Antiviral activity of Chinese medicine–derived phytochemicals against avian influenza A (H5N1) virus

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KEY MESSAGES

1. A screening platform was established to investigate antiviral agents from extracted herbal ingredients against infectious viruses, including avian influenza A (H5N1) virus.
2. More than 30 antiviral herbal fractions and compounds were screened for antiviral activity against H5N1 virus. Three proteins isolated from *Pandanus amaryllifolius* (PYM2), *Narcissus tazett*, and *Polygonatum odoratum* (POL) were identified to have most prominent anti-H5N1 potency. The efficacy of these proteins as antiviral products was investigated, as was molecular cloning and transgenic expression of both PYM2 and POL in bacteria (*Escherichia coli*) and POL in rice plants.
3. The proteins isolated from POL and the soluble seed protein from Gt1/SPPOL/POL transgenic rice showed a significant effect on inhibiting virus infection. This study provides the scientific basis for the use of anti-H5N1 ingredients as chicken feed.

Hong Kong Med J 2014;20(Suppl 4):S37-41
RFCID project number: 05050242

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Introduction

In 1997, a highly pathogenic avian influenza A virus (H5N1) in Hong Kong caused death in 6 of 18 infected persons.[1] Subsequent outbreaks of H5N1 avian influenza in several other countries indicated that the virus may be more widely established among the bird population and the environment than previously thought. Human deaths from avian influenza infections have been reported in Indonesia, Vietnam, Thailand, and China. Fortunately, there was no evidence of large-scale human-to-human transmission of the virus. To date only two classes of anti-influenza drugs (amantadine and its derivatives and neuraminidase inhibitors) have been clinically used, but their use may result in the emergence of resistant variants.[2] Phytochemicals such as phenolic compounds, flavonoids[3,4] and proteins such as cyanovirin-N[5] from natural sources may have significant inhibitory effects on influenza A viruses. Our laboratory has isolated, purified, experimentally tested, and identified more than 30 potent antiviral herbal extracts and active compounds that significantly inhibit various human viruses. Three proteins isolated from *Pandanus amaryllifolius* (PYM2), *Narcissus tazett* (NTL), and *Polygonatum odoratum* (POL) were identified to have most prominent anti-H5N1 potency. Their antiviral efficacy and potential as a product was investigated.

Methods

This study was conducted from September 2006 to August 2008. Antiviral activity against influenza A (H1N1) and/or (H3N2) viruses was evaluated in a P2 (Biosafety Level 2) laboratory, whereas that against avian influenza A (H5N1) virus was evaluated in a P3 (Biosafety Level 3) laboratory. A platform for screening antiviral activity against influenza viruses was set up to assess more than 30 experimentally proven antiviral chromatographic fractions and components from Chinese medicinal herbs (Table). The virus yield reduction assay was adopted. Antiviral activity was estimated. Triplicate cultures of Madin-Darby canine kidney cells in 60 mm plastic dishes infected with 100 plaque forming units/0.2 mL of H1N1 or H5N1 virus were set up. The infected cells were fixed and stained, and the virus titre was determined by a cytopathic effect reduction assay and/or plaque assay, which enabled identification of potent anti-H5N1 agents. Molecular characterisation and cloning of the potent antiviral proteins and their transgenic expression in bacteria as the bioreactor was then performed, and the immune modulatory potency of the active antiviral proteins was evaluated. Induction of production and gene expression of the immunomodulatory cytokines by the active antiviral proteins from herbs

was examined in mouse macrophages using specific cytokine primers, total RNA isolated from induced macrophages, and reverse transcription-polymerase chain reaction (RT-PCR). Their effects on the serum level of specific cytokines in mice were evaluated using the enzyme-linked immunosorbent assay (ELISA) 48 hours after an intraperitoneal injection of 5 mg/kg of the proteins.

Results

Of the 30 antiviral chromatographic fractions and components from Chinese medicinal herbs, 10 purified herbal compounds exhibiting potent inhibitory effects against H5N1 virus were identified. Among these, three proteins isolated from PYM2, NTL, and POL had the most prominent anti-H5N1 activity. The results of plaque reduction assays indicated that the proteins significantly inhibited the infectivity of H1N1, H3N2, H5N1, and influenza B viruses. For example, the antiviral effect of PYM2 was dose-dependent, with IC_{50} values of 3.75 μg/mL for H1N1, 0.13 μg/mL for H3N2, 26.03 μg/mL for H5N1, and 0.03 μg/mL for influenza B (Fig 1).

The anti-H5N1 efficacy of these proteins was further investigated for potential product development, possibly as antiviral chicken feed. Molecular cloning and transgenic expression

TABLE. Anti-H5N1 activity of purified phytochemicals and proteins that have been isolated and experimentally proven to have antiviral potential against other viruses*

Purified phytochemicals	Medicinal herbs	Anti–herpes simplex virus	Anti–respiratory syncytial virus	Anti–H5N1 virus
Amentoflavone	*Selaginella sinensis*		√	+
Wogonin	*Scutellaria baicalensis*		√	+
Anagyrine	*Sophora flavescens*		√	ND
Secoiridoid glucoside	*Ligustrum lucidum*	√	√	ND
Luteollin-7-O-glucoside	*Youngia japonica*		√	ND
Genkwanol B	*Wikstroemia indica*		√	ND
Orientin	*Trollius chinensis*		√	ND
7-O-galloyltricentifavan	*Pithecellobium clypearia*		√	+
7,4-di-O-galloyltricentifavan	*Pithecellobium clypearia*		√	+
Daphnoretin	*Wikstroemia indica*		√	+
Cassanefuranoditerpenoid	*Caesalpinia minax*	#	√	+
Friedelane triterpenoid	*Caesalpinia minax*	#	√	+
Lupane-type triterpenoid	*Schefflera heptaphylla*		√	+
Sesquiterpene	*Schefflera heptaphylla*		√	+
3,4-di-O-caffeoylquinic acid	*Schefflera heptaphylla*	√	√	++
3-O-caffeoylquinic acid	*Youngia japonica*		√	+
Cinnamaldehyde	*Cinnamomum cassia*	√		+
Lignin-CHO complex	*Prunella vulgaris*	√		++
Beta-glucan	*Pleurotus tuber-regium*	√		+
Polysaccharide	*Ganoderma lucidum*	√		+
Sulfated polysaccharide	*Sargassum patens*	√		++
Sulfated polysaccharide	*Hydroclathrus clathratus*	#		++
Polysaccharide	*Ardisia chinensis*		#	+
Lectin	*Pandanus amaryllifolius*	√	√	+++
Lectin	*Narcissus tazetta*	√	√	+++
Lectin	*Allium tuberosum*	√		++
Lectin	*Smilax glabra*	√	√	++
Lectin	*Dendrobium nobile*	√		++
Lipid-transfer protein	*Narcissus tazetta*	#	√	+
Lectin	*Polygonatum odoratum*	√	√	+++

* √ denotes having antiviral activity, # having potent activity against other viruses, + weak activity, ++ moderate activity, +++ prominent activity, and ND no detectable activity

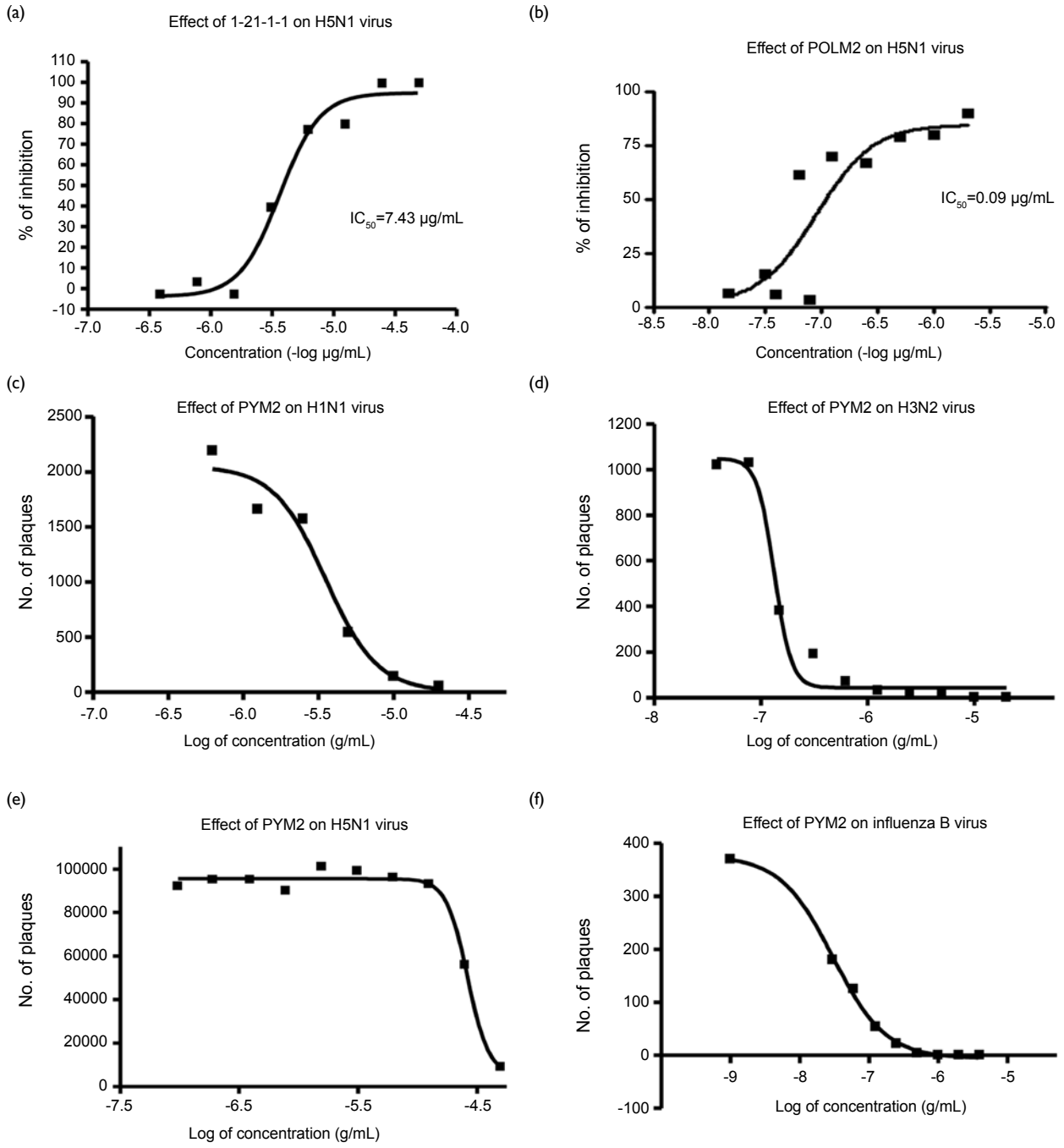
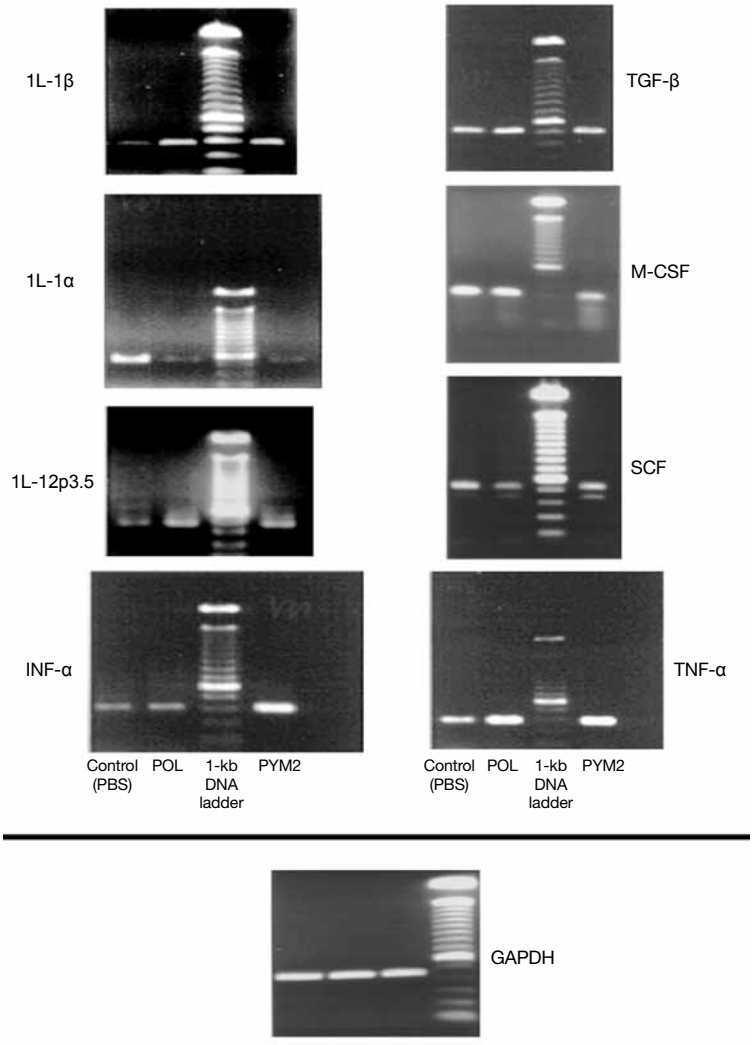


FIG 1. Inhibitory effects of (a) soluble *Polygonatum odoratum* (POL) protein from transgenic rice seed (0.39-100 μg/mL) and (b) purified POL from rhizome of POL (0.010-10 μg/mL) against H5N1 virus, and *Pandanus amaryllifolius* (PYM2) [0.001-50 μg/mL] against (c) H1N1, (d) H3N2, (e) H5N1, and (f) influenza B viruses. Results are based on an extracellular virus yield reduction assay and triplicate cultures of Madin-Darby canine kidney cells

of PYM2 and POL in bacteria (*Escherichia coli*) was investigated for potential application of the expressed proteins against H5N1 virus. For comparison, the rice-derived POL-containing soluble protein (obtained from another project) was confirmed to possess significant potency in terms of inhibiting H1N1 virus with an IC_{50} of 125.5 μg/mL, and H5N1 virus with an IC_{50} of 74.4 μg/mL, whereas POL isolated and purified directly from the leaves of POL exhibited anti-H5N1 activity with an IC_{50} of 6.23 μg/mL (Fig 1). In contrast, the soluble protein from wild type rice sample had no viral inhibitory effect on the three viruses tested, even though up to 1000 μg/mL soluble protein was applied on the viral culture. This suggested that the wild type rice seeds did not contain proteins that could inhibit the viral infections or that its antiviral activities were too low to be measured by this assay (Fig 1).

(a)



(b)

Cytokines	PYM2	POL
IL-1β	↑	↑
IL-1α	↓	↓
IL-2	ND	ND
IL-4	ND	ND
IL-10	+	+
IFN-γ	↑	↑
IL-12p35	↑	↑
TNF-α	↑markedly	↑slightly
TGF-β	↑slightly	+
M-CSF	↓	↑
SCF	↓	+

FIG 2. (a) Induction of cytokine gene expression by Pandanus amaryllifolius (PYM2) and Polygonatum odoratum (POL) in peritoneal macrophages; (b) modulation of cytokines by PYM2 and POL as in (a)

GAPDH is used as an internal standard. The proportion of densities of DNA bands is measured by a densitometer under ultraviolet light. ↑ denotes up-regulated, ↓ down-regulated, ND not detectable, + detectable but remains unchanged as compared to the control

The potency of the active antiviral proteins for modulating the immune system was evaluated. After induction of PYM2 and POL in mouse macrophages 48 hours after intraperitoneal injection of 5 mg/kg of the proteins, production and gene expression of IL-1β, IL-12p35, and TNF-α were substantially up-regulated, but TGF-β was only slightly up-regulated (Fig 2). Production of IFN-γ was more prominent in PYM2- than POL- induced macrophages. In blood serum, up-regulation of several cytokines was demonstrated. Compared with the control group (treated with only PBS), the up-regulation of IL-1β, IL-12, IFN-γ, and TNF-α was pronounced. A similar pattern appeared both in the induction by the proteins in cytokine production and gene expression in macrophages and the level of cytokines in blood serum of the mouse (Fig 2).

Discussion

Three proteins, namely PYM2, NTL, and POL, showed most activity against H1N1 and H5N1 viruses. As molecular cloning and characterisation of NTL had been extensively studied, investigation was focused on the expression of PYM2 and POL in bacteria (*E coli*). The proteins were confirmed to have significant inhibitory effects against H5N1 virus. For comparison, the soluble seed protein from Gt1/SP_{POL}/POL transgenic rice (obtained from another

project) was compared with purified POL for their effects on inhibiting virus infection. POL expressed in rice retains its potency against the virus. New application of herbal proteins was explored for the feasibility of rice-derived proteins against H5N1 virus and other human viruses. Further anti-viral activity assays such as plaque reduction assay should be performed using chromatographic column-purified rice protein.

The immune modulatory potency of the active antiviral substances was also evaluated for product development potential. The immunomodulatory effect of PYM2 and POL were investigated for their ability to induce production and gene expression of macrophage cytokines and the level of cytokines in blood serum of the mice after intraperitoneal injection of the test samples (PYM2 or POL or PBS as a control). The immunomodulation by PYM2 and POL on the cytokine profiles was monitored by the ELISA. The production and gene expression of IL-1β, IL-12p35, IFN-γ, and TNF-α were substantially up-regulated, but TGF-β was only slightly up-regulated. There was up-regulation of several cytokines in blood serum. Compared with the control group (treated with only PBS), the up-regulation of IL-1β, IL-12, IFN-γ, and TNF-α in mouse blood serum was prominent. This indicated that PYM2 or POL had some immunomodulatory effects in vivo, although its mechanism awaits elucidation.

Acknowledgement

This study was supported by the Research Fund for the Control of Infectious Diseases, Food and Health Bureau, Hong Kong SAR Government (#05050242).

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