Anti-influenza activity of *Alchemilla mollis* extract: Possible virucidal activity against influenza virus particles

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ABSTRACT: Influenza virus infection is a major public health problem that leads to significant morbidity and mortality. The emergence of resistance to the currently available anti-influenza agents has necessitated the development of new drugs with novel targets. Studying known ethno-medicinal plants is a promising approach for the discovery of new antiviral compounds. *Alchemilla mollis* is used in traditional medicine in Europe for different indications, including minimizing the symptoms of a sore throat. In this study, we found that *A. mollis* extract has anti-influenza activity, and investigated the mechanism underlying its inhibition of influenza virus replication. Plaque assays demonstrated that treatment of cells with *A. mollis* extract prior to infection did not inhibit influenza virus infection. However, plaque formation was markedly reduced when infected cells were overlaid with an agarose gel containing *A. mollis* extract. In addition, exposure of the virus to *A. mollis* extract prior to infection and treatment of cells during virus infection significantly suppressed plaque formation. Influenza virus-induced hemagglutination of chicken red blood cells was inhibited by *A. mollis* extract treatment. The inhibitory effect was observed against influenza A virus subtypes H1N1, H3N2, and H5N2. These findings suggest that *A. mollis* extract has virucidal or neutralizing activity against influenza virus particles. Furthermore, inhibitory effect of zanamivir synergistically increased after combination with *A. mollis* extract. Our results suggest that *A. mollis* extract has the potential to be developed as an anti-influenza agent.

Keywords: *Alchemilla mollis*, virucidal, synergistic effect, antiviral.

1. Introduction

Influenza viruses are enveloped, negative-strand RNA viruses with a segmented genome that belong to the Orthomyxoviridae family. Influenza pandemics or seasonal epidemics cause significant morbidity and mortality in both humans and animals. Currently, there are two main classes of anti-influenza drugs 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 due to the rapid emergence of drug resistance, side effects, and the drugs’ lack of effect on influenza B viruses (1), while neuraminidase inhibitor resistance in clinical isolates is also rapidly increasing (2, 3). These factors necessitate the development of novel anti-influenza drugs with reduced chances of resistance emergence.

Natural products have been in the limelight in the search for anti-viral compounds due to their abundance and the possibility for their inclusion in diet. There are numerous recent reports on plant extract constituents that possess potent antiviral activity, as reviewed by Kitazato, *et al.* (4). Several plant-derived compounds exert potent anti-influenza activity by blocking virus entry into cells, inactivating the virus, or inhibiting extracellular-signal-regulated kinase phosphorylation (5-7). We have demonstrated that valtrate and 1′-acetoxychavicol acetate derived from Valerianae Radix and *Alpinia galanga*, respectively, inhibit influenza virus replication by preventing the nuclear export of viral ribonucleoprotein (8).

*Alchemilla* plants (lady’s mantle) are used in traditional medicine for different indications, including minimizing the symptoms of sore throat, promoting wound healing, stopping bleeding, and alleviating nausea and vomiting. Herbal tea, Lady’s Mantle Herbal Tea bags Alchemilka Herba Alchemillae, constituted from *Alchemilla* species is commercially available (9). Different studies have indicated that *A. mollis* and other *Alchemilla* species have potent free radical scavenging activity (10), attributed to the phenolic compounds, tannins, and flavonoid glycosides present in the plants.
(11,12). *A. mollis* is a constituent of "Herba Alchemillae", a commercial drug with astringent, diuretic, and antispasmodic properties used in traditional medicine for the treatment of excessive menstruation and wounds (13). Despite numerous reports on the pharmacological importance of *A. mollis* and other *Alchemilla* species, their antiviral activity has not been investigated.

In this study, we investigated the anti-influenza activity of *A. mollis* extracts. We observed that *A. mollis* alkaline extract had virucidal activity against influenza virus particles and produced a synergistic effect upon co-treatment with zanamivir. This is the first report demonstrating the antiviral activity of *A. mollis* extract.

### 2. Materials and Methods

#### 2.1. Cells, viruses, and chemicals

Madin-Darby Canine Kidney (MDCK) cells were grown in Minimum Essential Medium (MEM) supplemented with 5% fetal bovine serum (FBS) at 37°C in 5% CO2. Influenza A virus strains A/WSN/33 (H1N1), A/PR/8/34 (H1N1), A/HK/8/68 (H3N2), and A/duck/Pennsylvania/84 (H5N2) were propagated in 10-day-old embryonated chicken eggs for 4 days. Allantoic fluid was then harvested and stored at −80°C. Zanamivir was purchased from GlaxoSmithKline (Middlesex, UK), dissolved in HEPES (pH 7.0) to a concentration of 10 mM, and stored at −20°C.

#### 2.2. Extraction of *A. mollis* plant material

One gram of dried and powdered plant material was extracted in 15 mL of different solutions using the extraction methods shown in Table 1. Next, the extracts were centrifuged at 12,100 × g for 15 min and the supernatant was collected and sterilized using a 0.45-pore size filter.

#### 2.3. Crystal violet assay

MDCK cells (3 × 10^4 cells/well) were seeded in 96-well tissue culture plates and incubated at 37°C for 24 h. For cytotoxicity assays, cells were washed and treated with serial dilutions of *A. mollis* extract in MEM. Virus inocula of 25 TCID50 (50% tissue culture infective dose) were added per well for the antiviral activity assay. Cells were fixed with EtOH and stained with 0.5% crystal violet after incubation for 48 h. The optical density (OD) at 560 nm was measured with Infinite M200 Tecan plate reader (Wako Pure Chemical Industries, Osaka, Japan). The OD value of treated wells was compared with that of untreated controls, calculated as a percentage relative cell density and plotted against the concentration of *A. mollis* extract. Three independent experiments were performed. Linear regression analysis to calculate the 50% cytotoxic concentration (CC50) and 50% inhibitory concentration (IC50) was performed with Microsoft Excel software. The selectivity index (SI) was determined from the CC50 to IC50 ratio.

#### 2.4. Plaque formation assay

Confluent MDCK cells cultured in 6-well plates were washed with serum-free medium and infected with 0.5 mL of virus solution (300 pfu/mL – A/WSN/33 virus) in serum-free MEM for 1 h at 37°C. Cells were washed with serum-free MEM and overlaid with MEM containing 0.8% agarose, 0.1% bovine serum albumin (BSA), 1% 100× MEM vitamin solution, and 0.03% glutamine. After incubation at 37°C for 72 h, plaques were visualized by fixing the cells with acetic acid:ethanol (1:1) for 1 h and staining with 0.5% amido black 10B for 3 h. Plaques were counted by visual examination. Results were represented as ratio of plaque number in the presence of extract or zanamivir to that in the absence of extract or zanamivir.

#### 2.5. Hemmaglutination inhibition (HI) assay

HI assay was performed as previously described by Ehrhardt (14). Briefly, in 96-well micro titer plates, serial dilutions of samples (50 μL) were prepared and mixed with an equal volume of virus suspension as follows: 40 HA units - A/WSN/33 (H1N1), 256 HA units - A/HK/8/68 (H3N2), and 128 HA units - A/duck/Pennsylvania/84 (H5N2). After incubation at 4°C for 1 h, 50 μL of 5% (v/v) chicken red blood cells (RBCs) (Nippon Bio-Test Laboratories, Tokyo, Japan) in phosphate-buffered saline (PBS) (−) was added and then incubated for 1 h at room temperature (RT) to allow for hemagglutination to occur.

#### 2.6. Heat treatment of *A. mollis* extract

Extract (400 μL) was aliquoted into 1.5-mL tubes and heated at 60°C or 100°C for 30 min. The samples were cooled at RT and stored at −30°C. The antiviral activity of the samples was determined with the crystal violet assay.

#### 2.7. Ultra-filtration of *A. mollis* extract

Four hundred microliters of extract were added to the filter cup of 10 kDa and 30 kDa Ultrafree™-MC units (Millipore, Bedford, USA), and centrifuged at 2,500 × g.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Method of extraction</th>
<th><em>SI</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>Shaking at RT for 24 h</td>
<td>25</td>
</tr>
<tr>
<td>Water containing 1% (w/v) NaHCO3</td>
<td>Shaking at RT for 24 h</td>
<td>5</td>
</tr>
<tr>
<td>50% ethanol</td>
<td>Shaking at RT for 24 h</td>
<td>42</td>
</tr>
</tbody>
</table>

*Selectivity index*
Combination treatment with A. mollis extract and zanamivir

Combination treatment with A. mollis extract and zanamivir was performed to investigate whether they have additive or synergistic effects on inhibiting influenza virus replication. MDCK cells were inoculated with A/WSN/33 virus at a multiplicity of infection (MOI) of 0.001 in the presence of mock medium or zanamivir (50 nM to 0.1 nM) mixed with 0.01%, 0.02%, 0.04%, 0.08%, 0.16%, and 0.31% of A. mollis extract. Cells were incubated at 37°C for 48 h and cell density was determined using the crystal violet assay. The percentage inhibitory activity of the zanamivir/A. mollis extract combination was calculated relative to the cell density in untreated controls. The combination treatment effect was analyzed by calculating the interaction index at 50% inhibitory activity using the isobole method as described by Tallarida (15). The following equation is used to calculate the interaction index (γ) for a combination of drugs A and B: 

\[ \gamma = \frac{Ae/Be + Bc/Ac}{\gamma_A + \gamma_B} \]

where \( \gamma_A \) and \( \gamma_B \) correspond to the concentrations of A and B when used in combination, and Ac and Be correspond to the concentrations that can produce an effect of the same magnitude if used alone. If \( \gamma < 1 \), the effect of the combination is synergistic, whereas if \( \gamma = 0 \) or >1, the effect is additive or antagonistic, respectively.

2.9. Statistical analysis

Results are represented as the mean ± standard error of the mean from three independent experiments. The difference between test samples and untreated controls was evaluated using Student’s t test. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Cellular toxicity and anti-influenza activity of A. mollis extract

The cellular toxicity and anti-influenza activity of different A. mollis extracts were evaluated using the crystal violet assay after incubating MDCK cells with the extract only or the extract and virus (A/WSN/33) for 48 h. All extracts inhibited influenza virus replication and had different levels of cytotoxicity (data not shown). A. mollis alkaline extract showed the highest anti-influenza activity. Alkaline extraction of plant materials has been shown to improve the extraction efficiency of hydrophilic compounds (16). The selectivity index of the alkaline extract was 42 in contrast to 25 of the water extract at RT (Table 1). The alkaline extract was not cytotoxic at concentrations of < 2.5% (CC50 = 5%, Figure 1). Influenza virus replication was inhibited at non-cytotoxic concentrations with an IC50 of 0.12%. The alkaline extract of A. mollis was used in subsequent experiments to elucidate its inhibitory mechanism on influenza virus replication.

3.2. Sensitivity of other influenza A strains to A. mollis extract

The effect of A. mollis extract on the replication of influenza A virus strains A/WSN/33 (H1N1), A/PR/8/34 (H1N1), A/HK/8/68 (H3N2), and A/duck/Pennsylvania/84 (H5N2) was evaluated using the crystal violet assay. Infections with A/PR/8/34 (H1N1), A/HK/8/68 (H3N2), and A/Duck Pennsylvania/84 (H5N2) were performed in the presence of 2.5 μg/mL trypsin. The extract suppressed replication of the four strains in a dose-dependent manner, although there was a slight difference in sensitivity between the strains (Table 2). These findings indicate that the antiviral activity of A. mollis extract is strain-independent.

3.3. Mechanism of action of A. mollis extract

Plaque formation assays with different cells and/or virus treatments were performed to determine the mode of action of A. mollis extract. Treatment of MDCK cells with either A. mollis extract or zanamivir (100 nM) for

Figure 1. Cytotoxicity and anti-influenza activity of A. mollis alkaline extract. MDCK cells (3 × 10^4 cells/well) in a 96-well culture plate treated with serial dilutions of A. mollis extract were incubated at 37°C for 48 h in the presence of mock medium or zanamivir (100 nM) for 48 h. All extracts inhibited influenza virus replication and had different levels of cytotoxicity (data not shown). A. mollis alkaline extract showed the highest anti-influenza activity. Alkaline extraction of plant materials has been shown to improve the extraction efficiency of hydrophilic compounds (16). The selectivity index of the alkaline extract was 42 in contrast to 25 of the water extract at RT (Table 1). The alkaline extract was not cytotoxic at concentrations of < 2.5% (CC50 = 5%, Figure 1). Influenza virus replication was inhibited at non-cytotoxic concentrations with an IC50 of 0.12%. The alkaline extract of A. mollis was used in subsequent experiments to elucidate its inhibitory mechanism on influenza virus replication.

Table 2. Sensitivity of different influenza A virus strains to A. mollis extract

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>IC50 (% )</th>
<th>SI</th>
</tr>
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<tbody>
<tr>
<td>A/WSN/33 (H1N1)</td>
<td>0.12 ± 0.02</td>
<td>42</td>
</tr>
<tr>
<td>A/PR/8/34 (H1N1)</td>
<td>0.15 ± 0.05</td>
<td>33</td>
</tr>
<tr>
<td>A/HK/8/68 (H3N2)</td>
<td>0.08 ± 0.02</td>
<td>63</td>
</tr>
<tr>
<td>A/duck/PA/84 (H5N2)</td>
<td>0.10 ± 0.03</td>
<td>50</td>
</tr>
</tbody>
</table>

a 50% Virus growth inhibitory concentration; b Selectivity index.
1 h at 37°C prior to infection with influenza virus did not inhibit plaque formation (Figure 2A), suggesting that the extract does not interfere with cell receptors for influenza virus. However, plaque formation was reduced by 53% after treatment with 1.6% A. mollis extract when the extract or zanamivir (100 nM) and virus were added to the cells at the same time (Figure 2B). When infected cells were overlaid with MEM containing different concentrations of A. mollis extract or zanamivir (100 nM), plaque formation was significantly inhibited in a dose-dependent manner. Plaque formation was reduced by 32%, 51%, and 92% in the presence of 0.1%, 0.4%, and 1.6% A. mollis extract, respectively, suggesting that the extract inhibited influenza virus infectivity. Furthermore, no plaques were observed in the presence of zanamivir, which blocks the release of progeny virions from infected cells (Figure 2C). Next, we examined the effect of virus exposure to A. mollis extract prior to infection. Influenza virus particles (6 × 10⁶ pfu) were incubated with different concentrations of the A. mollis extract or zanamivir (100 nM) at RT for 1 h or 3 h and then diluted (300 pfu/mL) for the plaque formation assay.

A significant reduction in plaque formation was observed after incubation with 1.6% A. mollis extract (Figure 2D and 2E), indicating that the extract directly affects virus particles. Collectively, these results suggest that the inhibitory effect of A. mollis extract is due to a direct virucidal or neutralizing activity of the extract against influenza virus particles.

### 3.4. Inhibition of hemagglutination by A. mollis extract

The results of the plaque reduction assay suggest that A. mollis extract inhibits influenza virus particle infectivity. It is well known that influenza virus hemagglutinin (HA) plays an essential role in virus infection. Influenza A virus HA can bind to N-acetylneuraminic acid on the surface of RBCs, causing agglutination. Thus, to examine whether A. mollis extract blocks the ability of virus particles to bind to cell receptors, we performed an HI assay. Influenza A viruses A/WSN/33, A/HK/8/68, and A/duck/Pennsylvania/84 were pretreated with the extract for 1 h before the addition of RBCs and further incubation at RT for 1 h. A. mollis extract inhibited binding of the three strains to RBCs in a concentration-dependent manner, indicating that the extract directly acts on influenza virus particles (Figure 3A). Moreover, sodium bicarbonate solution (1%, w/v), an extraction solvent for A. mollis, did not inhibit the hemagglutination activity of A/WSN/33 virus particles (Figure 3B).

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**Figure 2.** Effect of A. mollis extract and zanamivir on plaque formation. Plaque formation assays were carried out as described in the "Materials and Methods" section. (A) Treatment of cells prior to infection. (B) Treatment of cells during virus infection. (C) Treatment of cells after virus infection. (D) Treatment of virus prior to infection at RT for 1 h. (E) Treatment of virus prior to infection at RT for 3 h. The results are presented as the percentage of plaques formed in each treatment relative to the plaques formed in untreated controls, an average of 160 plaques. The results are represented as the mean ± SD obtained from three independent experiments. * indicates statistical significance (p < 0.05, Student's t test).

**Figure 3.** Hemagglutination inhibition assay. (A) A. mollis extract was diluted as indicated in 96-well micro titer plates using (PBS) (--). Fifty microliters of virus diluted in (PBS) (--), were added to 50 μL of the extract dilutions and incubated for 1 h at 4°C. Chicken RBCs (5% v/v) in (PBS) (--), were mixed with the pre-treated virus and incubated again at RT for 1 h. (B) The hemagglutination inhibition activity of 1% (w/v) NaHCO₃ was tested in a similar manner.
3.5. Thermal stability and molecular size of A. mollis active ingredient(s)

To determine the properties of the active ingredient(s) present in A. mollis extract, heat treatment and ultrafiltration of the extract were performed. Heat stability was determined by heating the extract at 60°C or 100°C for 30 min prior to testing for antiviral activity. Figure 4A shows that heat-treated and untreated extracts showed similar antiviral activities, suggesting that the active ingredient(s) of the extract are heat stable. Additionally, the molecular weight of active ingredient(s) in the extract was determined. Filtrates from 10, 30, and 50 kDa ultra-filtration columns and unfiltered control were tested for antiviral activity. The 10-kDa filtrate did not have antiviral activity, whereas the 30- and 50-kDa filtrates did, although they were less potent than the unfiltered control (Figure 4B). This can be explained by the fact that compounds with molecular weights close to the nominal molecular weight limit of the column may be partially retained. These results indicate that the active ingredient(s) in the extract have molecular weights greater than 10 kDa and less than 30 kDa.

3.6. Synergistic effect of zanamivir and A. mollis extract combination

Zanamivir and A. mollis extract have different mechanisms of action. Zanamivir blocks the release of progeny virions from infected cells, while A. mollis extract inhibits the infectivity of influenza particles. Therefore, a combination study was performed to determine the effect of zanamivir and A. mollis extract co-treatment on influenza virus replication. The inhibitory effect of zanamivir against influenza virus increased in the presence of the extract. Using the isobole method, the interaction index at 50% inhibitory activity was calculated to determine whether the observed effect was additive or synergistic. Table 3 shows that different zanamivir and extract combinations had interaction indices of < 1, indicating synergistic effects. Increasing extract concentrations decreased synergism, and maximal synergy (interaction index = 0.36) was observed in the 0.01% A. mollis extract and 2.2 nM zanamivir combination.

4. Discussion

In this study, we demonstrated the anti-influenza activity of A. mollis extract. We found that the extract acts on influenza virus particles directly and inhibits their infectivity. Plaque assays where drugs are added to the overlay gels are one of the most reliable methods for screening compounds with antiviral activity. When infected confluent MDCK cell monolayers were overlaid with 0.8% agarose in MEM containing A. mollis extract, plaque formation by the A/WSN/33 virus was significantly inhibited. Exposure of the virus to the extract prior to infection and treatment of the cells during infection significantly reduced plaque formation, whereas pre-treatment of the cells did not affect plaque formation. Further, A. mollis extract inhibited the hemagglutination ability of influenza virus. Therefore, it is conceivable that the inhibitory effect of the extract is a result of its direct interaction with the virus. A. mollis extract was effective against four different strains of influenza A virus with slight differences in sensitivity, suggesting that the extract may be a wide range inhibitor against influenza virus infections. We also showed a synergistic effect on influenza virus replication when zanamivir and A. mollis extract were used in combination.

Alchemilla species extracts have significant radical scavenging activity because of their high levels of flavonoid glycosides and tannins. In addition to other biological activities, the flavonoid glycosides isoquercitrin, miquelianin, and hyperoside present in A. mollis (10) have demonstrated anti-influenza activity (17-19). However, antiviral activity of Alchemilla
species-derived flavonoid glycosides has not been reported. Flavonoid glycosides are soluble in water and alcohols, and if the aglycone has a free phenolic group, the compound is soluble in alkaline solutions (20), unlike most tannins, which are unstable in alkaline conditions. Since an alkaline extraction method was used to produce the A. mollis extract in this study, it is possible that the active ingredient(s) in the extract could be flavonoid glycosides. Moreover, most flavonoids exist as glycosides or polymers and are thermally stable (21); this is in agreement with our findings that the active ingredient(s) in A. mollis extract have molecular weights between 10 and 30 kDa and exhibit thermal stability.

Synergistic interactions are being widely studied in phytomedicine. The presence of many bioactive constituents and their by-products in plant extracts is claimed to be responsible for the high effectiveness of many extracts. Synergistic effects are produced when extract constituents affect different targets or interact with one another to improve the solubility and thereby increase the bioavailability of one or several substances in the extract (22). Combination therapy with a highly active antiretroviral treatment regimen has proven to be quite effective in HIV management and in drug resistance reduction (23). The combination of currently available anti-influenza drugs showed the potential for additive or synergistic antiviral activity and inhibition of drug resistance in in vitro and in vivo studies. These reports point out the benefits of combination therapy. Furthermore, a combination of drugs can also reduce side effects.

In conclusion, we have demonstrated the anti-influenza activity of A. mollis by direct association with influenza virus particles. In addition, the inhibitory effect of A. mollis was shown to be strain-independent. Because Alchemilla plants (lady's mantle) is available as an herbal tea (Lady's Mantle Herbal Tea bags, Alchemilka Herba Alchemillae) it could easily be used for influenza infection management together with zanamivir. Collectively, these findings indicate that A. mollis could be a potential anti-influenza agent, although more studies are necessary to confirm its anti-influenza effect in vivo.

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References


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