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Antiviral activity of five Asian medicinal pant crude extracts against highly pathogenic H5N1 avian influenza virus

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ABSTRACT

Objective: To study the antiviral properties of the five Asian medicinal plants against *in vitro* infection by the highly pathogenic avian influenza virus (H5N1).

Methods: Crude extracts of *Andrographis paniculata*, *Curcuma longa* (*C. longa*), *Gynostemma pentaphyllum*, *Kaempferia parviflora* (*K. parviflora*), and *Psidium guajava* obtained by both water and ethanol extractions were investigated for their cytotoxicity in the Madin–Darby canine kidney cells. Thereafter, they were investigated *in vitro* for antiviral activity and cytokine response upon H5N1 virus infection.

Results: The results revealed that both water and ethanol extracts of all the five studied plants showed significant antiviral activity against H5N1 virus. Among these plants, *C. longa* and *K. parviflora* showed strong anti-H5N1 activity. Thus, they were selected for further studies on their cytokine response upon virus infection. It was found that ethanol and water crude extracts of *C. longa* and *K. parviflora* induced significant upregulation of *TNF-α* and *IFN-β* mRNA expressions, suggesting their roles in the inhibition of H5N1 virus replication.

Conclusions: To the best of the authors' knowledge, this study is among the earliest reports to illustrate the antiviral property of these Asian medicinal plants against the highly pathogenic avian H5N1 influenza virus. The results of this study shed light on alternative therapeutic sources for treatment of H5N1 influenza virus infection in the future.

1. Introduction

The influenza disease caused by infection with the highly pathogenic avian influenza H5N1 virus is one of the most devastating, zoonotic viral infectious diseases affecting humans worldwide [1]. Generally, the H5N1 virus infects wild and domestic birds, and, occasionally, mammals, including humans [2–4]. H5N1-infected patients usually show the symptoms in the respiratory system, with occasional intestinal and nervous system infections [5,6]. Currently, treatments of the influenza virus-infected patients are mainly based on supportive treatment, depending on the patient's conditions, together with an application of antiviral drug therapy [7,8]. The approved anti-influenza drugs, so far, are classified in groups of neuraminidase inhibitors, such as oseltamivir and zanamivir, and an M2 ion channel inhibitor, such as amantadine and rimantadine [9–11]. Although anti-influenza drugs have been used effectively against influenza A virus infection in humans, increased reports of drug-resistant influenza viruses have brought the attention back on the disease which has become a matter of public concern [12–16]. Therefore, seeking out alternative anti-influenza agents is needed and is of importance in the formation of strategies in the future for treatment of influenza diseases.

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Medicinal plants have long been known for their protective effects against a broad range of bacteria, protozoa, parasites, and viruses [17–19]. Using extracts from medicinal plants, a number of studies have shown their protective effects against infection with H1N1, H6N1, and H3N8 influenza viruses [13,20–22]. However, only rare reports have described the effects of medicinal plants against H5N1 virus infection [23,24]. Since Thailand is situated in the tropical regions where a large variety of medicinal plants grow naturally, crude extracts of five native Asian medicinal plants, including *Andrographis paniculata* (*A. paniculata*), *Curcuma longa* (*C. longa*), *Gynostemma pentaphyllum* (*G. pentaphyllum*), *Kaempferia parviflora* (*K. parviflora*), and *Psidium guajava* (*P. guajava*), were used to study the antiviral effects against infection with the H5N1 virus. Furthermore, plants that showed strong antiviral activity were subsequently investigated for their ability to induce cytokine mRNA expression in the tested cell line. The results obtained from the present study indicate that crude extracts of the studied plants may be used as an alternative therapeutic compound in the treatment of H5N1 influenza-infected patients or animals in the future.

2. Materials and methods

2.1. Plants and preparation of extracts

Naturally growing *A. paniculata*, *C. longa*, *G. pentaphyllum*, *K. parviflora* and *P. guajava* were collected from Muang District of Chiang Mai Province, Thailand, from April through June, 2013. Crude extracts of dried leaves of *A. paniculata*, *G. pentaphyllum*, and *P. guajava*, and roots of *C. longa* and *K. parviflora* were obtained by maceration with either 95% ethanol or water, as previously described [19]. The extract solutions were filtered with Whatman No. 1, concentrated under a rotary evaporator (Buchi, Flawil, Switzerland), and finally lyophilized (Snijders Scientific, Tilburg, The Netherlands) to obtain the dried crude extracts. These dried crude extracts were resuspended with 1% dimethyl sulfoxide (DMSO, Bio Basic Inc., NY, USA), aliquoted to a final dilution of 100 mg/mL, and kept at −20 °C for use as study reagents in further experiments.

2.2. Cell culture and viruses

The Madin–Darby canine kidney (MDCK) cells were grown in Dulbecco's modified Eagle medium (DMEM; PAA Laboratories GmbH, Pasching, Austria), and supplemented with 10% fetal bovine serum (FBS; PAA Laboratories GmbH, Pasching, Austria) and 1% Pen/Strep under standard culture conditions (37 °C, 5% CO_2). The highly pathogenic avian H5N1 influenza virus (A/Chicken/Thailand/CUK2/04; kindly provided by Prof. R. Thanawongnuwech, Faculty of Veterinary Sciences, Chulalongkorn University, Bangkok, Thailand) was propagated on MDCK cells. The virus titer was determined by the Reed and Muench method as 50% tissue culture infectious dose ($TCID_{50}$)/mL.

2.3. Cytotoxicity testing

Cytotoxicity of the study reagents on MDCK cells was studied by both staining with crystal violet and MTT assay (Sigma–Aldrich Co., St. Louis, MO, USA) as previously described [19]. In brief, MDCK cells were incubated in 96-well microtiter plates (Nunc®, Roskilde, Denmark) for 24 h under standard culture conditions. Two-fold serial dilutions of the study reagents were mixed in DMEM/10% FBS and added in triplicate to the 90% confluent cells. The medium without any study reagent served as the control. After 72 h, the cells were either fixed or stained with crystal violet, or their viability was assessed by the MTT test. The 50% cytotoxicity dose (CD_{50}) of the crude extracts that did not kill the cells was chosen and used for further studies.

2.4. Antiviral activity test

Antiviral test of the crude extracts of medicinal plants was performed in the MDCK cells, as previously described [19]. Briefly, MDCK cells were grown in 96-well microtiter plates, and were infected with the H5N1 virus at a multiplicity of infection (MOI) of one, for 1 h, at 37 °C. Then, unbound viruses in the supernatants were removed, and a medium (DMEM/10% FBS) containing *A. paniculata*, *C. longa*, *G. pentaphyllum*, *K. parviflora* and *P. guajava* study reagents was added. The control was a medium with DMSO added to 1% concentration, but without the study reagents. The plates were incubated under standard culture conditions; the supernatant containing the virus was collected at 24 h, 48 h, and 72 h post infection (hpi), and quantified for virus titer, as described previously [25].

2.5. RNA extraction and quantitative RT-PCR

The total RNA of the H5N1-infected MDCK cells in the presence of the study reagents was obtained and determined using Nucleospin® RNA II (Machery-Nagel GmbH, Dauren, Germany), as suggested by the manufacturer. The control group was H5N1-infected MDCK cells in the presence of DMSO alone. The cDNA was synthesized from 500 ng total RNA with poly (dT) primers and high capacity cDNA reverse transcriptase kits (Applied Biosystems, CA, USA). To quantify the expression of cytokines in the MDCK cells, real time PCR (SYBR® green Selected Master Mix, Life Technologies, CA, USA) was performed on the ABI7300 thermo cycler (Applied Biosystems, CA, USA) in a total reaction volume of 20 L. The oligonucleotide primers used in this study, including *TNF-α, IFN-α, IFN-β, IL-6, IL-10,* and *GAPDH,* have been reported as used in previous studies [26–28]. The PCR conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. The threshold cycles (Ct) of all genes were used for the calculation of the gene expression by the $2^{-\Delta\Delta CT}$ method [29], normalized to that of *GAPDH* gene at the corresponding time points compared to the control groups. The data were obtained from triplicate wells from at least two independent experiments, and shown as mean fold changes ± standard errors.

2.6. Statistical analysis

Statistical analyses were performed with GraphPad Prism 5 (Graph Pad Inc., La Jolla, CA, USA). Student *t*-test was used to compare the means of two independent groups. Statistical significance was designated as $P < 0.05$.

3. Results

3.1. Plant preparation and determination of its toxicity on MDCK cells

To investigate the anti-H5N1 virus activity of the five Asian medicinal plants, including *A. paniculata*, *C. longa*, *G. pentaphyllum*, *K. parviflora* and *P. guajava*, crude extracts using water and ethanol extractions were obtained. The plant part used and the percentage yield obtained by the two different solvents are shown in Table 1. The cytotoxicity test on the MDCK cells of the ethanol extracts of *A. paniculata*, *C. longa*, *G. pentaphyllum*, *K. parviflora* and *P. guajava* study reagents revealed that at high concentrations of 8.2 g/mL, 69.3 g/mL, 135.6 g/mL, 2.2 g/mL, and 90.7 g/mL, respectively, these ethanol extracts did not show any cytotoxic effect to the cells (Table 1). On the other hand, the water extracts of *A. paniculata*, *C. longa*, *G. pentaphyllum*, *K. parviflora* and *P. guajava* at high concentrations of 380.3 g/mL, 142.3 g/mL, 468.2 g/mL, 438.4 g/mL, and 195.6 g/mL, respectively, showed no toxicity on the tested cells (Table 1). These concentrations of the extracts were, therefore, chosen for further studies on their antiviral activity *in vitro*.

3.2. Screening of extracts for anti-H5N1 virus activity

The results of the antiviral test show that crude extracts obtained by water and ethanol extractions of *A. paniculata*, *C. longa*, *G. pentaphyllum*, *K. parviflora* and *P. guajava* significantly ($P < 0.05$) inhibited H5N1 virus replication in MDCK cells when compared to the control group (Figure 1). Among these plants, inhibition of the H5N1 virus replication was predominantly observed as early as 24 hpi by the ethanol extract of *C. longa* (Figure 1). Furthermore, at 48–72 hpi, the water and the ethanol crude extracts of *C. longa* and *K. parviflora* showed significant antiviral activity ($P < 0.05$) against the H5N1 virus. The plants were, therefore, chosen for further investigation of their induction of cytokine response in the tested cells after H5N1 virus infection. Here, it needs to be emphasized that the antiviral property of *A. paniculata*, *C. longa*, *G. pentaphyllum*, *K. parviflora* and *P. guajava* study reagents were not due to DMSO since the control group, the DMSO-containing medium, did not affect the virus growth kinetic (Figure 1).

3.3. Effect of plant extracts on cytokine mRNA expression

To investigate whether the antiviral effect of *C. longa* and *K. parviflora* crude extracts relates to the expression of cytokines in MDCK cells, H5N1-infected cells in the presence or absence of the extracts were collected and tested for their mRNA expression by quantitative RT-PCR. The results indicated that crude extracts of *C. longa* and *K. parviflora* significantly induced upregulation of the *TNF-α* and the *IFN-β* mRNA expressions in the MDCK cells upon inoculation with H5N1 virus (Figure 2). It should be noted that the expression of *IFN-β* in H5N1-infected MDCK cells was observed mainly only at 24 hpi, while the expressions of TNF-α by *K. parviflora* and *C. longa* crude extracts were found to have significant upregulation, up to 48 hpi and 72 hpi, respectively. On the other hand, the expressions of *IL-6* and *IL-10* mRNA by *K. parviflora* and *C. longa* crude extracts seemed to be significantly downregulated compared to the control group (Figure 2).

Table 1
Percent yield and CD_{50} of the five medicinal plants obtained by two different solvents on MDCK cells.

Botanical name	Parts	Family	Percent yield (%)		CD_{50} (μg/mL)	
			water[a]	EtOH[b]	water[a]	EtOH[b]
A. paniculata	Leaves	Acanthaceae	7.17	4.62	380.3	8.2
G. pentaphyllum	Leaves	Cucurbitaceae	3.79	2.25	468.2	135.6
C. longa	Roots	Zingiberaceae	6.31	7.12	142.3	69.3
P. guajava	Leaves	Myrtaceae	7.82	3.42	195.6	90.7
K. parviflora	Roots	Zingiberaceae	5.93	6.52	438.4	2.2

[a]Water extraction; [b]Ethanol extraction.

4. Discussion

During the past few years, outbreak of avian influenza virus infection in humans has been a matter of serious concern with regard to public health because of its potential pandemic proportions in human's society [30–32]. Moreover, an increase in the cases of drug-resistant influenza A virus [14,33,34] has brought to the fore the urgent need for alternative and abundantly available anti-influenza agents. In the present study, it has been shown for the first time that ethanol and water crude extracts of five Asian medicinal plants, including *A. paniculata*, *C. longa*, *G. pentaphyllum*, *K. parviflora* and *P. guajava*, possess antiviral properties against H5N1 influenza virus infection *in vitro*, and could be used as alternative antiviral compounds against H5N1 influenza virus infection. The five Asian medicinal plants used in this study were particularly chosen since there have been reports of their antiviral activity against herpes simplex virus type 1 [35], Newcastle disease virus [36], hepatitis B virus [37], influenza H1N1 virus [38], human immunodeficiency virus (HIV), and human cytomegalovirus virus [39], exhibited especially by *A. paniculata*, *G. pentaphyllum*, *C. longa*, *P. guajava*, and *K. parviflora* crude extracts, respectively. Despite reports that these five medicinal plants inhibit pathogenic H5N1 influenza virus replication, the results of this study indicate that among these plants, only the crude extracts of *C. longa* and *K. parviflora* obtained by both water and ethanol extractions showed strong antiviral activity against the H5N1 virus.

It has been shown that the chemical constituents of ethanol extract of *K. parviflora* are 5,7-dimethoxyflavone, trimethylapigenin, and tetramethylluteolin, and that these compounds bear anti-inflammatory activity and inhibition of nitric oxide synthase (NOS) expression in RAW 264.7 cells [40]. Furthermore, it has been shown that 5-hydroxy-7-methoxyflavone and 5,7-dimethoxyflavone obtained from *K. parviflora* are the most potent inhibitors of HIV-1 protease activity [39]. In addition to *K. parviflora* crude extract, it has been shown that curcumin (diferuloylmethane), the active ingredient of *C. longa*, is a potent compound for anti-tumorigenic, anti-bacterial, antiviral, and anti-inflammatory activities, both *in vivo* and *in vitro* [41–43]. Although the effect of curcumin against H1N1 and H6N1 influenza has been reported [22], this study is among the earliest to investigate anti-H5N1 virus activity and cytokine response of infected cells after virus infection.

The effects of anti-H5N1 virus activity by *C. longa* and *K. parviflora* crude extracts were clearly demonstrated by the upregulation of the *TNF-α* and the *IFN-β* mRNA expressions in the tested MDCK cells. IFN-β, a subset of type 1 interferon, is

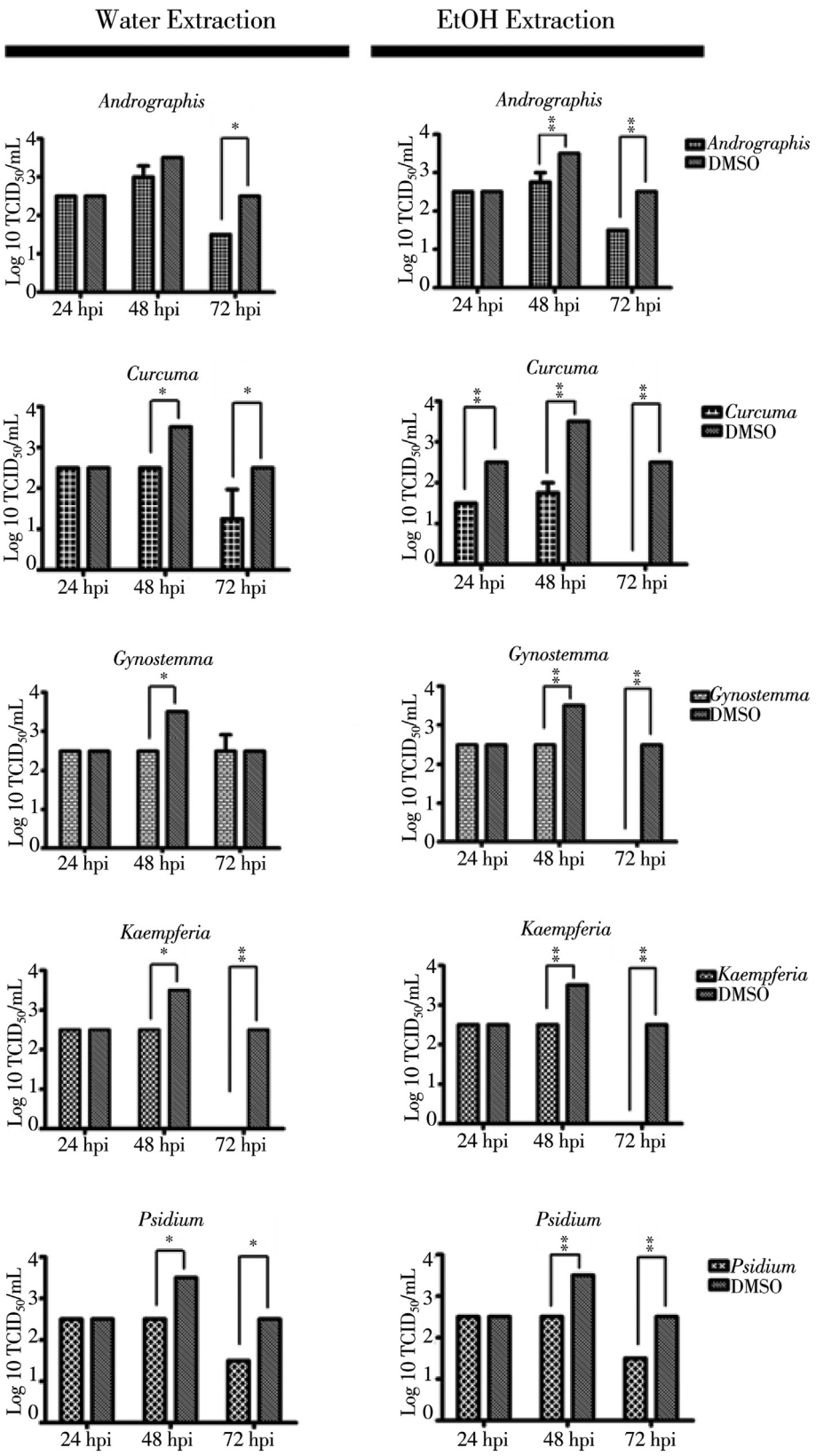

Figure 1. The antiviral activity of *A. paniculata*, *C. longa*, *G. pentaphyllum*, *K. parviflora*, and *P. guajava* crude extracts on the H5N1 virus at the multiplicity of infection (MOI).
The representative data are mean and standard error. Asterisks indicate statistical significance (* = P <0.05 and ** = P <.001).

known for its potent antiviral cytokines [44,45]. The application of IFN-β therapy has been shown to reduce disease severity and viral load of the hepatitis C virus, both *in vivo* and *in vitro* models [46–48]. In addition to IFN-β, a previous study indicates, it is TNF-α that is the first line of defense against avian, swine, and human influenza virus infections in the natural host [49]. The powerful attack against *in vitro* anti-influenza virus by TNF-α is in a dose-dependent manner, and the antiviral effect is greater than that of IFN-γ and IFN-α [49]. The antiviral activity of TNF-α has been shown to be regulated

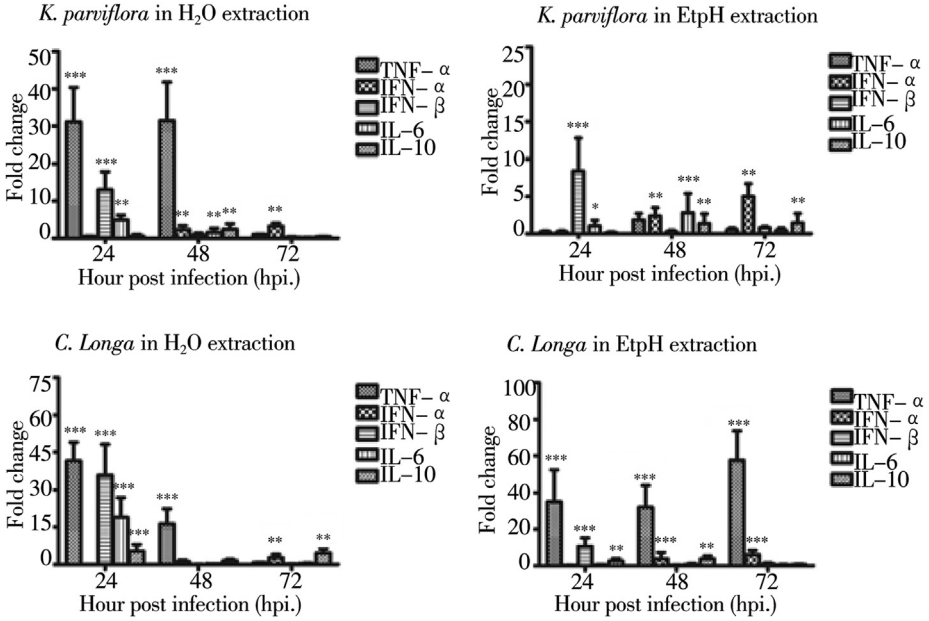

Figure 2. Quantitative assessment of cytokine expression in H5N1-infected MDCK cells in the presence of different crude extracts. The representative data are mean and standard error.
Asterisks indicate statistical significance (* = P <.005, ** = $P \leq 0.01$, and *** = P <.0001).

by the expression of the NF-κB-activating inhibitor of the κB kinase complex IKK-α/β, and over-expression of the latter inhibits replication of the hepatitis B virus [50]. Although it is unclear whether downregulation of IL-6 and IL-10 gene expressions involves in the anti-H5N1 virus activity in this study, there has been demonstrated that these cytokines are highly upregulated in the fatality cases of H5N1-infected humans [51]. To summarize, though the mechanism of the anti-H5N1 influenza virus by an upregulation of the *IFN-β* and the *TNF-α* mRNA expressions in MDCK cells by *C. longa* and *K. parviflora* crude extracts remains to be determined, the results of this study shed light on the use of medicinal plants as alternative antiviral compounds against influenza virus infection.

In conclusion, the present study demonstrates that crude extracts of *A. paniculata*, *C. longa*, *G. pentaphyllum*, *K. parviflora* and *P. guajava* at appropriate concentrations potentially inhibit H5N1 virus replication *in vitro*. The results of this study may be useful for application of these five medicinal plants in the control and prevention of H5N1 virus in the future.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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