VIROLOGICA SINICA, February 2009, 24 (1):19-27
DOI 10.1007/s12250-009-2983-x

CLC number: R373 Document code: A Article ID: 1674-0769 (2009) 01-0019-09

Anti-Influenza A Virus Effect of *Hypericum perforatum L.* Extract*

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Abstract： To study the antiviral effect of *Hypericum perforatum L.* extract (HPE) on influenza A virus (IAV) (H_1N_1) *in vitro* and *in vivo*. Cytopathic effect (CPE) and neutral red (NR) dye uptake were used to examine the antiviral effect of HPE on Madin Darby Canine Kidney (MDCK) cells which were infected with IAV *in vitro*. HPE was effective against influenza A virus (IAV) *in vitro*, with a 50% effective concentration (EC_{50}) of 40 μg/mL. The mean 50% cytotoxic concentration (CC_{50}) in the MDCK used in these experiments was 1.5 mg/mL. Ribavirin was run in parallel with EC_{50} values of 5.0 μg/mL; the mean CC_{50} for ribavirin was 520 μg/mL. Oral gavage administrations of HPE or ribavirin to mice infected with the IAV were highly effective in preventing death, slowing the decline of arterial oxygen saturation, inhibiting lung consolidation and reducing lung virus titers. The minimum effective dose of HPE in these studies was 31.25 mg/kg/day, which was administered twice daily for 5 d beginning 4 h prior to virus exposure. Below a dosage of 2000 mg/kg/day, almost all treated mice survived, which suggests that HPE is of low toxicity. Ribavirin's minimum effective dose was 40 mg/kg/day with the LD_{50} determined to be 200 mg/kg/day. Delay of the initiation of either HPE or ribavirin therapy, using approximately 1/3 LD_{50} dose each time, could still be protective as late as 48 h after exposure to the IAV. While both agents appeared to have similar efficacy against IAV infections, HPE was considered to be less toxic and may warrant further evaluation as a possible therapy for influenza.

Key words: Influenza A virus (IAV); *Hypericum perforatum L.* extract (HPE); Ribavirin

Influenza infection is a common respiratory disease, causing high morbidity in the general population, a high mortality in at-risk groups, as well as considerable cost of hospitalization and treatment and loss in productivity (38). According to WHO data, 10% -20% of the world population fall victims to influenza yearly. Although influenza usually proceeds as a 'self-limited' infection, it can cause severe extra-pneumonic complications (20). The possibility of both pneumonic and extra-pneumonic complications increases dramatically

Received: 2008-07-31, Accepted: 2008-11-28

* Foundation items: One Hundred Person Project of The Chinese Academy of Sciences (2008-287) ; The Project of Basic Scientific Research Fund for Central Public-Welfare of Institute of Sciences (BRF070402).

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in less healthy and senior patients. An efficient protection against influenza is vaccination which, in patients above 65 years of age, could reduce the probability of death by up to 80% (26). This is particularly true in situations in which vaccines are unavailable or ineffective due to viral antigenic changes or poor host immune responses. In this regard, the development of antiviral drugs provides another important way to combat influenza.

Despite recent success in the development of new antiviral agents, the need for effective therapies for influenza virus infections continues to exist. The M_2 channel inhibitors are effective against influenza strains, but their use is limited because of central nervous system and gastrointestinal side effects, emergence of viral resistance and lack of effectiveness against influenza B (6). Meanwhile the broad-spectrum antiviral, ribavirin, is a recognized inhibitor of influenza virus infections *in vitro* and in animal models (30, 12, 13), but ribavirin has a relatively small therapeutic index, causes hemolytic anemia induced at high doses and produces potential teratogenic effects (8, 31).The search for viral inhibitors with plant origins is a promising approach in the development of new therapeutic agents. In this respect a large number of extracts and pure substances have been tested and some of them have been proved to have a selective antiviral effect (5, 39).

The plant *Hypericum perforatum L* belongs to the genus Guttiferae which contains about 400 species, and is found in Europe, West Asia, North Africa and North America. It is used in herbal medicine externally for the treatment of skin wounds, eczema and burns and internally for several applications such as diseases of the alimentary tract (21, 9). Many studies have shown that *Hypericum perforatum L.* extract (HPE) has antiviral activity (1, 29) and can also have considerable effect against cytomegalovirus (CMV) and human immunodeficiency virus (HIV) (2, 10, 11, 40, 27, 28). The antiviral action of HPE has been studied on lipid enveloped and non-enveloped DNA and RNA viruses (14, 15, 16, 36) and also, clinical studies have been conducted in HIV infected patients (4, 37).

Besides the above-mentioned activities, HPE has been reported to possess marked antimicrobial (17) and antiretroviral activity (15). However, scientific information is rarely available for the mechanism of antiviral activity of HPE at cellular and molecular levels. Therefore, the present study was designed to study the effect of HPE at cellular level using the culture of MDCK cells infected with IAV *in vitro*, and the animal model of influenza A virus infection by comparing its therapeutic effects with those of Ribavirin, whose mechanisms of antiviral activities are well known. The results of these experiments are reported here.

MATERIALS AND METHODS

Agents

Hypericum perforatum L. extract was provided by Lanzhou Institute of Animal and Veterinary Pharmaceutics Sciences, Chinese Academy of Agricultural Sciences and the ribavirin (H19993462) was purchased from Tianjin Pharmaceutical Group Xinzheng Co., Ltd, Henan, China.

Virus and cell

The human influenza A virus (IAV) strain (H_1N_1) (A/Gansu/1771/2006, Gansu Center for Disease Control and Prevention, Lanzhou, China) was used in this

study. The virus used in cell culture experiments was passaged through MDCK cells at least once to prepare pools. The pools were then titrated in MDCK cells before use and were then propagated in the allantoic cavities of 9-day-old chicken eggs for 72 h at 35℃. Then the virus was passaged five times through mice, after which the recovered virus from the lungs was used to prepare a pool in MDCK cells, which were provided by the Gansu Center for Disease Control and Prevention. MDCK cells were grown in DMEM (Dulbecco's Modified Eagle Medium) (from Sigma Company) supplemented with 0.55% w/v $NaHCO_3$ (Jianghai Biological Engineering Co., Ltd, Beijing, China), 100 units/mL penicillin, 100 mg/mL streptomycin and 10% fetal calf serum (FCS) (Sijiqing Company, Hangzhou, China). FCS was reduced to 2% for the viral infection. The cell suspension containing 100 000 cells per mL was obtained after enzymatic dissociation with trypsin (1 g/L, 1:250, Lvshengyuan Biotechnology). 0.1 mL of this cell suspension was added to each well of a 96-well cell culture plate (Corning, USA, NY. 14831). The cells were incubated (at 37℃ in a 5%CO_2 incubator) for 3 d and after forming a continuous layer, they were inoculated with the virus.

Animals

Female specific pathogen-free BALB/c mice weighing 18-21 g were purchased from The Cancer Hospital of Gansu Province, China.

Arterial oxygen saturation (SaO_2) determination

SaO_2 was determined using the Ohmeda Biox 3800 pulse oximeter (Medi Tech Group Co., Ltd, Qingdao, China). The ear probe attachment was used with the probe placed on a thigh of the mice. Readings were made after a 30 s stabilization time on each animal (32). All SaO_2 determinations were made on days 3 through 11 after virus exposure.

Lung virus titer determination

Each mouse lung was homogenized and varying dilutions assayed in triplicate for infectious virus in MDCK cells grown in 96-well flat-bottomed microplates (34). Each lung homogenate was centrifuged at 2500 rpm for 30 min and the supernatants were used in these assays.

***In vitro* antiviral evaluation**

Three methods were used to assay antiviral activity *in vitro*: inhibition of virus-induced cytopathic effect (CPE) determined by visual (microscopic) examination of the cells, increase of neutral red (NR) dye uptake into cells and virus yield reduction (35). Eight concentrations of the test agents, each concentration varying in 2X steps, were evaluated in MDCK cells. Standard virus controls, toxicity controls and normal medium controls were included in all assays. CPE inhibition data were expressed as 50% effective (viral CPE-inhibitory) concentration (EC_{50}), 50% cytotoxic (cell-inhibitory) concentration (CC_{50}) and selectivity index (SI), determined as the CC_{50}/EC_{50}. Virus yield reduction data were expressed as the concentration inhibiting virus yield by one log10 (EC_{90}); the SI for virus yield results was calculated as CC_{50}/EC_{90}.

***In vivo* toxicity determination**

HPE and ribavirin were each evaluated for the dose considered lethally toxic to mice, which was done by treating ten mice per dose with HPE and ribavirin. The ribavirin doses studied were 320,160, 80, 40, and 20 mg/kg/day and HPE doses were 2000, 1000, 500, 250 and 125 mg/kg/day. The mice were treated by oral gavage (po) twice daily for 5 days and their weights were determined prior to the first treatment

and again 18 h after the final treatment. Animals were checked daily for 14 d to check whether any had died.

Initial *in vivo* antiviral studies

The general procedure of initial evaluation for the relative efficacies of HPE and ribavirin was to infect groups of mice intranasally with the IAV. This was done by anesthetizing them with aether and instilling 50 μL of 25 LD_{50} concentration of virus on the nares. The human influenza A virus (IAV) strain was studied *in vivo*. The mice were treated with varying dosages (as described in Table 3) of either drug po twice daily for 5 d beginning 4 h prior to virus exposure. Parameters for determining the effects of treatment included prevention of death through 14 d, slowing of SaO_2 decline, inhibition of lung consolidation [scores ranging from 0 (normal) to 4 (maximal plum coloration) and lung weight], and lessening of lung virus titers. The lung parameters were assayed on d 3, 6 and 9 of the infection, with 3-4 mice being killed at each time point. Ten mice were used per treated group to assay for death and SaO_2. Virus control was used in each experiment with the same numbers of animals used for each disease parameter. Toxicity control was also run in parallel for each dose; three mice were used per dose, with their weights taken before the start of the treatment and again 18 h after the last treatment and the animals were observed for overt signs of toxicity and death for 14 d. A normal control was run in parallel; these animals were weighed along with the toxicity controls and SaO_2 levels were ascertained on the same days as the animals were infected. The normal control was sacrificed along with the infected mice to provide background lung data.

***In vivo* antiviral effects of delay in therapy initiation**

To determine the relative efficacy of ribavirin and HPE against the IAV infection when initiation of therapy was delayed, groups of 10 mice infected with the IAV were treated po twice daily for 5 d with either agent beginning 4, 12 or 20 h post-virus exposure. Effects on mortality and slowing of SaO_2 decline were determined, with the numbers of animals per group described in Table 4. The dose of ribavirin was equivalently non-toxic (1/3 LD_{50} dose), which was 70 mg/kg/day, and the dose of HPE was equiva- lently nontoxic, 500 mg/kg/day. The animals were held for 14 d. When it was determined that all periods of treatment were highly effective, the experiment was repeated with treatments delayed until 28, 36 or 48 h post-virus exposure.

Statistical evaluations

Increases in number of survivors were evaluated using χ^2 analyses. Differences in the mean days to death, mean SaO_2 values, lung scores and mean lung virus titers were analyzed by t-test.

RESULTS

***In vitro* antiviral effects**

Both HPE and ribavirin were inhibitory to the influenza A virus evaluated, the EC_{50} values for ribavirin

Table 1. *In vitro* inhibition of influenza A virus by HPE and ribavirin

Agents	Neutral red EC_{50} (μg/mL)	Virus yield EC_{90} (μg/mL)
HPE	40	36
Ribavirin	5.0	6.5

Note: CC_{50} value for HPE was 1.5 mg/mL and CC_{50} value for ribavirin was 520 μg/mL. EC_{50}: 50% virus-inhibitory (effective) concentration. EC_{90} : 90% virus yield-inhibitory (effective) concentration (concentration inhibiting virus yield by 1 log10).

being 5.0 μg/mL, whereas HPE was approximately five-fold less potent, its EC_{50} being 24 μg/mL (Table 1). Multiple cytotoxicity assays using neutral red uptake indicated the mean CC_{50} values for ribavirin and HPE was 520 μg/mL and 1.5 mg/mL respectively.

Comparison of murine toxicities

Oral gavage treatment with ribavirin and HPE using therapies twice daily for 5 d indicated that the approximate LD_{50} dose of ribavirin was 200 mg/kg/day, whereas HPE was better tolerated since below a dosage of 2 000 mg/kg/day, almost all treated mice survived. It should also be noted that some weight gain was seen at dosages below 2000 mg/kg/day. No attempt was made to determine the cause of death in the mice in this range finding study (Table 2).

Comparison of the *in vivo* anti-influenza A virus efficacies of ribavirin and HPE

Multiple dosages of ribavirin and HPE were utilized and both were evaluated against the IAV in young adult mice. The results of each treatment against the IAV infections are summarized in Table 3. Due to the large amount of data available, only the last day of SaO_2 determination, day 11, is shown in the tables; it was on this day that the values usually reached their lowest levels in virus control animals and thus differences resulting from therapy were the most pronounced. The day 6 lung consolidation data are shown in the tables, since it was at this time that lung scores and weights were maximal; day 3 lung virus titers are indicated because these titer differences are usually best seen early in the infection. According to these parameters each agent was significantly inhibitory to the virus.

Against the influenza A virus infection, HPE was effective in inhibiting death and SaO_2 decline at doses down to 31.25 mg/kg/day. Ribavirin's inhibition of this virus infection was seen down to 40 mg/kg/day.

Each agent has approximately the same therapeutic index. But HPE represented the minimum effective dose and had the least toxicity in the mice, as described in Table 3.

Effects of delay in therapy initiation

The results of delaying the initiation of therapy with ribavirin and HPE on the influenza A virus infection are presented in Table 4. In this experiment, 500 mg/kg/day of HPE and 70 mg/kg/day of ribavirin were used, including a nontoxic dose of HPE and an approximately one-third of the LD_{50} dose of ribavirin.

Table 2. Comparison of toxicity of po administered HPE and ribavirin in mice

Agent	Dosage (mg/kg/day)	Survived/total	MDD[a] ± S.D.	Mean host weight changed[b] (g)	Estimated LD_{50}[c] (mg/kg/day)
HPE	2000	10/10	＞14	-0.4	
	1000	10/10	＞14	0.1	
	500	10/10	＞14	0.7	Lower toxicity
	250	10/10	＞14	1.2	
	125	9/10	12.2±0.8	0.9	
Ribavirin	320	2/10	3.7±2.3	-1.6	
	160	6/10	8.4±1.1	-1.1	
	80	9/10	11.9±0.5	0.2	200
	40	10/10	＞14	0.4	
	20	8/10	12.0±0.3	0.5	
Normal control	-	10/10	＞14	2.4	-

[a] Mean day to death of mice dying prior to day 14. [b] Difference between weight prior to the start of treatment and weight 18 h after the end of therapy. [c] Determined by line of regression.

Table 3 Inhibition of influenza A virus infections in mice treated po with HPE or ribavirin

Agent	Dose (mg/kg/day)	Survived/total	MDD[a]±S.D.	Mean day 11 SaO_2 (%±S.D.)	Mean lung parameters		
					Day 6 score±S.D.	Day 6wt (mg± S.D.)	Day 3 virus titer (log10±S.D.)
HPE	125	10/10 [a]	＞14 [a]	86.9±3.4 [a]	0.0±0.0 [a]	144±17 [a]	4.9±1.5 [a]
	62.5	10/10 [a]	＞14 [a]	85.7±3.3 [a]	0.0±0.0 [a]	159±2 [a]	4.6±1.0 [a]
	31.25	8/10 [a]	12.0±1.2 [a]	82.1±3.1 [a]	0.3±0.6 [a]	193±11 [a]	5.0±0.8 [a]
	15.63	4/10	7.6±2.8	76.6±4.2	1.3±1.7	237±23	6.1±1.1
Ribavirin	160	5/10	8.4±1.1	83.5±4.6 [a]	0.0±0.0 [a]	138±12 [a]	5.1±0.2 [a]
	80	9/10 [a]	12.7±2.8 [a]	82.6±1.7 [a]	0.4±0.5 [a]	166±10 [a]	5.7±1.0
	40	10/10 [a]	＞14 [a]	81.6±2.5 [a]	0.3±0.4 [a]	192±33 [a]	5.9±0.7
	20	3/10	6.4±0.3	75.7±1.8	1.7±0.8	217±24	6.4±0.4
Normal control	-	10/10	＞14	87.2±2.3	0.0±0.0	128±13	-
Virus control	-	2/10	7.4±1.3	72.3±0.0	2.9±0.4	264±17	6.9±1.3

Mean day to death of mice dying prior to day 14. [a] $P < 0.01$ compared to virus control

Both agents remained highly protective of the mice despite the delay in starting the treatment, and a highly significant difference in reduction of mortality was seen as late as the 20 h post-virus exposure initiation time, which was the longest delay evaluated in the first experiment. One infected animal treated with ribavirin and HPE respectively died at this latest therapy initiation time. All mice survived in the other infected and treated groups. In the second experiment, with the therapy initiation being further delayed, both agents were highly protective of the mice even when the treatment was begun 48 h after virus exposure, which was the latest time evaluated.

DISCUSSION

As a herbal remedy known since Greek and Roman times, *Hypericum perforatum L.*(HP) (Fam: Cluciaceae = Guttiferae) has been used against ulcers, diabetes mellitus, the common cold, gastrointestinal disorders, jaundice, hepatic and biliary disorders in Turkish medicine (3). Antidepressant actions of HP has been demonstrated in experimental (22, 23, 24)

Table 4. Effect of delay of p.o. HPE or ribavirin treatment on an influenza A virus infection in mice

Start of treatment (h post-virus exp.)	Agent	Dosage (mg/kg/day)	Survived/total	MDD[a] ±S.D	Mean day 11 SaO_2 ±S.D
4	HPE	500	10/10 [a]	＞14 [a]	85.9±3.1 [a]
	Ribavirin	70	10/10 [a]	＞14 [a]	85.1±2.7 [a]
12	HPE	500	10/10 [a]	＞14 [a]	84.6±2.2 [a]
	Ribavirin	70	10/10 [a]	＞14 [a]	84.1±0.8 [a]
20	HPE	500	9/10 [a]	12.9±0.7 [a]	83.7±1.8 [a]
	Ribavirin	70	9/10 [a]	12.3±0.1 [a]	82.9±2.6 [a]
4	Saline	-	1/10	5.9±2.1	76.1±1.4
28	HPE	500	10/10 [a]	＞14 [a]	84.1±2.9 [a]
	Ribavirin	70	10/10 [a]	＞14 [a]	83.6±1.0 [a]
36	HPE	500	10/10 [a]	＞14 [a]	83.3±2.4 [a]
	Ribavirin	70	10/10 [a]	＞14 [a]	83.0±1.4 [a]
48	HPE	500	10/10 [a]	＞14 [a]	82.7±1.7 [a]
	Ribavirin	70	9/10 [a]	11.9±0.7 [a]	81.9±2.5
28	Saline	-	1/10	6.2±0.6	76.1±2.0

Mean day to death of mice dying prior to day 14. [a] $P < 0.01$ compared to Saline

and clinical (37) studies. Antitumoral effects are also cited (19). Hepatoprotective activity of its extracts on rodent species has been reported too (7, 25).

HPE was considered highly effective against influenza A virus in this study, as was shown both *in vitro* and in experimentally infected mice. And its cytotoxicity was less than that of ribavirin (CC_{50} values of 1.5 mg/mL for HPE versus 520 μg/mL for ribavirin)

Efficacy in infected mice was demonstrated by all disease parameters studied in these experiments, including prevention of death, slowing of the mean day to death, lessening of SaO_2 decline, inhibition of lung consolidation as seen by lessened lung scores and lower lung weights and reduction in titers of infectious virus recovered from the lungs. In some experiments, the lung virus titers were not markedly affected by therapy with either agent, although a strong disease inhibitory effect was observed. It is probable that in such cases, examination of the lungs at an earlier time, e.g. day 1, would have demonstrated a greater effect. This was shown in the case with neuraminidase inhibitors (33). This early time period was not selected for the present study because significant lung consolidation is not manifested at this time.

The relative toxicities of the two agents in mice did not correlate well with what was seen *in vitro*; in the animals, HPE was approximately no more toxic than ribavirin (below the dosage of 2000 mg/kg/day, HPE was almost non-toxic, while an LD_{50} of 200 mg/kg/day was observed for ribavirin).

Both agents appeared to work equally well when the initiation of therapy was delayed as late as 48 h after virus exposure. This was the latest time considered. In a previously study, ribavirin was not efficacious against another IAV infection when treatment began 24 h after virus exposure; however, in that study the viral challenge was highly lethal to the placebo-treated animals, causing 100% mortality and a mean day to death of 4.9 ± 1.1 days. It was apparent that when the viral challenge resulted in reduced mortality, such as in the present experiment where 90% of the placebo treated mice had a greater mean time to death (6.2 ± 0.6 days), treatment could be delayed much longer and still be efficacious.

The high efficacy and tolerability of the herb's extract is unquestionable and, above all, HPE does not produce major side effects, making it a safe herbal therapeutic agent. But hypericin, one of the active ingredients within HPE, was a sort of photosensitizer, which might cause allergic reaction. The daily dose of the herb, which is about 500 mg extract, corresponds to a total dose of 5 mg of hypericins, the main photosensitizers in the herb. These doses of hypericins, given orally, do not provoke skin phototoxicity to patients.

The use of HPE as a novel agent for viral disease offers the advantages of easy production, low cost, extensive availability, adequate water solubility and minimal side effects.

The present study suggests that HPE may have potentials for treatment of human influenza A virus infections. The agent's *in vitro* efficacy against the strain of the IAV would suggest that it may be of use against the current outbreaks of influenza occurring in human populations in Asia (18). The lack of sufficient supplies of active drugs for treatment of such infections, in the event of a pandemic occurrence, would justify further studies on HPE to investigate into this possibility.

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