are presented. (B) Effect of manuka honey on plaque numbers. Plaques in Figure 2A were counted, and the percentage plaque inhibition relative to infected controls (virus only) was determined for each drug concentration. Closed bar, 6.25 mg/mL of manuka honey; open bar, 25 mg/mL of manuka honey; gray bar, without manuka honey. Means of duplicate samples are shown as relative plaque numbers. Data are presented as mean ± SD. (C) Time-dependent virucidal activity of manuka honey. Manuka (closed square), acacia (open circle), or honeydew (open triangle) honey samples were mixed with virus preparations to a final concentration of 6.25 mg/mL and incubated at room temperature for the indicated time periods. The mixtures were subsequently diluted, and plaque assays were immediately performed. Plaque numbers are expressed as a percentage of the number of plaques obtained in the absence of honey. Data are presented as mean ± SD of duplicate measurements.

63.2 ± 17.3% and 25 mg/mL, 5.6 ± 1.1%; Figure 2B). The anti-influenza drug zanamivir was added after infection (100 nM) as a positive control, and caused a significant decrease in plaque numbers (31.3 ± 1.9%; Figure 2B). Moreover, among the honey samples tested, only manuka honey exhibited time-dependent virucidal activity (Figure 2C). These data suggest that manuka honey has strong virucidal activity.

Synergistic Antiviral Effects of Manuka Honey in Combination with Neuraminidase Inhibitors

A combined use of synergistically active antiviral compounds that have different mechanisms of action may provide advantages over single-agent treatments. To determine whether manuka honey and neuraminidase inhibitors have synergistic effects on influenza virus replication prevention, 2-fold serial dilutions of manuka honey and the neuraminidase inhibitors zanamivir or oseltamivir were added to MDCK cells before infection (Figure 3). In the presence of manuka honey at 0.20, 0.39, 0.78, 1.56, and 3.13 mg/mL concentrations, the relative IC_{50} value of zanamivir was reduced to 12.1 ± 10.1%, 9.2 ± 4.5%, 4.6 ± 2.3%, 3.8 ± 0.9%, and 0.3 ± 0.3%, respectively (Figure 3A and 3B). Similarly, the relative IC_{50} value of oseltamivir was reduced to 10.7 ± 5.1%, 4.7 ± 1.2%, 3.1 ± 1.0%, 1.3 ± 0.4%, and 0.1 ± 0.1%, respectively (Figure 3C and 3D). These results suggest that the antiviral action of manuka honey has a synergistic effect with that of zanamivir and oseltamivir. Similar synergistic effects were also observed in the presence of amantadine hydrochloride (data not shown).

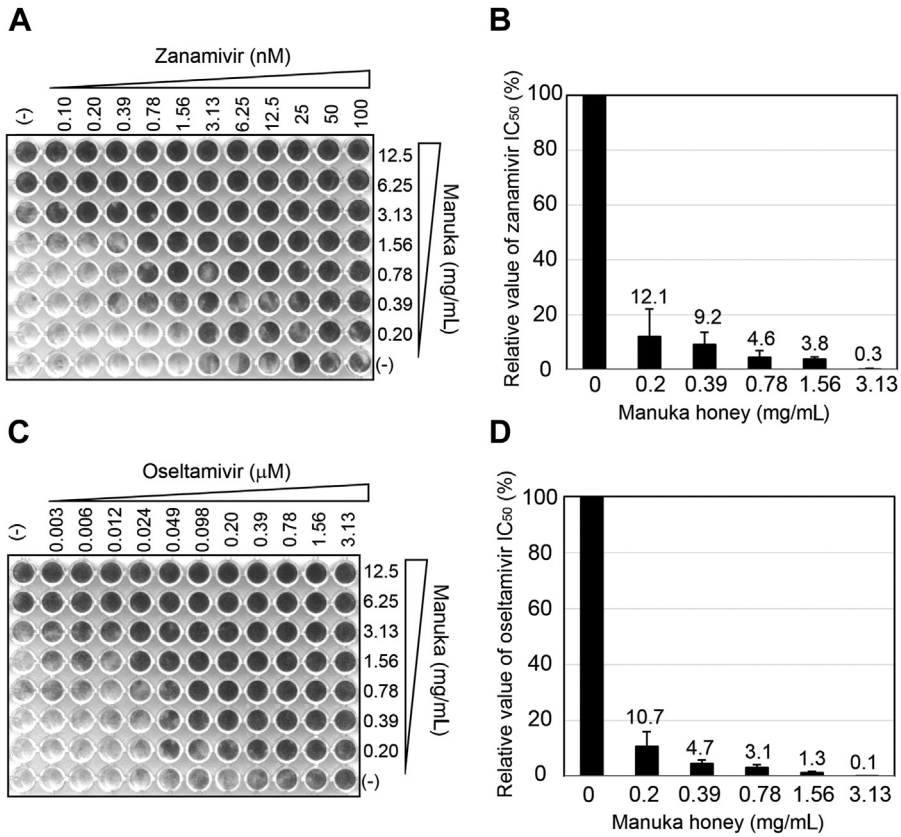

Figure 3. Synergistic effects of honey and NA inhibitors. Serially diluted manuka honey and neuraminidase inhibitors were added to MDCK cells, and immediately incubated with influenza virus for 2 days. The plates were fixed, stained with CV, and OD values were determined as described in the Materials and Methods. Representative data of zanamivir (A) and oseltamivir (C) are shown. The IC_{50} values of zanamivir (B) and oseltamivir (D) in the absence of manuka honey are shown as 100%. Data are presented as mean ± SD from two independent experiments.

Discussion

Influenza viruses are a serious threat to human health. Thus, there is an urgent requirement for the development of novel anti-influenza virus drugs. With numerous reports about the anti-microbial activities and composition of honey in mind, we hypothesized that honey is effective against influenza virus and evaluated the anti-influenza viral activity from various sources. The presented data indicates that manuka honey has the strongest anti-influenza viral activity among the honey samples tested (Figure 1 and Table 1), which is most likely because of its virucidal activity, as suggested by the plaque inhibition experiments (Figure 2).

Previous reports show that manuka honey displays antibacterial activities against *S. aureus*, *Eschericia coli* (13), and *H. pylori* (28) at concentrations of 10%, 10%, and 5%, respectively, whereas its antiviral activity against VZV had an IC_{50} value of 45 mg/mL (24). In the present study, the IC_{50} of manuka honey for the influenza virus was observed to be 3.6 mg/mL (Table 1). Moreover, in the presence of 25 mg/mL manuka honey, viral plaque formation was completely suppressed, suggesting that manuka honey is more effective against influenza virus than against VZV or bacteria.

Although the influenza-inhibitory constituents of honey remain unknown, a number of antibacterial constituents have been reported. High sugar concentrations of ~80% cause osmotic shock in bacteria; however, osmotic shock is unlikely to contribute to the antiviral effects of honey because influenza virions can be purified using high concentrations of sucrose (up to 60%). The CC_{50} values of the honey samples tested in this study were extremely similar in MDCK cells, ranging from 80.6 to 82.8 mg/mL (Table 1), possibly reflecting the nonspecific osmotic pressure exerted by the sugars in honey. This is in agreement with previous observations that treatment with 100 mg/mL acacia honey has a cytotoxic effect in A375 cells (29). Some bacteria are sensitive to the low pH (~4) of honey; however, in our study, the honey samples were diluted with MEM before use, ensuring that cells were cultured at physiological acceptable pH. Proteomic analyses showed that both honey (30) and royal jelly (31) contain the bactericidal peptide bee defensin-1 (32). However, our preliminary results show that royal jelly did not exhibit cytotoxicity and anti-influenza viral activity (CC_{50} > 10 mg/mL, IC_{50} > 10 mg/mL). Taken together, it is unlikely that

previously identified antibacterial constituents such as high sugar concentrations, low pH, and bee defensin-1 contributed to the observed anti-influenza viral activity of honey. Hydrogen peroxide (H_2O_2) is yet another constituent of honey, which displays both bactericidal activity (32) and virucidal activity against influenza virus (33). However, there is a discrepancy in the reported concentrations of H_2O_2 in honey. Schnider et al. reported that manuka honey contains H_2O_2 (13), whereas another study showed that it does not (34). Therefore, we cannot exclude the possibility that H_2O_2 in honey contributes to its antiviral activity.

It is known that honey contains a wide range of phytochemicals such as phenolic acids and flavonoids. The flavonoid rutin has been reported to exhibit antibacterial activity (35). Rutin-containing honey has been shown to display antimicrobial activity against pathogenic bacteria such as *S. aureus* and *E. coli* (36). Moreover, the anti-influenza viral activity of rutin has also been reported (37). In the current study, soba (buckwheat) honey exhibited the second highest anti-influenza viral activity (Table 1), and buckwheat inflorescences are known to have high rutin content (38). Acacia honey particularly contains the flavonoid chrysin. Whereas chrysin does not have antimicrobial activity against pathogenic bacteria or yeast (39), the anti-influenza viral activity of chrysin-rich fractions from *Scutellaria baicalensis* has been reported (40). These observations suggest that rutin and chrysin are possible constituents that contribute to the anti-influenza viral activity of buckwheat and acacia honey, although the flavonoid content in these honey samples has not been investigated in the present study.

Manuka honey was observed to exhibit the highest anti-influenza viral activity among the honey samples tested in this study (Table 1). The α-ketoaldehyde compound methylglyoxal (MGO) is present in extremely high concentrations in manuka honey (41). It was recently shown that MGO is the major determinant of the antibacterial activities of manuka honey (13,42). A previous study indicated that MGO has antiviral activities against the foot-and-mouth disease virus (43). Moreover, our preliminary results showed that MGO concentration was ~20–160 fold higher in manuka honey than in the other honey samples tested in this study (data not shown). Therefore, it is possible that MGO in manuka honey contributes to its anti-influenza viral activity (44). Although honey was less potent than zanamivir against influenza virus (Table 1), we observed that a combined use of anti-influenza drugs with manuka honey resulted in synergistic anti-influenza virus effects. The presence of manuka honey (3.13 mg/mL) markedly decreased the IC_{50} values of oseltamivir (0.1% vs. control) and zanamivir (0.3% vs. control; Figure 3). This is in agreement with the synergy observed between antibiotics and manuka honey in the inhibition of MRSA (45,46).

The oseltamivir-resistant seasonal H1N1 influenza viruses spread extremely rapidly throughout the world. In Japan, for example, the oseltamivir-resistant seasonal H1N1 virus increased from 2.6% in 2007/2008 to >99% in 2008/2009 (47). At present, although neuraminidase inhibitors including oseltamivir are effective against most 2009 pandemic (H1N1) and highly pathogenic H5N1 and H7N9 viruses, it is easy to speculate that a major population of these viruses and other future pandemic influenza viruses may become neuraminidase inhibitor-resistant any time. Because compounds or plant extracts that exhibit virucidal activity have broad-spectrum (48,49), it is possible that the virucidal activity of manuka honey is effective against H5N1 and H7N9 viruses.

In conclusion, the results obtained showed that honey, in general, and particularly manuka honey, has potent inhibitory activity against influenza virus, demonstrating a possible medicinal value. Further investigations are required to identify the active antiviral components in manuka honey and to determine its synergistic effects with known antiviral drugs.

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References

1. Claas EC, Osterhaus AD, van Beek R, et al. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet 1998;351:472–477.
2. Kawano H, Haruyama T, Hayashi Y, et al. Genetic analysis and phylogenetic characterization of pandemic (H1N1) 2009 influenza viruses that found in Nagasaki, Japan. Jpn J Infect Dis 2011;64:195–203.
3. Watanabe T, Kiso M, Fukuyama S, et al. Characterization of H7N9 influenza A viruses isolated from humans. Nature 2013;501:551–555.
4. Hayden FG. Antiviral resistance in influenza viruses–implications for management and pandemic response. N Engl J Med 2006;354:785–788.
5. Stephenson I, Democratis J, Lackenby A, et al. Neuraminidase inhibitor resistance after oseltamivir treatment of acute influenza A and B in children. Clin Infect Dis 2009;48:389–396.
6. Hai R, Schmolke M, Leyva-Grado VH, et al. Influenza A(H7N9) virus gains neuraminidase inhibitor resistance without loss of *in vivo* virulence or transmissibility. Nat Commun 2013;4:2854.
7. Elton D, Simpson-Holley M, Archer K, et al. Interaction of the influenza virus nucleoprotein with the cellular CRM1-mediated nuclear export pathway. J Virol 2001;75:408–419.
8. Liu G, Xiong S, Xiang YF, et al. Antiviral activity and possible mechanisms of action of pentagalloylglucose (PGG) against influenza A virus. Arch Virol 2011;156:1359–1369.
9. Kim M, Kim SY, Lee HW, et al. Inhibition of influenza virus internalization by (-)-epigallocatechin-3-gallate. Antiviral Res 2013;100:460–472.
10. Serkedjieva J, Velcheva M. *In vitro* anti-influenza virus activity of the pavine alkaloid (−)-thalimonine isolated from *Thalictrum simplex* L. Antivir Chem Chemother 2003;14:75–80.
11. Watanabe K, Takatsuki H, Sonoda M, et al. Anti-influenza viral effects of novel nuclear export inhibitors from Valerianae Radix and *Alpinia galanga*. Drug Discov Therap 2011;5:26–31.

12. Makau JN, Watanabe K, Kobayashi N. Anti-influenza activity of *Alchemilla mollis* extract: possible virucidal activity against influenza virus particles. Drug Discov Ther 2013;7:189−195.
13. Schneider M, Coyle S, Warnock M, et al. Anti-microbial activity and composition of manuka and portobello honey. Phytother Res 2013;27: 1162−1168.
14. Eteraf-Oskouei T, Najafi M. Traditional and modern uses of natural honey in human diseases: a review. Iran J Basic Med Sci 2013;16: 731−742.
15. Tonks AJ, Cooper RA, Jones KP, et al. Honey stimulates inflammatory cytokine production from monocytes. Cytokine 2003;21:242−247.
16. Bergman A, Yanai J, Weiss J, et al. Acceleration of wound healing by topical application of honey. An animal model. Am J Surg 1983;145: 374−376.
17. Jaganathan SK, Mandal SM, Jana SK, et al. Studies on the phenolic profiling, anti-oxidant and cytotoxic activity of Indian honey: *in vitro* evaluation. Nat Prod Res 2010;24:1295−1306.
18. Swellam T, Miyanaga N, Onozawa M, et al. Antineoplastic activity of honey in an experimental bladder cancer implantation model: *in vivo* and *in vitro* studies. Int J Urol 2003;10:213−219.
19. Jenkins R, Burton N, Cooper R. Proteomic and genomic analysis of methicillin-resistant *Staphylococcus aureus* (MRSA) exposed to manuka honey *in vitro* demonstrated down-regulation of virulence markers. J Antimicrob Chemother 2014;69:603−615.
20. Lusby PE, Coombes AL, Wilkinson JM. Bactericidal activity of different honeys against pathogenic bacteria. Arch Med Res 2005; 36:464−467.
21. Manyi-Loh CE, Clarke AM, Munzhelele T, et al. Selected South African honeys and their extracts possess *in vitro* anti-*Helicobacter pylori* activity. Arch Med Res 2010;41:324−331.
22. Ansari MJ, Al-Ghamdi A, Usmani S, et al. Effect of jujube honey on *Candida albicans* growth and biofilm formation. Arch Med Res 2013; 44:352−360.
23. Zeina B, Othman O, al-Assad S. Effect of honey versus thyme on Rubella virus survival *in vitro*. J Altern Complement Med 1996;2:345−348.
24. Shahzad A, Cohrs RJ. *In vitro* antiviral activity of honey against varicella zoster virus (VZV): a translational medicine study for potential remedy for shingles. Transl Biomed 2012;3:1−7.
25. Al-Waili NS. Topical honey application vs. acyclovir for the treatment of recurrent herpes simplex lesions. Med Sci Monit 2004;10: MT94−MT98.
26. Sun X, Tse LV, Ferguson AD, et al. Modifications to the hemagglutinin cleavage site control the virulence of a neurotropic H1N1 influenza virus. J Virol 2010;84:8683−8690.
27. Haruyama T, Nagata K. Anti-influenza virus activity of *Ginkgo biloba* leaf extracts. J Nat Med 2012;67:636−642.
28. McGovern DP, Abbas SZ, Vivian G, et al. Manuka honey against *Helicobacter pylori*. J R Soc Med 1999;92:439.
29. Pichichero E, Cicconi R, Mattei M, et al. Acacia honey and chrysin reduce proliferation of melanoma cells through alterations in cell cycle progression. Int J Oncol 2010;37:973−981.
30. Di Girolamo F, D'Amato A, Righetti PG. Assessment of the floral origin of honey via proteomic tools. J Proteomics 2012;75:3688−3693.
31. Schonleben S, Sickmann A, Mueller MJ, et al. Proteome analysis of *Apis mellifera* royal jelly. Anal Bioanal Chem 2007;389:1087−1093.
32. Chen C, Campbell LT, Blair SE, et al. The effect of standard heat and filtration processing procedures on antimicrobial activity and hydrogen peroxide levels in honey. Front Microbiol 2012;3:265.
33. Neighbor NK, Newberry LA, Bayyari GR, et al. The effect of microaerosolized hydrogen peroxide on bacterial and viral poultry pathogens. Poult Sci 1994;73:1511−1516.
34. Kwakman PH, Te Velde AA, de Boer L, et al. Two major medicinal honeys have different mechanisms of bactericidal activity. PLoS One 2011;6:e17709.
35. Basile A, Sorbo S, Giordano S, et al. Antibacterial and allelopathic activity of extract from *Castanea sativa* leaves. Fitoterapia 2000; 71(Suppl 1):S110−S116.
36. Pimentel RB, da Costa CA, Albuquerque PM, et al. Antimicrobial activity and rutin identification of honey produced by the stingless bee *Melipona compressipes manaosensis* and commercial honey. BMC Complement Altern Med 2013;13:151.
37. Ibrahim AK, Youssef AI, Arafa AS, et al. Anti-H5N1 virus flavonoids from *Capparis sinaica* Veill. Nat Prod Res 2013;27:2149−2153.
38. Kalinova J, Triska J, Vrchotova N. Distribution of Vitamin E, squalene, epicatechin, and rutin in common buckwheat plants (*Fagopyrum esculentum* Moench). J Agric Food Chem 2006;54:5330−5335.
39. Mamadalieva NZ, Herrmann F, El-Readi MZ, et al. Flavonoids in *Scutellaria immaculata* and *S. ramosissima* (Lamiaceae) and their biological activity. J Pharm Pharmacol 2011;63:1346−1357.
40. Hour MJ, Huang SH, Chang CY, et al. Baicalein, ethyl acetate, and chloroform extracts of *Scutellaria baicalensis* inhibit the neuraminidase activity of pandemic 2009 H1N1 and seasonal influenza A viruses. Evid Based Complement Alternat Med 2013;2013:750803.
41. Kwakman PH, Zaat SA. Antibacterial components of honey. IUBMB Life 2012;64:48−55.
42. Adams CJ, Boult CH, Deadman BJ, et al. Isolation by HPLC and characterisation of the bioactive fraction of New Zealand manuka (*Leptospermum scoparium*) honey. Carbohydr Res 2008;343:651−659.
43. Ghizatullina NK. Effect of methyl glyoxal on infectivity and antigenicity of foot-and-mouth disease virus. Acta Virol 1976;20:380−386.
44. Talukdar D, Ray S, Ray M. Further testing of antiviral activity of methylglyoxal urgently needed. Med Hypotheses 2006;67:673−674.
45. Jenkins R, Cooper R. Improving antibiotic activity against wound pathogens with manuka honey *in vitro*. PLoS One 2012;7:e45600.
46. Jenkins RE, Cooper R. Synergy between oxacillin and manuka honey sensitizes methicillin-resistant *Staphylococcus aureus* to oxacillin. J Antimicrob Chemother 2012;67:1405−1407.
47. Ujike M, Shimabukuro K, Mochizuki K, et al. Oseltamivir-resistant influenza viruses A (H1N1) during 2007-2009 influenza seasons, Japan. Emerg Infect Dis 2010;16:926−935.
48. Kramer A, Galabov AS, Sattar SA, et al. Virucidal activity of a new hand disinfectant with reduced ethanol content: comparison with other alcohol-based formulations. J Hosp Infect 2006;62:98−106.
49. Shin WJ, Lee KH, Park MH, et al. Broad-spectrum antiviral effect of *Agrimonia pilosa* extract on influenza viruses. Microbiol Immunol 2010;54:11−19.