The antiviral mechanism of the crude extract from the flowers of *Trollius chinensis* based on TLR 3 signaling pathway

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Abstract: The effects of crude extract from the flowers of *Trollius chinensis* on expressions of mRNA and proteins related to vital genes (TLR 3, TBK 1, IRF 3 and IFN β) in TLR 3 signaling pathway were investigated in the presence/absence of Polyinosinic acid-polycytidylic acid (PolyI: C) to ascertain the antiviral mechanism of these flowers. Real-time PCR and western blot were applied to determine the expressions of mRNA and proteins, respectively, and immunofluorescence assay was employed to study the effect on IRF 3 distribution between nuclei and cytoplasma. In the absence of PolyI:C, the crude extract reduced the mRNA expression of TLR 3, IRF 3 and IFN β and the protein expression of TLR 3, and increased the protein expression of IRF 3 and the distribution of IRF 3 in nuclei. In the presence of PolyI:C, the extract reduced the mRNA and protein expressions of TLR 3 and the mRNA expression of IFN β, meanwhile inhibited the translocation of IRF 3 into nuclei. The antiviral mechanism of the crude extract from the flowers of *T. chinensis* is to protect the host from inflammatory damage through intervening the TLR 3 signaling pathway and reducing the secretion of inflammatory factors.

Keywords: *Trollius chinensis*, antiviral mechanism, TLR 3 signaling pathway, mRNA.

INTRODUCTION

Influenza, which is caused by influenza virus, is an infectious disease threatening human beings seriously. It is of high incidence, strong infection and significant mortality, and has erupted a global pandemic in the 20th century. According to World Health Organization, there is an estimation of three to five million cases of severe human influenza with approximately 250,000 to 500,000 deaths every year. Although some agents have been approved by US Food and Drug Administration to treat influenza, their side-effects can not be ignored (Meijer *et al.*, 2009; Astrahan *et al.*, 2011). By contrast, vaccines are the mainstay of prophylactic treatment for influenza, but problems still exist since the development of vaccines requires the identification and characterization of viruses, which may not be available during the early stages of a pandemic (Law *et al.*, 2017). This means that almost no drug or treatment has ideal effect against influenza virus (Guo, 2010), and it highlights the urgent need for antiviral drugs. Over the past few decades, traditional Chinese medicine has been recognized by the world, and its curative effect and antiviral mechanism have also become a hot topic. Various investigations found that febrifuge and detoxifying herbs have a good effect against virus, and they are highly expected in the development of influenza treatment drugs (Xing *et al.*, 2014). The flowers of *Trollius chinensis* Bunge, which have a long history as a drug to treat infections of the upper respiratory tract due to their heat-clearing and toxic-resolving efficacy (Wang *et al.*, 2014; Pharmacopoeia Commission of the People's

Republic of China, 1977; Wang *et al.*, 2012). Judging from the early researches of our laboratory and other scholars, it is well accepted that both the crude extract from the flowers of *T. chinensis* and the components of flavonoids have strong inhibitory effect against influenza virus. In addition, they showed lower toxicity to normal tissues and cells (Cai *et al.*, 2006; Zhao *et al.*, 2010). However, their antiviral mechanism has not been elucidated yet. Antiviral immunity is made of innate immunity and acquired immunity, and the former plays a significant role in the defense against invasion of pathogenic microorganisms owing that it belongs to the first line (Janeway *et al.*, 2002). The innate immunity depends on different recognition systems to detect pathogen-associated molecular patterns (PAMPs), and Toll-like receptors (TLRs) are a group of highly conservative molecules that play a critical role in the recognition of PAMPs and in the activation of innate immune responses to infectious agents (Beutler *et al.*, 2006; Miyake *et al.*, 2004; Zhang *et al.*, 2015). They consist of extracellular, transmembrane, and intracellular regions. The extracellular region contains leucine-rich repeat (LRR) motifs, of which the function is mainly to recognize the PMARs, whereas the intracellular region is TIR domain, which is curial for signal transduction on account of mediating protein interactions. TLR 3 is one of the important members of the TLR family, which is mainly expressed in dendritic cells through identification of virus dsRNA, and activation of NF-κB, IRF3 and AP-1 in antiviral response. Firstly, TLR 3 recognizes the ligand dsRNA, actives the downstream signal protein TBK1 and induces the phosphorylation. Secondly, the phosphorylated TBK 1 further activates IRF 3 and the

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latter is phosphorylated to form dimers. Thirdly, P-IRF 3 is translocated into nuclei and induces the secretion of cytokine IFNβ against viral infection (Kawai *et al*., 2008; Chang *et al*., 2009; Hacker *et al*., 2006; Matsumoto *et al*., 2004; Elisabeth *et al*., 2008). Influenza virus belongs to ssRNA virus, which first produces dsRNA when invading into the host cells (Hou *et al*., 2017). Polyinosinic acid-polycytidylic acid (PolyI:C) is an analog of dsRNA and can replace influenza virus to trigger TLR 3 signaling pathway in the investigation of antiviral mechanism (Singh *et al*., 2012; Alexopoulou *et al*., 2001; Mosmann *et al*., 1983; Okahira *et al*., 2005). Thus, In order to illuminate the antiviral mechanism of the extract from the flowers of *T. chinensis*, its effects on the expressions of mRNA and proteins of the vital genes (TLR 3, TBK 1, IRF 3 and IFN β) in TLR 3 signaling pathway with or without PloyI:C were investigated.

MATERIALS AND METHODS

The flowers of T. chinensis, which were authenticated by Professor Rufeng Wang, were purchased from the drug markets in Anguo, Hebei province. A voucher specimen has been deposited at the Herbarium of the School of Life Sciences, Beijing University of Chinese Medicine. The flowers were extracted 2 times by reflux, then evaporated and lyophilized to gain the powder. The powder was dissolved in the medium before use. PolyI:C was the product of Sigma (St. Louis, Missouri, USA). Trizol reagent and Super Signal (R) West Pico Trial Kit were supplied by Thermo Fisher Scientific Inc. (Waltham, MA, USA). The Prime Script™ 1st strand cDNA Synthesis Kit was produced by TAKARA (Najihigashi, Kusatsu, Shiga, Japan). The iTaq Universl SYBR Green supermix was manufactured by Bio-Rad (Hercules, California, USA). The RIPA and BCA protein assay kits were purchased from the Beijing Solarbio Biotechnology Co. Ltd. (Beijing, China). The primary antibodies for TLR 3 and TBK 1 were from Novus (Littleton, CO, USA), and those are specific to IRF 3 and β-actin were from Santa Cruze (Dallas, Texas, USA) and Beijing Zhongshan Jinqiao Co. Ltd. (Beijing, China), respectively. All of the secondary antibodies used in this research were also supplied by Santa Cruze (Dallas, Texas, USA). The 96-well plates, 6-well plates, and 6cm dishes were supplied by Coring Coaster (Cambridge, MA, USA/Coring, NY, USA). The 35 mm dishes specifically used for confocal scanning were obtained from Nest (Wuxi, Jiangsu, China). The CFX96 real-time PCR instrument, the Mini PROTEAN® Tetra cell electrophoresis, and the trans-Biot® SD Cell Semi-electric gyroscope were all the products of Bio-Rad (Hercules, California, USA). The fluoview FV1000 laser scanning confocal microscope was produced by Olympus (Nishi-Shinjuku, Shinjuku-Ku, Japan). JB-CJ-1500 super clean bench (Beijing Great Wall Air Purification Co. Ltd., Beijing, China), the Epoch microplate reader (Bio Tek Instruments, Inc., Winooski, VT, USA), the MCO-18AIC

CO2 incubator (SANYO Inc., Osaka, Japan), and the TGL-20M low temperature and high speed centrifuge (Shanghai Luxiangyi instrument Co. Ltd., Shanghai, China) were also used.

Cell culture
The canine kidney cell MDCK (No. 3111C0001CCC 000078) was obtained from the Cell Resource Center, Peking Union Medical College (Beijing, China). The cells were cultured in the 25cm2 flasks in DMEM supplemented with 10% (v/v) fetal bovine serum and they were split 1:3 to 1:6 when the confluent was reached 80 to 90%.

Cell viability
The MDCK cells were dispensed into 96-well plates at the density of 5000 cells/well at the logarithmic growth phase. When cells were adhered in an appropriate density, they were cultured with 100μL of fresh medium in the presence of different concentrations of the crude extract in quadruplicate for 24h. Then the drug-containing medium was replaced by MTT solutions at the final concentration of 0.5 mg·mL-1. About 4 h later, the purple crystals were dissolved in 150μL of DMSO. After 10 min of shaking, the OD values were determined on a microplate reader at 490 nm. The inhibitory rate was calculated by the formula: inhibitory rate % = 1- A/A0%; wherein, A represented the average OD value of the treatment group, and A0 was the average OD value of control group.

Real-time PCR analysis
Total RNA was extracted with Trizol reagent when cells were treated with crude extract ($0.5 mg·mL^{-1}$) or positive drug (ribavirin, $1.25 mg·mL^{-1}$) in the presence/absence of PolyI:C ($50 μg·mL^{-1}$). The cDNA was synthesized with the PrimeScript™ 1st strand cDNA Synthesis Kit according to the manufacturer's protocol. The real-time PCR was carried out on the CFX96 real-time PCR instrument with amplification for 40 cycles at 95 for 5s, and annealing and extending at 58 for 30s as per the manufacture's protocol of the iTaq Universl SYBR Green supermix supplied by the Bio-Rad company. The Genbank accessions, primers and product size of each tested genes are listed in table 1.

Western blot assay
Cells were lysed with the RIPA lysis buffer supplemented with protease inhibitor cocktail, PMSF, NaF and NaVO3 for 40 min, then the total proteins were obtained by centrifugation at 12000g for 5 min. The concentrations were detected by BCA protein assay kit according to instruction. The proteins were analyzed by electrophoresis on 8% SDS-polyacrylamide (SDS-PAGE) gels and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were subsequently probed with their own antibodies against TLR 3, TBK 1 and IRF 3, respectively. Then, they were washed and incubated with the corresponding secondary antibodies, and enlightened before detecting the fluorescence intensity.

Table 1: The gene-specific primers for real-time PCR

Gene name	Accession No.	Primers sequences		Product size
GAPDH	NM_001003142	Forward : TTCCACGGCACAGTCAAG		115
		Reverse : ACTCAGCACCAGCATCACC		
TLR3	JF681167	Forward : CAGAGAACCTTTGTTTGGAACTG		119
		Reverse : GGTGAGATTTGTATGCTTTAGTCC		
TBK1	XM_538266	Forward : CCCTCCCTAAAGTTCATCCA		85
		Reverse : CAGGCATAACACACGACTCC		
IRF3	XM_005616307	Forward : CCCAAGGATGAAGATGGAGA		100
		Reverse : AGAGGGTGTAGCGTGGTGAG		
IFNβ	FJ194477	Forward : TCCTGTTGTATTTCTCCACCAT		147
		Reverse : CGAAGTTTATCCTGTCCTTGAG		

Immunofluorescence Assay
After treated for 24h, cells were washed twice with cold PBS and fixed with 4% paraformaldehyde in PBS at room temperature. Then, they were blocked with 5% BAS in TBS containing 0.1% Trixon-100 (TBSTx) for 60 min at room temperature, and incubated with anti-IRF3 overnight at 4°C. The dishes were washed three times with TBSTx, and incubated with the secondary antibody rabbit anti-Cys anti-goat IgG. The cell nuclei were stained with DAPI. The slides were washed three times, mounted, and viewed with an Olympus confocal laser scanning microscope.

STATISTICAL ANALYSIS

Experiments were repeated three times and all results were presented as means ± standard deviation. Differences were evaluated with one-way ANOVA using SPSS (version 12) and $p < 0.05$ was regarded as statistically significant.
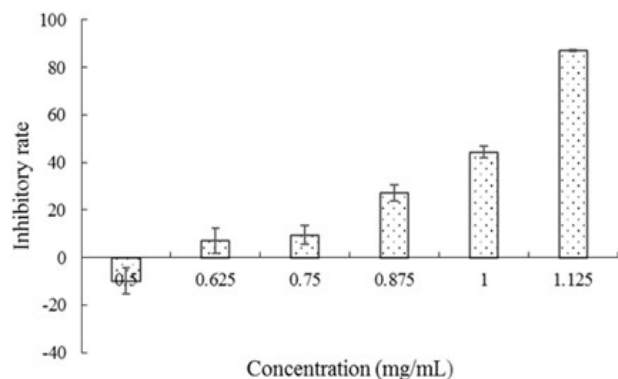

Fig. 1: The inhibitory rate of the crude extract from the flowers of *T. chinensis* on MDCK cells.

RESULTS

Cell Viability
The inhibitory rate of the crude extract on MDCK cells was increased along with the concentration (fig. 1). The maximal nontoxic concentration (TC0) and the median toxic concentration (TC50) were 0.53 and 1.00mg·mL^{-1}, respectively. They were calculated by linear fitting and the function was as follows:
$$y = 1.056x - 0.561$$
wherein, y was the inhibitory rate, and x was the concentration of the crude extract. Based on these results, the tested concentration was designed at 0.5mg·mL^{-1} in the later experiments.

Effect on mRNA expression
The effects of the crude extract on the mRNA expression of TLR 3, TBK 1, IRF 3 and IFNβ are shown in fig. 2. For TLR 3, the ligand of PolyI:C or dsRNA of virus (fig. 2A) was up-regulated by PolyI:C when stimulated for 6 and 12h, and the degree at 6 h was much higher than that at 12h. However, it had no effect at 24h, which indicated that the influence of PolyI:C on TLR 3 expression was related to the stimulation time. In the absence of PolyI:C, both the crude extract and the positive drug ribavirin down-regulated the expression of TLR 3 when compared with the normal control group. In contrast, in the presence of PolyI:C, the crude extract and ribavirin reduced the expression of TLR 3, which was up-regulated by PolyI:C. As shown in fig. 2B, TBK 1, the signal transduction protein, had no effect in mRNA expression when stimulated with PolyI:C either at 6 h or at 24h. However, the crude extract at 6 h and ribavirin at 6 and 24h down-regulated the expression of mRNA when PolyI:C was absent. The mRNA expression of IRF 3 was not affected by both PolyI:C and the crude extract, whereas it was down-regulated by ribavirin. All of these descriptions are

represented in fig. 2C. IFN β is a type interferon, which plays an important role in antiviral effect. As exhibited in fig. 2D, the expression of IFN β was up-regulated by

PolyI:C stimulation for 6 and 12h, and its level at 12h was higher than that at 6h. Nevertheless, it showed no influence when the stimulation was prolonged to 24h. In
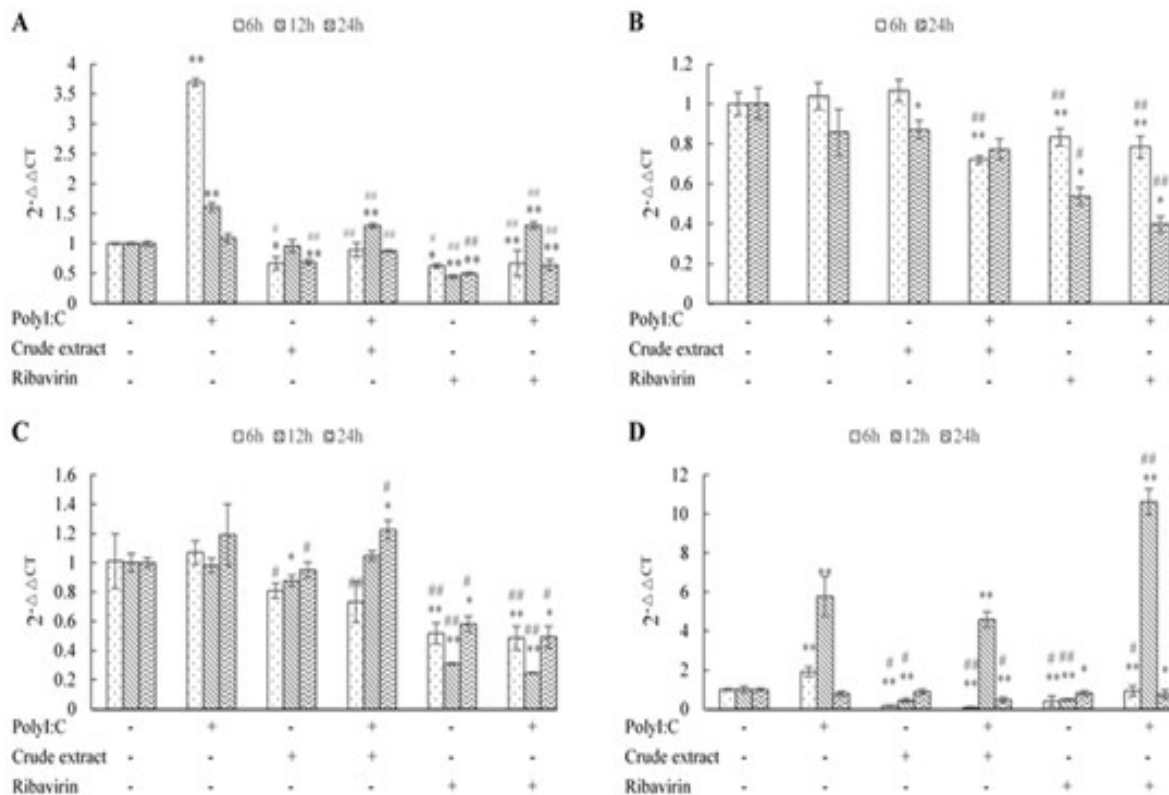
* P<0.05, ** P<0.01, compared with normal group; # P<0.05, ## P<0.01, compared with PolyI:C group
Fig. 2: Effects of the crude extract on mRNA expression of vital genes. (A: TLR3; B: TBK1; C: IRF3; D: IFNβ)
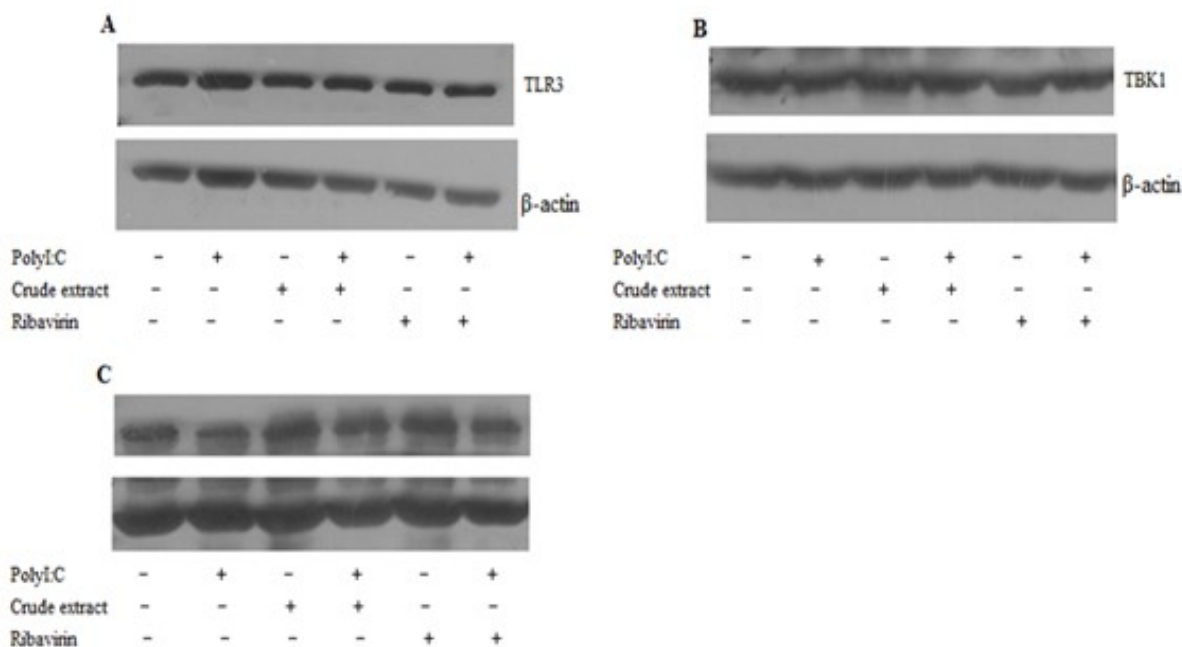

Fig. 3: Effects of the crude extract on protein expression of vital genes. (A: TLR3; B: TBK1; C: IRF3)

the absence of PolyI:C, the crude extract and ribavirin decreased the expression of IFN β at 6 and 12h. In the presence of PolyI:C, the expression of IFN β was also down-regulated by the test drugs.
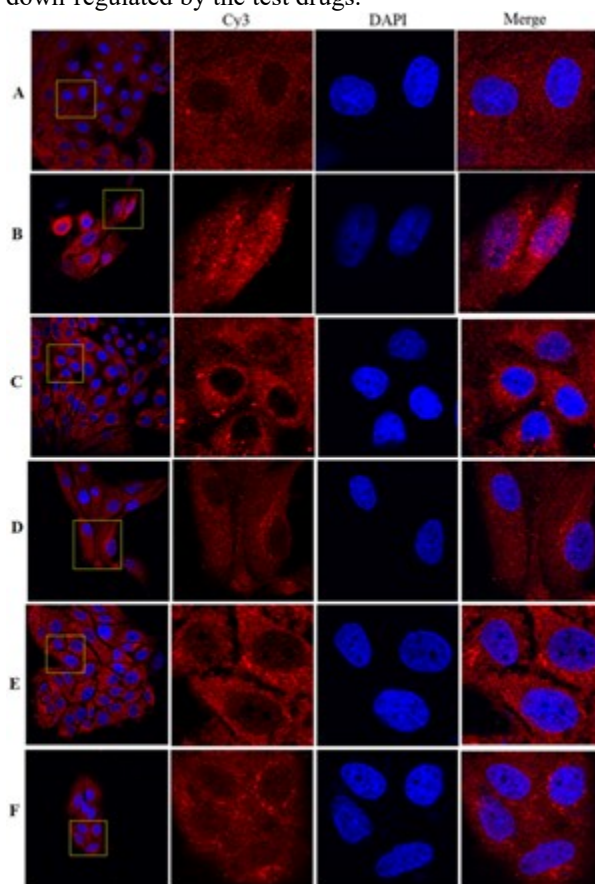

Fig. 4: Effects of the crude extract on translocation of IRF3 into nucleus. (A: Normal control group; treated with B: PolyI:C; C: The crude extract; D: PolyI:C and the crude extract; E: Ribavirin; F: PolyI:C and ribavirin)

Effect on protein expression
The effects of the crude extract on the protein expression of TLR 3, TBK 1, and IRF 3 are displayed in fig. 3. When stimulated with PolyI:C for 12h, the expression of TLR 3, TBK 1 and IRF 3 in protein level was down-regulated. In the absence of PolyI:C, the crude extract significantly up-regulated the protein expression of IRF 3. However, it could down-regulated the protein expression of TLR 3 and TBK1, respectively. The expression of TLR3 and IRF 3 was increased by ribavirin, whereas the expression of TBK 1 was reduced by this positive drug. In the presence of PolyI:C, the crude extract up-regulated the protein expression of TLR 3, TBK 1 and IRF 3. Meanwhile, ribavirin up- and down -regulated the expression of TLR 3 and IRF 3, respectively and had no influence on TBK 1.

Effect on IRF 3 translocation
IRF 3, namely interferon regulatory factor 3, could be translocated into nuclei when activated by upstream transduction protein. In order to study the effects of the crude extract on the distribution of IRF 3 between nuclei and cytoplasma in the presence/absence of PolyI:C, the immunofluorescence assay was carried out using confocal microscopy. The cells were stained with Cys-labeled anti-IRF3 antibody (red) and the nuclei were stained with DAPI (blue). Since the pictures exhibited were too small to be identified clearly, the regions in yellow were magnified. As illustrated in fig. 4, IRF 3 was mainly distributed in cytoplasma in normal control group. When stimulated with PolyI:C for 24h, a large amount of IRF 3 was found in nuclei, which indicated that it was translocated into the nuclei. In the absence of PolyI:C, the amount of IRF 3 in nuclei was a little more than normal control group when treated with the crude extract, which showed that it promoted the translocation of IRF 3 into nuclei. Unlike the crude extract, ribavirin had no this effect due to the unchanged distribution of IRF 3. In the presence of PolyI:C, the IRF 3 in nuclei was much less when treated with the crude extract along with PolyI:C compared with the group treated with PolyI:C alone, yet this effect was not found in the group treated with ribavirin along with PolyI:C.

DISCUSSION

Under the conditions of the experiment, the effects of the crude extract on the expression of TLR 3, TBK 1 and IRF 3 in mRNA and protein levels and the translocation of IRF 3 in the absence/presence of PolyI:C were evaluated. According to the above results, the crude extract down-regulated the mRNA expression of TLR 3, IRF 3 and IFN β and the expressions of TLR 3 protein level in the absence of PolyI:C, while the expression of IRF 3 in protein level was on the contrary. Meanwhile, it promoted the translocation of IRF 3 into nuclei. As we all know, mRNA is the template for protein translation. Thus, the influences of the crude extract on the expressions of those two should be in consistence, while the results of this study self-contradicted. However, the translation process from mRNA into protein is very complex and time-consuming owing to the modification needed, and phosphorylated IRF 3 is the real active formation; therefore, the contradiction could be explained and understood. Proteins are the undertaker of life, so the influences on the expression and translocation of protein are more reliable. As a result, we can consider that the crude extract up-regulated IRF 3 protein expression preliminarily. In the presence of PolyI:C, the crude extract down-regulated the expression of TLR 3 in mRNA and up-regulated TLR 3 protein levels and inhibited the translocation of IRF 3 into nuclei, meanwhile decreased the expression of IFN β in mRNA level which was up-regulated by PolyI:C. Although it increased the protein expression of TLR 3 and IRF 3, only P-IRF 3 could play a role in transducing signal in TLR 3 signaling pathway. So, the increase of TLR 3 and IRF 3 in protein level could

not be an evidence to show that the crude extract up-regulated the TLR 3 signaling pathway.

CONCLUSION

In conclusion, the crude extract from the flowers of *T. chinensis* has effect on TLR 3 signaling pathway not only under physiological condition but also under pathological condition. It exerts antiviral action through intervening the TLR 3 signaling pathway and reducing the secretion of inflammatory factors so as to protect the host from inflammatory damage.

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1748

Pak. J. Pharm. Sci., Vol.34, No.5, September 2021, pp.1743-1748